Biosynthesis of Amphotericin Derivatives Lacking Exocyclic Carboxyl Groups*5

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Amphotericin B is a medically important antifungal antibiotic that is also active against human immunodeficiency virus, Leishmania parasites, and prion diseases. The therapeutic use of amphotericin B is restricted by severe side effects that can be moderated by liposomal formulation or structural alteration. Chemical modification has shown that suppression of charge on the exocyclic carboxyl group of amphotericin B substantially reduces toxicity. We report targeted deletions of the amphN cytochrome P450 gene from the chromosome of the amphotericin-producing bacterium Streptomyces nodosus. The mutant strains produced amphotericin analogues in which methyl groups replace the exocyclic carboxyl groups. These compounds retained antifungal activity and had reduced hemolytic activity.

There are increasingly urgent requirements for new antibiotics to treat infectious disease (1). The need is especially acute with systemic fungal infections that are increasing in incidence and are often fatal (2). Of the few antifungals available at present, the most reliable is amphotericin B (compound 1 in Fig. 1), a polyene macrolide synthesized by Streptomyces nodosus (3). Amphotericin B disrupts ergosterol-containing fungal membranes and has a broad spectrum of activity. Whereas resistance to most non-polypeptide antifungal agents has appeared rapidly, the most prevalent fungal pathogens of humans have been slow to develop resistance to amphotericin B, even after more than three decades of clinical use (4). The advantages of amphotericin B are offset by toxicity that results from low water solubility and interactions with cholesterol in mammalian membranes. Toxicity has also prevented full exploitation of the antiviral, antiprion, and antiparasitic properties of amphotericin B (3). The adverse effects of the drug can be reduced by liposomal formulation, heat-induced superaggregation, or structural alteration (5). Chemical modification has shown that suppression of charge on the exocyclic carboxyl group reduces toxicity and improves antifungal specificity (6). Further improvements have been made by derivatization with additional sugars (7).

Substantial reduction in amphotericin B toxicity would minimize adverse side effects on patients and also allow high dose treatment of infections by emerging fungal pathogens that are sensitive only to high concentrations of the drug. Non-toxic amphotericin analogues and formulations may also be useful in other therapeutic areas. Polyene antibiotics delay the onset of prion diseases in animal models (8). These effects are thought to result from interactions of polyenes with cholesterol-rich membrane microdomains that contain glycosylphosphatidylinositol-anchored prion proteins (3, 9). Amphotericin B formulations are gaining favor for treatment of cutaneous and visceral leishmaniasis, particularly as resistance to other anti- Leishmania drugs continues to increase (10). Polyenes may also become important in treatment of viral infections. Polyenes interfere with the sterol-rich membranes of enveloped viruses such as human immunodeficiency virus (HIV) (3). Recently, amphotericin B has been found to reactivate latent HIV in macrophages, and these polyene-stimulated cells in turn induce viral reactivation in T lymphocytes (11). Although the current highly active antiretroviral therapy effectively eliminates free virus, reservoirs of provirus must be reactivated to allow complete clearance (11). Amphotericin B derivatives may form part of a strategy for eradication, rather than suppression, of the virus in AIDS patients.

Although total synthesis of amphotericin B has been achieved (12), the synthetic route toward production of improved antibiotics is not economically feasible. The starting material for lipid formulation or chemical modification is amphotericin B isolated from cultures of S. nodosus. Recent studies on amphotericin biosynthesis in this organism have led to the possibility of obtaining non-toxic analogues as primary fermentation products (13–15).

The carbon chain of the amphotericin B macrolactone is assembled from acetate and propionate units by a modular polyketide synthase (13). The polyketide precursor undergoes three modifications to form the final antibiotic, namely oxidation of a methyl branch to a carboxyl group, glycosylation with GDP-mycosamine, and hydroxylation at C-8 (13). Amphotericin A (compound 2 in Fig. 1), a less active tetrane,2 is produced as a co-metabolite. The enoyl reductase domain in module 5 of the polyketide synthase is thought to be sluggish, reducing only some of the enoyl-acyl carrier protein 5 oligoketide intermediates so that amphotericin A is produced as well as amphotericin B (14). The biosynthetic gene cluster contains two cytochrome P450 genes, amphL and amphN (13). Phage KC515-mediated disruption of amphL led to synthesis of 8-deoxymycolamide. Recent studies on amphotericin biosynthesis in this organism have led to the possibility of obtaining non-toxic analogues as primary fermentation products (13–15).

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5 The on-line version of this article (available at http://www.jbc.org) contains further analysis of S. nodosus mutants and amphotericin derivatives in the form of supplemental Figs. 8–13.

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2 The term “tetrane” is used to describe amphotericin A and derivatives in which the C-28=C-29 double bond is reduced. Amphotericin B and derivatives with a series of seven conjugated double bonds are described as heptanes.
EXPERIMENTAL PROCEDURES

Genetic Procedures—Escherichia coli XL1-Blue was used for routine cloning. E. coli ET 12567 was used to obtain non-methylated DNA for digestion with BclI. Streptomyces lividans 66 (John Innes strain 1326) was used as the host for the construction of recombinant phages. Construction of phages KC-DI and KC-ΔNM was carried out as described previously (16, 17). PCR was used to identify recombinant phages. Oligonucleotide primers D1F (5'-AAAAGATCTTTGGAGAACGCAGGATCC-3') and D1R (5'-AGGACATATGTGACCTTGCTACGTTGTCGGTCAGTCGTTC-3') were used to detect recombinant phage KC-DI, and primers KCNM-F (5'-GCGGGGATCCGAGGGTGACCAGCTTTGGACGCATTC-3') and KCNM-R (5'-TGACAGATCGGACCGAGGATTGTCATCCAAGC-3') were used to identify KC-ΔNM phages. Isolation of genomic DNA and Southern hybridization were carried out as described (14, 15).

S. nodosus lysogens were selected with thiostrepton. To obtain revertants resulting from a second cross-over recombination event, the lysogen was cultured in the absence of thiostrepton for several generations and protoplasted. Thioestrepton-sensitive revertants were identified by using toothpicks to place individual regenerated protoplast colonies onto tryptone soya agar and tryptone soya agar containing thiostrepton.

Production and Analysis of Polyenes—For polyene production, S. nodosus mutants were grown on fructose dextrin soya medium as described previously (15). Four 500-ml cultures were centrifuged, and the sedimented mycelium was extracted with 400 ml of acetone to recover the polyenes. After concentration by evaporation, polyenes were extracted from the aqueous residue with 50 ml of butan-1-ol. The extract was evaporated to dryness, and the polyenes were dissolved in 4 ml of methanol. The methanol-soluble polyene sample was applied to an Amberlite XAD16 column (2.5 × 50 cm). Polyenes were recovered after isocratic elution with methanol at a flow rate of 2 ml/min. Fractions were scanned in the wavelength range 250–450 nm, and those containing the highest concentrations of polyene were pooled and concentrated by rotary evaporation. The sample was then purified by gel filtration on a lipophilic Sephadex LH20 column. Batches of 2 ml containing up to 6 mg of total polyene (the heptaene/tetraene ratio varied between 1:3 and 10:1) were applied to a Sephadex LH20 column (50 × 2.5 cm), equilibrated with methanol and eluted at a flow rate of 1 ml per min. The absorption spectrum of each fraction was measured in the wavelength range 250–450 nm. This result revealed that gel filtration partially resolved the tetraene from the heptaene. With amphotericin-derived tetraenes, reduction of the double bond between C-28 and C-29 alters the molecular shape by making the polyene chain more flexible. This could assist resolution of tetraenes and heptaenes. The usefulness of Sephadex LH20 gel permeation for separating hydroxy lipids has been reported (18).

The concentrations of heptaene and tetraene solutions were determined by measuring absorbance values at 405 nm and 305 nm, respec-
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tively. An extinction co-efficient of 161,000 liters mol⁻¹ cm⁻¹ was used for amphotericin B and heptaene analogues. An extinction coefficient of 79400 liters mol⁻¹ cm⁻¹ was used for tetrane analogues of amphotericin A (13).

Electrospray mass spectrometry (ESMS)³ was performed with a Quattro Micro tandem quadrupole mass spectrometer (Waters Corporation, Micromass Ltd., Manchester UK) in positive and negative ion modes. Exact mass measurements of the analytes were determined using an on-line liquid chromatography to a time-of-flight instrument (Waters Corporation, Micromass Ltd., Manchester UK).

N-Acetylated heptaene compound 3 (Fig. 1) was prepared as follows. A crude polyene extract from the Δnm strain containing 24 mg of heptaene was dried in vacuo. The residue was suspended in 3 ml of methanol/Me₂SO (2:1). Acetic anhydride (0.1 ml; 1.06 mmol) was added with stirring at 0 °C for 20 min. The mixture was then stirred at room temperature for 1 h. A 100-ml volume of diethyl ether was added, and the resulting polyene precipitate was sedimented by centrifugation. About 11 mg of modified heptaene was recovered. This was purified by high-performance liquid chromatography on a Supelco silica preparative column (25 cm × 21 mm; 5 µm) with a gradient of 10–15% methanol in ethyl acetate. Approximately 5 mg of N-acetylated heptaene compound 3 was obtained. This material was used for structural characterization by NMR. Spectra were recorded on a Bruker DRX-400 instrument.

Assessment of Biological Activities of Polyenes—Bioassays were carried out as described previously (15). Candida albicans was obtained from the culture collection of the Department of Industrial Microbiology, University College Dublin. Horse erythrocytes were used to determine minimum hemolytic concentrations (MHCs). Serial 2-fold dilutions were prepared from concentrated stock solutions of polyenes in methanol. A 50-µl volume of each polyene dilution was mixed with 250 µl of 2.5% (v/v) defibrinated horse blood in phosphate-buffered saline. The mixtures were incubated at 37 °C for 1 h, and hemolysis was assessed visually or by sedimenting undyed erythrocytes and determining the absorbance of the supernatant fluid at 545 nm. The MHC was recorded as the lowest concentration that gave complete hemolysis.

RESULTS

Deletion of the amphDII and amphN Genes—Within the amphotericin biosynthetic gene cluster, the amphN PA50 and amphM ferredoxin genes are located downstream from the amphDII and DI genes that encode mycosamine synthase and mycosaminyl transferase (14). Targeted disruption of amphN was found to be problematic, with phage constructs failing to integrate or giving unwanted chromosomal deletions that abolished polyene production. The reasons for these failures were not understood, but it was possible that the DNA sequence of the amphN region was only weakly recombinogenic or that disruption of amphN might cause lethal accumulation of toxic polyene biosynthetic intermediates. To gain further insights, a recombinant phage was constructed failing to integrate or giving unwanted chromosomal deletions.

A 1611-bp BclI-PstI fragment (nucleotides 63699–65712 of the amphotericin cluster) was cloned between the BamHI and PstI sites of the KC-UCD1 phage vector (16) to give KC-DI (Fig. 2A). Correct integration of this phage should leave intact copies of amphDI and amphDII in the chromosome. However, it was anticipated that the integrated prophage DNA might separate the downstream amphDII and amphN genes from their promoter, which is thought to be located upstream of amphDI (14).

Propagation of phage KC-DI on S. nodosus yielded two thiostrepton-resistant lysogens, DI-1 and DI-2. Genomic DNA from these strains was analyzed by Southern hybridization. The 1611-bp BclI-PstI fragment is located within a 2-kb Ncol fragment of S. nodosus chromosomal DNA (nucleotides 63699–65712 of the amphotericin cluster). Phage integration should result in formation of new 10.6- and 3.2-kb Ncol fragments that contain this sequence (Fig. 2B). However, Southern analysis revealed that deletions had occurred around the integration site in both lysogens. S. nodosus DI-2 had lost the 10.6-kb band containing the amphDII region but retained the 3.2-kb band containing amphDI. In contrast, S. nodosus DI-1 had the 10.6-kb band (amphDII region) but not the 3.2-kb band (amphDI region) (Fig. 2C).

S. nodosus DI-1 produced no polyenes. Extracts from S. nodosus DI-2 had a UV absorption spectrum characteristic of a tetraene/heptaene mixture with heptaene as the major product. These extracts had antifungal activity that was ~10 times lower than that of amphotericin B (see below).

Analysis of S. nodosus DI-2 genomic DNA by PCR confirmed that the amphDII and amphN genes were deleted (data shown in supplemental Fig. 8, available in the on-line version of this article). The production of a heptaene by this strain suggested that the amphK and amphA polyketide synthase genes on either side of this region remained intact. The amphL gene is located at a distance of 3 kb from amphM and reads in the opposite orientation. This gene was detected by PCR and was presumably still functional.

S. nodosus DI-2 lacks the amphN gene and was expected to be deficient in oxidation of the polyene C-16 methyl group to a carboxyl group. This strain also lacks the amphDI gene and should be deficient in the formation of mycosamine. A heptaene-enriched fraction was purified by chromatography on Amberlite XAD16 and by gel filtration on lipophilic Sephadex LH20. Analysis by ESMS revealed a compound with a mass appropriate for compound 5 (Fig. 1), an amphotericin analogue with a methyl group instead of the exocyclic carboxyl and a neutral deoxyhexose replacing mycosamine (Fig. 3; calculated molecular

³ The abbreviations used are: ESMS, electrospray mass spectrometry; MHC, minimum hemolytic concentration; MIC, minimum inhibitory concentration.
mass = 894.5; observed [M + Na]$^+$ = 917.5, [M + K]$^+$ = 933.6, [M – H]$^-$ = 893.5, and [M + Cl]$^-$ = 929.5. High resolution MS in negative ion mode gave 893.4943, which is consistent with the molecular formula C$_{47}$H$_{74}$O$_{16}$ (calculated exact mass 893.4899 for [M – H]$^-$). With samples that contained tetranea, a species with a mass of 903.5 was also detected in negative ion mode, and additional species with masses of 879.5 and 915.6 were detected in negative ion mode (supplemental Fig. 9 in the online version of this article). These masses are appropriate for compound 6 (Fig. 1), the 8-deoxytetraene analogue of compound 5 (Fig. 1) (calculated molecular mass = 880.5; observed [M + Na]$^+$ = 903.5, [M – H]$^-$ = 879.5, and [M + Cl]$^-$ = 915.6).

The production of compound 5 (Fig. 1) is consistent with the genotype of S. nodosus DI-2. Mycosamine biosynthesis is thought to involve conversion of GDP-mannose to GDP-4-keto-6-deoxymannose, a keto-isomerization to form GDP-3-keto-6-deoxymannose, and AmphDII-catalyzed transamination to form GDP-mycosamine (14). The amphDII-deficient S. nodosus DI-2 is capable of synthesizing either GDP-4-keto-6-deoxymannose or GDP-3-keto-6-deoxymannose. Either of these activated deoxyhexuloses could be reduced and transferred to the modified aglycone by the glycosyl transferase encoded by the intact gene. GDP-4-keto-6-deoxy-3,5-d-mannose reductases have been found to function in the biosynthesis of GDP-3,5-d-mannose in other bacteria (19). It is possible that S. nodosus has a ketoreductase that can act on the GDP-deoxyhexulose that accumulates in the absence of AmphDII.

Previous chemical modification experiments have shown that a positive charge on the amino sugar is important for antifungal activity of amphotericin B (6). The presence of a neutral sugar is consistent with the positive charge on the amino sugar is important for antifungal activity of amphotericin B (6).

**Targeted Deletion of the amphN Cytochrome P450 and amphM Ferredoxin Genes**—The polynes produced by S. nodosus DI-2 suggested that amphotericin analogues lacking C-16 carboxyl groups were not toxic to the producing cell. Renewed efforts were made to achieve a targeted disruption of the amphN gene that would leave the glycosylation genes intact.

A 1200-bp Stul fragment was deleted from a pUC118 plasmid clone of the 6-kb PstI fragment containing the amphN region of the amphotericin biosynthetic gene cluster (nucleotides 58981–65544). This deletion removed the 3'-end of the amphN gene, the adjacent amphM ferredoxin gene, and part of open reading frame 3, which has no role in amphotericin biosynthesis (14). The ligated flanking sequences were cloned as a BclI-PstI fragment into the KC-UCD1 phage vector to generate the recombinant phage KC-ΔNM (Fig. 4A). Propagation of this phage on S. nodosus yielded two thioestrepton-resistant lysogens. Analysis of genomic DNA by Southern hybridization revealed that one of these had a correctly integrated prophage (not shown). Thioestrepton-sensitive revertants were identified by screening. Analysis of genomic DNA by PCR revealed that several had undergone gene replacement. Results from a typical deletion mutant are shown in Fig. 4B. Further analysis by Southern hybridization revealed that the 5.3-kb NcoI fragment containing the amphnm region had been replaced by a smaller 4.1-kb fragment (supplemental Fig. 10, available in the on-line version of this article). The deletion mutant was designated S. nodosus ΔNm.
strain gave total polyene yields of up to 0.05 g/liter in unoptimized batch cultures. Under the same growth conditions the wild type *S. nodosus* produced 1 g/liter of polyene. The ratio of tetraene/heptaene was variable in both strains.

**Analysis of Polyenes from *S. nodosus* ΔNM—**Polyenes were extracted from mycelium and purified by chromatography on Amberlite XAD16 and by gel filtration on Sephadex LH20. The tetraene eluted ahead of the heptaene, so that with tetraene-rich extracts the tetraene could be separated from lower levels of heptaene. The heptaene could be purified from extracts of cultures that produced this compound exclusively.

Absorption spectra of heptaene and tetraene fractions are shown in supplemental Fig. 11 (available in the on-line version of this article).

Analysis of the tetraene fraction by ESMS revealed a major compound with a mass appropriate for compound 7 (Fig. 1) ([M + H]+ = 880.5), the chloride adduct of compound 7 was detected when the tetraene was analyzed in negative ion mode ([M + Cl]− = 914.6). The intensity of the M + 2 peak at 916.6 is increased as a result of the high natural abundance of 37Cl relative to 35Cl.

The purified heptaene fraction had a major species with a mass appropriate for compound 3, 16-descarboxyl-16-methyl amphotericin B (Fig. 6; calculated molecular mass 893.5; observed [M + H]+ = 894.6, [M + Na]+ = 916.6, [M − H]− = 892.6, and [M + Cl]− = 928.6). A dehydrated form of the heptaene was also detected ([M + H]+ = 876.6 and [M − H]− = 874.6). High resolution ESMS gave the molecular formula C47H77NO14 (calculated exact mass for [M + H]+ = 880.5, observed mass = 880.5).

It is unclear why the heptaene compound 3 is apparently hydroxylated at C-8, whereas the tetraene compound 7 (Fig. 1) is not. *S. nodosus DI-2* also appeared to produce a heptaene compound 5 that is hydroxylated and a tetraene compound 6 (Fig. 1) that is not (see above). It is possible that the AmphM ferredoxin normally co-operates with both the AmphL and AmphN cytochrome P-450s. In the *amphNM* mutant,
AmphL may recruit a different ferredoxin to form a complex that acts less efficiently on the tetraene.

Heptaene compound 3 (Fig. 1) was also characterized by NMR. The mycosamine amino group was acetylated to facilitate further purification. Analysis by ESMS revealed molecular ions with masses appropriate for N-acetylated forms of heptane compound 3 (Fig. 1) ([M + Na]⁺ = 958.5 and [M + Cl]⁻ = 970.5) (data not shown). Final purification was achieved by high-performance liquid chromatography. Examination by proton NMR (400 MHz, d4-methanol) gave a spectrum reminiscent of that for amphotericin B with four doublets (supplemental Fig. 12, available in the on-line version of this article). However, the integration of the upfield doublet suggested two methyl doublets. Phase-sensitive proton-proton correlation spectroscopy revealed that the resonances at 1.02 were indeed two methyl doublets correlating with 1.24 and 1.82 ppm, the latter being consistent with coupling to the C-16 proton in the expected product (TABLE ONE, Fig. 7, and supplemental Fig. 13, available in the on-line version of this article). These data are consistent with the presence of a methyl group attached to C-16. The assignments are based upon the couplings observed in the correlation spectroscopy spectrum and by chemical shift analogy with amphotericin B.

**Biological Activities of Novel Polyenes—**Agar diffusion assays were used to test the activities of the novel polyenes against *C. albicans*. Under the conditions used, the tetraene 8-deoxy-16-descarbonyl-16-methyl-amphotericin A (compound 7) showed a minimum inhibitory concentration (MIC) of 5 μg/ml, whereas amphotericin B showed an MIC of 1.25 μg/ml. The tetraene compound 7 (Fig. 1) was therefore four times less active than amphotericin B. However, the tetraene showed a dramatic reduction in hemolytic activity. The minimum hemolytic concentration was 166 μg/ml compared with 5 μg/ml for amphotericin B, a 33-fold reduction. The purest active (non-acetylated) sample of compound 3 (Fig. 1) contained 16-descarbonyl-16-methyl amphotericin B and a dehydrated form of this heptane. This mixture had an MIC of 1 μg/ml total heptaene against *C. albicans*, indicating that the antifungal activity is comparable with that of amphotericin B. The MHC was 50 μg/ml, indicating that the heptane mixture was 10 times less hemolytic than amphotericin B. Previous studies have compared the MHCs and MHCs of amphotericin B, its methyl ester, and various semi-synthetic derivatives (6, 20). Our results for amphotericin B fall within the range of values obtained by other groups. Slight variations in these estimates presumably result from differences in assay conditions. However, our results indicate that the new analogues (compounds) 3, 5, and 7 (Fig. 1) have antifungal activities that are comparable with that of amphotericin B, whereas their hemolytic activities are lower.

**DISCUSSION**

The biosynthetic gene clusters for a number of polyenes have now been investigated. These include amphotericin (14), nystatin (21), candicidin/FR-008 (22, 23), pimaricin (24), and rimocidin (25). All of these clusters contain cytochrome P450 genes homologous to *amphN*. These P450 genes are thought to function in formation of the exocyclic carboxyl group that is characteristic of most glycosylated polyenes (26). The amphotericin, nystatin, and pimaricin clusters contain additional P450 genes (amphL, nysL, and pimD).

**TABLE ONE**

| Assignment | ppm   | Correlations (strong/weak) |
|------------|-------|----------------------------|
| 39         | 1.02  | (2H, d) 1.82               |
| 41         | 1.02  | (2H, d) 1.24               |
| 40         | 1.12  | (3H, d) 2.39               |
| 38         | 1.20  | (3H, d) 5.38               |
| 16         | 1.24  | (1H) 3.96                  |
| 6’         | 1.26  | (3H, d) 3.28               |
| 1.30       |       |                            |
| 1.35       |       |                            |
| 1.45       |       |                            |
| 1.49       |       |                            |
| 18a        | 1.72  | 4.33/3.96(3.65/2.22/2.22/1.45w) |
| 36         | 1.82  |                            |
| 1.97       | (1H)  | 1.30                       |
| N’Acetyl   | 2.02  | (3H, s) None                |
| 18b        | 2.22  | (1H) 1.72/4.42w             |
| 2.25       |       |                            |
| 36         | 2.38  |                            |
| 3.24       | (1H)  | 1.12                       |
| 32.2       | (2H)  | 1.72                       |
| 35         | 3.24  | (1H) 2.38                  |
| 3.28       | (1H)  | 1.26/3.83                  |
| 3.61       | (2H)  | 1.26w                      |
| 3.62       | (2H)  | 1.72s                      |
| 3.72       | (2H, ca t) | 1.49                    |
| 3.78       | (2H)  | None                       |
| 4’         | 3.83  | (3H) 3.28                  |
| 1’         | 3.96  | (2H, ca t) 1.24/1.72w       |
| 4.20       | (2H)  | 1.45m/2.25m                |
| 4.33       | (2H, ca t) | 1.72                    |
| 4.42       | (2H, ca t) | 6.03/2.22w                |
| 1’         | 4.61  | (1H, br s) None             |
| 33         | 5.38  | (1H) 6.18 (H-32)            |
| 37         | 5.38  | (1H) 1.20/3.28              |
| 31         | 6.01  | (ca dd) 6.18 (H-32)         |
| 20         | 6.03  | (ca dd) 4.42                |
| 21–32      | 6.15–6.55 (12H) | 6.01/5.38                |

**FIGURE 7.** Proposed structure of compound 3 with the five vicinal methyl-methine coupling correlations indicated by double-headed arrows. The complete numbering system for amphotericin B is given in Ref. 13.
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P450s are homologous to each other and are thought to act on the polyol chains of their respective polyene macrolactones. Inactivation of *pimD* resulted in the formation of 4,5-de-epoxypimaricin (27), whereas inactivation of *amphl* resulted in the formation of 8-deoxyamphotericin B (15). Here we show that deletion of *amphN* and *amphM* results in formation of novel polynes with masses appropriate for analogues in which the C-16 methyl group is not oxidized.

Modification of the exocyclic carboxyl group of amphotericin B is known to bring about a substantial reduction in its toxicity (6). When the polyene interacts with membrane sterols, this carboxyl group is thought to contribute to an extensive network of hydrogen bonds that involves the mycosamine amino group, a water molecule, and the sterol hydroxyl group (28). This system of bonds is equally strong in polyene-cholesterol and polyene-ergosterol complexes. With amphotericin B methyl ester, this network is weakened, so that the polyene binds more selectively to ergosterol through specific hydrophobic interactions within the lipid bilayer. The amphotericin analogues (compounds) 3 and 7 (Fig. 1) are expected to show similar improvements in specificity within the lipid bilayer. The amphotericin analogues retain good antifungal activity and have lower hemolytic activities than amphotericin B.

It is unclear why the yields of these new analogues are lower than that of amphotericin B. However, further strain improvement could lead to production of these compounds on a larger scale to provide material for further chemical modification or lipid formulation.

In our results establish the role of the AmphN protein in amphotericin biosynthesis. AmphN is unusual among the cytochrome P450s that modify macrolide antibiotics in that it appears to introduce two oxygen atoms into an unactivated methyl group. Directed evolution of *amphN* and *amphM* resulted in the formation of 4,5-de-epoxypimaricin (27), whereas inactivation of *amphN* in amphotericin biosynthesis. AmphN is unusual among the cytochrome P450s that modify macrolide antibiotics in that it appears to introduce two oxygen atoms into an unactivated methyl group. Directed evolution of *amphN* and *amphM* resulted in the formation of 4,5-de-epoxypimaricin (27), whereas inactivation of *amphN* and *amphM* results in formation of novel polynes with masses appropriate for analogues in which the C-16 methyl group is not oxidized.

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