Chemistry & Biology, Vol. 10, 1215–1224, December, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2003.12.001

Biosynthesis of Deoxyamphotericins and Deoxyamphoteronolides by Engineered Strains of *Streptomyces nodosus*

Barry Byrne,[1] Maria Carmody,[1] Emma Gibson,[2] Bernard Rawlings,[2] and Patrick Caffrey[1,*]
[1]Department of Industrial Microbiology
Centre for Synthesis and Chemical Biology
Conway Institute of Biomolecular and Biomedical Research
University College Dublin
Dublin 4
Ireland
[2]Department of Chemistry
University of Leicester
University Road
Leicester LE1 7RH
United Kingdom

Summary

Amphotericin B is an antifungal antibiotic produced by *Streptomyces nodosus*. During biosynthesis of amphotericin, the macrolactone core undergoes three modifications: oxidation of a methyl branch to a carboxyl group, mycosaminylation, and hydroxylation. Gene disruption was undertaken to block two of these modifications. Initial experiments targeted the *amphDIII* gene, which encodes a GDP-D-mannose 4,6-dehydratase involved in biosynthesis of mycosamine. Analysis of products by mass spectrometry and NMR indicated that the *amphDIII* mutant produced 8-deoxyamphoteronolides A and B. This suggests that glycosylation with mycosamine normally precedes C-8 hydroxylation and that formation of the exocyclic carboxyl group can occur prior to both these modifications. Inactivation of the *amphL* cytochrome P450 gene led to production of novel polyenes with masses appropriate for 8-deoxyamphotericins A and B. These compounds retained antifungal activity and may be useful new antibiotics.

Introduction

The heptaene macrolide amphotericin B (**1a**) (Figure 1) is a medically important antifungal agent that is produced by *Streptomyces nodosus* in combination with the tetraene amphotericin A (**1b**). The antifungal activity of polyenes results from specific interactions with ergosterol, the predominant sterol lipid in fungal membranes. Polyene-sterol complexes associate to form transmembrane channels that allow unregulated loss of ions and small molecules causing cell death [1]. Amphotericin B has very high activity and is the most effective drug for the treatment of life-threatening systemic fungal infections [2], and resistance still has not emerged as a serious problem even after more than 40 years of regular clinical use. However, amphotericin B does have severe side effects that include nephrotoxicity, cardiotoxicity, and neurotoxicity, due to low water solubility and interactions with cholesterol in mammalian cell membranes [3]. As the incidence of life-threatening fungal infections is increasing [4], there is an urgent need to develop effective new polyene-based antibiotics with reduced toxicity.

Numerous amphotericin analogs have been made by chemical modification of the carboxyl and amino groups [5, 6]. Some of these analogs show improved antifungal specificity, particularly amphotericin B methyl ester. This indicates that more extensive structural alterations could further improve the therapeutic index. However, it has proved difficult to chemically modify the macrolactone ring. An ability to understand and manipulate the biosynthetic pathway would enable production of large numbers of new analogs with alterations on the macrolactone and the sugar, any of which may prove to be a clinically important alternative to the toxic amphotericin B.

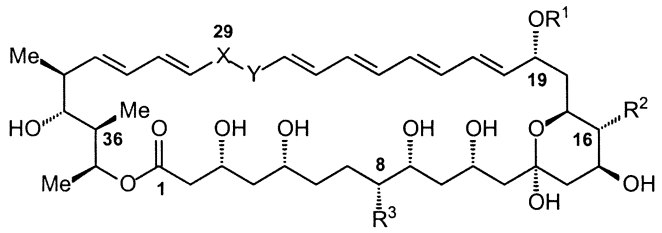
The biosynthesis of the amphotericins has been investigated by feeding experiments with ^{13}C-labeled precursors [7] and the biosynthetic gene cluster has been characterized [8]. A sluggish enoyl reductase (ER5) is believed responsible for the coformation of amphotericins A and B (**1b**, **1a**). The macrolactone core (**1i**, **1j**) is assembled from acetate and propionate units by a large modular polyketide synthase encoded within the gene cluster, as are enzymes that assemble the mycosamine sugar from GDP-D-mannose, and the glycosyl transferase to attach the sugar to the ring. The cluster also contains genes for two cytochrome P450 monooxygenases that are thought responsible for hydroxylation at C-8 and oxidation of the C-16 methyl branch to a carboxyl group. Techniques have been developed for replacement of *S. nodosus* genes to allow the engineered biosynthesis of amphotericin analogs [8]. Here, these developments are exploited to disrupt genes involved in the late steps of amphotericin biosynthesis. The results provide the first insights into the order in which these tailoring modifications occur and also generate previously unavailable analogs of amphotericins for therapeutic testing.

Results

Disruption of the *amphDIII* Gene

A 2091 base pair region of the amphotericin cluster (nucleotides 3474–5565; [8]) containing the *amphDIII* gene was amplified by PCR with oligonucleotides designed to introduce unique BamHI and PstI sites at opposite ends of the product. These sites were used to clone the amplified DNA into pUC118. The resulting plasmid was digested with BglII, which cuts a single site approximately in the center of the *amphDIII* gene. The cohesive ends were repaired with T4 DNA polymerase and ligated to create a frameshift mutation. The removal of the BglII site was verified by restriction analysis. The BamHI-PstI fragment was cloned into KC515 and recombinant phage were identified by PCR with primers

*Correspondence: patrick.caffrey@ucd.ie



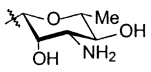
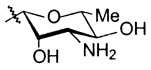
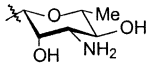
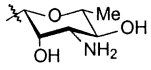
	R^1	R^2	R^3	X-Y	RMM	Name
1a		–COOH	–OH	*E* -CH=CH-	**923.5**	Amphotericin B
1b		–COOH	–OH	—CH_2CH_2—	**925.5**	Amphotericin A
1c		–COOH	–H	*E* -CH=CH-	**907.5**	8-Deoxyamphotericin B
1d		–COOH	–H	—CH_2CH_2—	**909.5**	8-Deoxyamphotericin A
1e	–H	–COOH	–H	*E* -CH=CH-	**762.4**	8-Deoxyamphoteronolide B
1f	–H	–COOH	–H	—CH_2CH_2—	**764.4**	8-Deoxyamphoteronolide A
1g	–H	–COOMe	–H	*E* -CH=CH-	**776.4**	Methyl-8-Deoxyamphoteronolide B
1h	–H	–COOMe	–H	—CH_2CH_2—	**778.4**	Methyl-8-Deoxyamphoteronolide A
1i	–H	–Me	–H	*E* -CH=CH-	**732.4**	8-Deoxy-16-descarboxyl-16-methylamphoteronolide B
1j	–H	–Me	–H	—CH_2CH_2—	**734.4**	8-Deoxy-16-descarboxyl-16-methylamphoteronolide A

Figure 1. Structure of Amphotericin B and Analogs

specific for the insert. The KC515-*amphDIII* phage was propagated on *S. nodosus*, and three thiostrepton-resistant lysogens were isolated and purified. One of the lysogens was cultured in the absence of thiostrepton to allow prophage excision and gene replacement to occur. The mycelial cells were converted to protoplasts to generate single cell units. After protoplast regeneration, single colonies were tested for thiostrepton sensitivity to identify clones in which a second crossover event had excised the prophage. Nineteen thiostrepton-sensitive revertants were obtained from 208 clones. Genomic DNA was isolated from these revertants, and the *amphDIII* region was amplified by PCR and subjected to restriction analysis. Two of the revertants gave PCR products that lacked a BglII site and were identified as *amphDIII* mutants. Figure 2 shows restriction analysis of the *amphDIII* region PCR products amplified from *S. nodosus* ATCC14899 and one of the replacement mutants.

Analysis of Polyenes Made by the *amphDIII* Mutant

The *amphDIII* mutant was grown on fructose-dextrin-soybean medium [7] and was shown by UV spectrophotometry to produce tetraenes (50–250 mg/L culture broth) and lower levels of heptaenes, predominantly in the sedimentable mycelial fraction of the cultures. This mycelial sediment was extracted with methanol, and the tetraenes were partially purified by selective precipitation (removing some heptaene and more polar polyene products). Analysis by ESMS indicated that the major products present were 8-deoxyamphoteronolides along

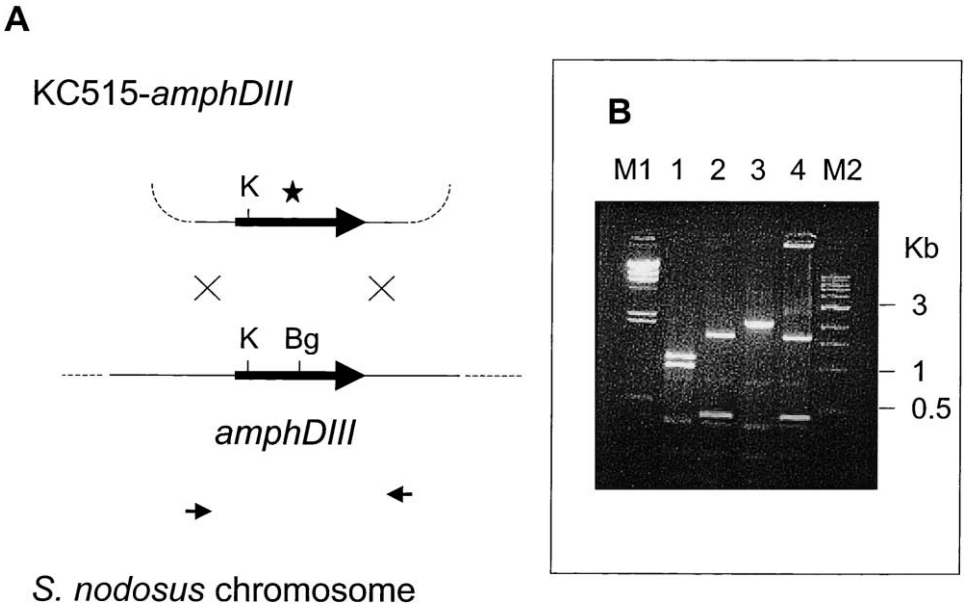


Figure 2. Targeted Inactivation of the *amphDIII* Gene

(A) A double crossover recombination event results in replacement of the *amphDIII* gene with a form containing a frameshift mutation and lacking a BglII site. K and Bg denote the unique KpnI and BglII sites within the 1035 base pair *amphDIII* gene. The star denotes the frameshift mutation. The small arrows represent the primers used to amplify the *amphDIII* region.

(B) Analysis of a typical replacement mutant. The oligonucleotide primers described in the text were used to amplify the *amphDIII* region from *S. nodosus* ATCC14899 and from the *amphDIII* mutant. The 2091 bp PCR products were treated with BglII or KpnI. The region amplified from wild-type *S. nodosus* was cut by BglII to give the expected 973 and 1118 bp fragments (lane 1), whereas the product amplified from the mutant was resistant to cleavage by BglII (lane 3). The DNA amplified from both strains gave the expected 446 and 1645 bp fragments when digested with KpnI (lanes 2 and 4).

with some amphoteronolides. Figure 3 shows spectra obtained with a sample enriched in 8-deoxyamphoteronolide A (**1f**) with some amphoteronolide A.

The partially purified 8-deoxyamphoteronolides were converted to methyl esters (**1g** and **1h**) and a fraction containing mainly tetraene products further purified by normal phase HPLC, from which two tetraenes were isolated, both with the expected molecular weight (778.4). Analysis of one of these components by proton, carbon, and proton-proton 2D NMR gave spectra consistent with methyl 8-deoxyamphoteronolide A (**1h**) (Figure 4A) [9]. Preliminary NMR analysis of the second component gave spectra consistent with either methyl 37-*epi*-8-deoxyamphoteronolide B or methyl 36-*epi*-8-deoxyamphoteronolide B (Figure 4B). This assignment is based upon a large upfield shift of H-37 in the proton and 2D NMR spectra from 5.2 in **1h** (coupled to methyl doublet at 1.2 ppm) to 4.0 ppm (coupled to methyl doublet at 1.2 ppm) in the epimer. These compounds are undergoing further characterization (B.R., unpublished data). Molecular modelling indicates that epimerization at either C-36 or C-37 results in a major change in macrolactone conformation. The stereochemistry at these centers is determined by module 1 of the amphotericin PKS. Literature evidence suggests that this module uses a (2*S*)-methylmalonyl extender unit (**2a**) and upon condensation with acetyl CoA initially generates a (2*R*)-2-methyl-3-oxoacyl chain (**2b**) [10, 11] that can readily epimerize at C-2. Based on the stereochemistry observed in amphotericin B, the Amph KR1 appears to select the 2-epimerized (2*S*)-2-methyl-3-oxobutanoyl thioester intermediate (**2c**) and to carry out stereospecific antireduction to form the (2*S*,3*S*)-3-hydroxy-2-methylbutanoyl thioester intermediate (**2e**) (Figure 5). 37-*epi*-8-deoxyamphoteronolides would result from KR1 generating the opposite alcohol stereochemistry to form **2f**. 36-*epi*-8-deoxyamphoteronolides would arise from the selection by KR1 of the unepimerized substrate (**2b**), with ketoreduction to form **2d** (Figure 5). The data do not exclude the possibility that antireduction still occurs, but on the unepimerized substrate, to give the bis-epimer (2*R*, 3*R*)-3-hydroxy-2-methylbutanoyl thioester. The literature to date is consistent with all of these possible mechanisms. Complete structural characterization of the products should help to resolve the issue.

epi-Amphoteronolides have not been reported, and their formation suggests that although the remaining extension modules can tolerate an "incorrect" *epi*-diketide and extend it to form the epimerized macrolactone and that the AmphN cytochrome P450 efficiently oxidizes the C-41 methyl to a carboxyl group (vide infra), the mycosaminyltransferase does not efficiently recognize the *epi*-8-deoxyamphoteronolide as substrate. *S. nodosus* ATCC14899 was subsequently found to produce a minor polyene with chromatographic properties identical to those of the putative *epi*-8-deoxyamphoteronolide (B.R., unpublished data). This indicates that these compounds are normally generated at low levels by the amphotericin PKS but were only detected during characterization of 8-deoxyamphoteronolides formed by the *amphDIII* mutant. This is the first known example of a natural (nonchimaeric) polyketide synthase in which a

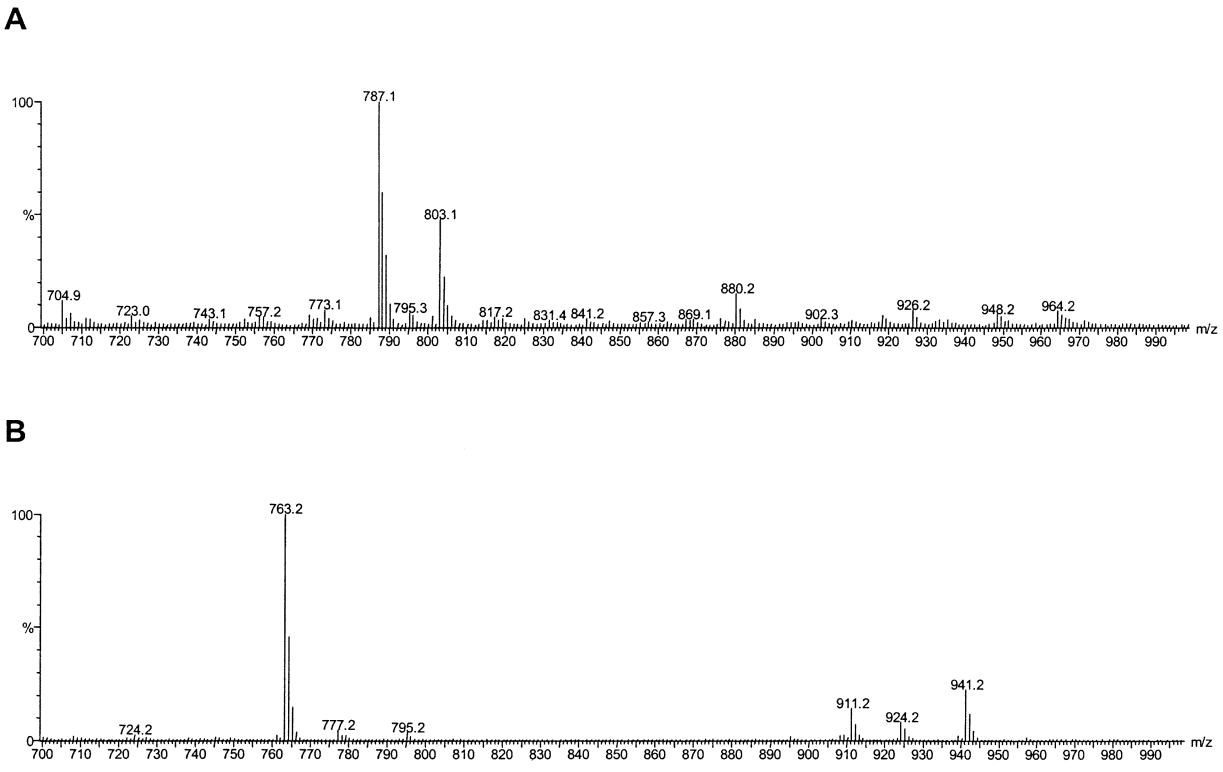


Figure 3. Analysis of Polyenes Produced by *amphDIII* Mutant by Mass Spectrometry

Typical extracts were found to contain 8-deoxyamphoteronolide A (**1f**) when analyzed by ESMS in positive ion mode (A) and negative ion mode (B). A sodium adduct was detected in positive ion mode. A lesser amount of amphoteronolide A ($M + Na^+ = 803$) was detected by positive ion ESMS only. Detection in this mode could be more sensitive for the 8-hydroxylated amphoteronolides if Na^+ coordinates to the 1,2 diol present in these compounds.

lapse in programming fidelity results in production of epimeric macrolactones.

Polyenes were also detected in culture supernatants of *amphDIII* strains, and more polar polyene fractions were obtained when mycelial extracts were chromatographed. These polyenes may represent analogs of amphotericin that are modified with alternative sugars. The absence of the "normal" glycosylation (which would allow rapid export from the cell), resulting in a slow build up of polyenes in the cell, may allow other relatively slow conversions to now occur to the macrolactone. In the absence of GDP-mycosamine, the AmphDI mycosaminyl glycosyl transferase might be capable of using GDP-D-mannose, dTDP-D-glucose, or even dTDP-L-rhamnose as substrate to convert a fraction of the aglycone to polar glycosylated minor products, with mannosyl, glucosyl, or rhamnosyl residues attached at C-19. Indeed, a minor compound with a mass appropriate for hexosyl-amphoteronolide A ($M - H^+ = 941.5$) was routinely detected in extracts from the *amphDIII* mutant (Figure 3B). To investigate this compound further, a polar tetraene fraction was methylated and purified further. Analysis by positive ion ESMS showed a large peak at 979.4, corresponding to the sodium adduct of desmycosaminyl *O*-mannosylamphotericin A methyl ester, or the *O*-glucosyl isomer, with no evidence of the 8-deoxy analogs (data not shown). Presumably, the AmphL cytochrome P450 recognizes the corresponding hexosyl 8-deoxy intermediate and hydroxylates it.

NDP-deoxysugar flexibility in macrolide glycosyltransferases has been documented. An oleandrosyltransferase OleG2 that normally utilizes dTDP-L-oleandrose when introduced into *Saccharopolyspora erythraea* EryBV mutant (lacking dTDP-L-oleandrose) was shown able to complement the missing EryBV mycarosyltransferase activity, but by attaching rhamnose to form 3-L-rhamnosyl-6-deoxyerythromycin B [12, 13]. The dTDP-L-rhamnose is believed to be present in the cytoplasm as a cell wall building block.

As 8-deoxyamphoteronolides may exist in *amphDIII* mutant cells for several days, other adventitious glycosylation processes may very slowly occur during this time, resulting in the glycosylation of other hydroxyl groups. The polyenes nystatin A3 and candidoin each have an additional dideoxyhexose sugar residue attached at the position corresponding to C-35 of amphotericin [14].

The polyenes produced by the *amphDIII* mutant were tested for antifungal activity in agar diffusion assays with *Saccharomyces cerevisiae* as an indicator organism. No inhibition zones were detected when extracts containing 14 μg total tetraene and 6 μg total heptaene per ml were assayed (data not shown). The amphoteronolides and their hexosyl analogs had dramatically reduced activity compared to amphotericins A and B extracted from wild-type *S. nodosus*. This observation is consistent with previous findings that a positive charge on the amino group of mycosamine is essential for significant antifungal activity [5].

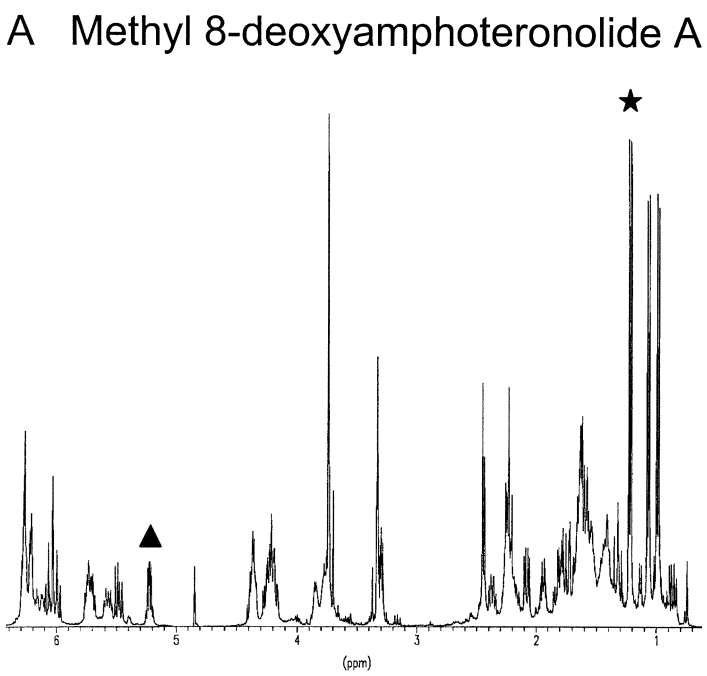


Figure 4. NMR Analysis of Polyenes Produced by the *amphDIII* Mutant

(A) Proton NMR (400 MHz) spectrum of methyl 8-deoxyamphoteronolide A (**1g**) showing the C-37 proton at 5.2 ppm (▲) and the coupled doublet at 1.2 ppm (*).

(B) Proton NMR spectrum of methyl *epi*-8-deoxyamphoteronolide A showing the C-37 proton at 4.0 ppm (▲) and the coupled doublet at 1.2 ppm (*). Residual 8-deoxyamphoteronolide H-37 proton indicated (Δ).

Disruption of the *amphL* Gene

The amphotericin cluster contains two cytochrome P450 genes, *amphL* and *amphN*. The AmphN protein shows a high degree of sequence identity with the NysN, PimG, and CanC proteins that are thought to function in formation of exocyclic carboxyl groups in nystatin, pimaricin, and candicidin [15]. The AmphL protein is homologous to the NysL P450, which is thought to introduce the C-10 hydroxyl group of nystatin [16], and to PimD, which introduces the epoxide in the polyol chain of pimaricin. Aparicio et al. disrupted *pimD* to produce a novel pimaricin, de-epoxypimaricin [17]. These considerations suggest that AmphL functions to introduce the C-8 hydroxyl group of amphotericin.

The *amphL* gene contains two SacI sites flanking the 1053 base pair region between nucleotides 56903 and 57956 of the amphotericin cluster. This internal SacI fragment of *amphL* was cloned between the SacI sites

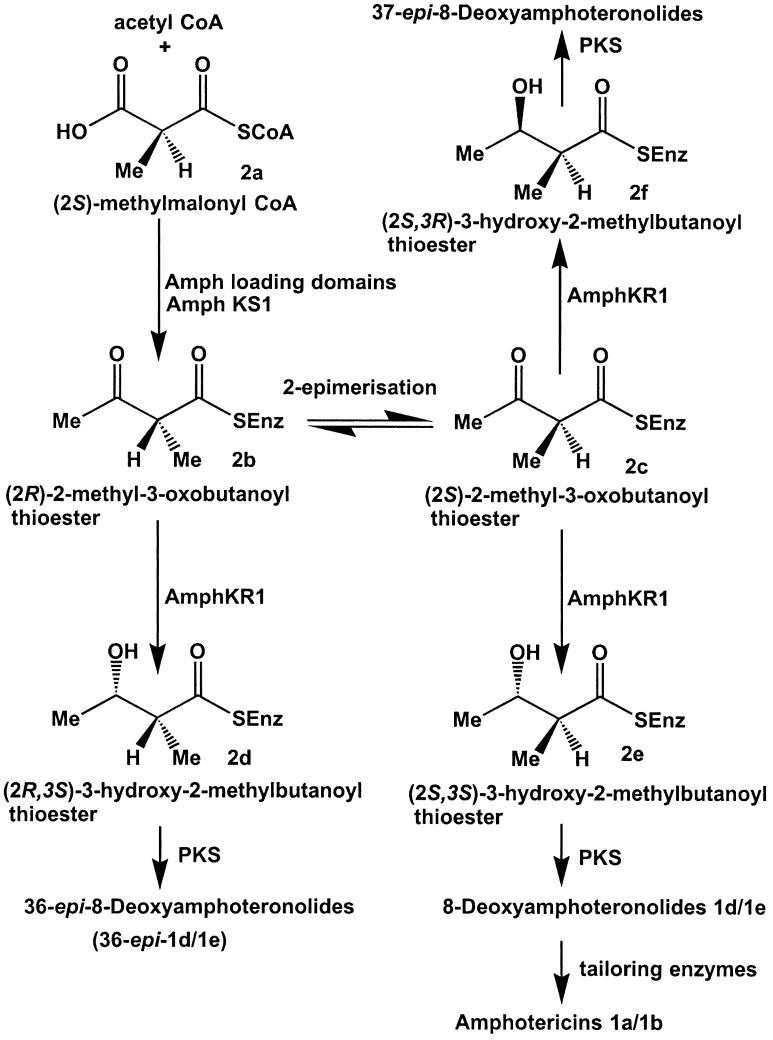


Figure 5. Proposed Routes for Production of *epi*-Amphoteronolides by the Amphotericin PKS

of KC515. Recombinant phage were identified by PCR with oligonucleotide primers specific for the insert. Since the *amphL* gene appears to be located at the end of a transcription unit, insert-directed integration of this phage into the chromosome was not expected to have polar effects on transcription of any other genes. A single crossover event would be sufficient to bring about gene disruption. The recombinant phage was plated on *S. nodosus*, and lysogens were selected by overlaying plates with soft agar containing thiostrepton. A typical lysogen was isolated and genomic DNA was analyzed for the presence of prophage DNA by Southern hybridization and by PCR. Analysis of hybridizing restriction fragments indicated that gene disruption had occurred as anticipated. A typical experiment is shown in Figure 6. Homologous recombination between the phage and chromosomal sequences resulted in loss of the 4.8 kb XhoI fragment that contains *amphL* and appearance of 3.4 and 43 kb junction fragments.

Analysis of Polyenes Made by the *amphL* Mutant

The *amphL* mutant produced heptaene and tetraenes that were found predominantly in the mycelial fraction of the culture. The yields were greatly reduced (2–5 mg/L) compared to amphotericin production by *S. nodosus* ATCC14899 [7] or 8-deoxyamphoteronolide production by the *amphDIII* mutant. The mycelium was extracted with methanol to give multigram quantities of extract, from which partially purified polyenes were obtained by selective precipitation and column chromatography for analysis by electrospray mass spectrometry. Analysis in negative ion mode gave compounds with masses of 906.2 and 920.2 (Figure 7; all compounds show an M:M + 1:M + 2 ratio of ca. 6:3:1 due to isotopic natural abundance). The $M - H^+$ of 906.2 is in good agreement with that expected for 8-deoxyamphotericin B (**1c**) (M, 907.5). The peak with a mass of 920.2 suggests the presence of a homolog with an additional methyl group. Analysis in positive ion mode yielded masses of 930.3 and 932.3, 944.3 and 946.3, consistent with the masses of sodium ion $(M + 23)^+$ adducts of 8-deoxyamphotericins A and B and homologs. NMR analysis of these samples revealed resonances indicating at least two polyenes present in large quantities of glycerides and an unidentified aromatic metabolite. Adequate purification of these compounds was hampered by their low abundance in mycelia and their relative resistance to

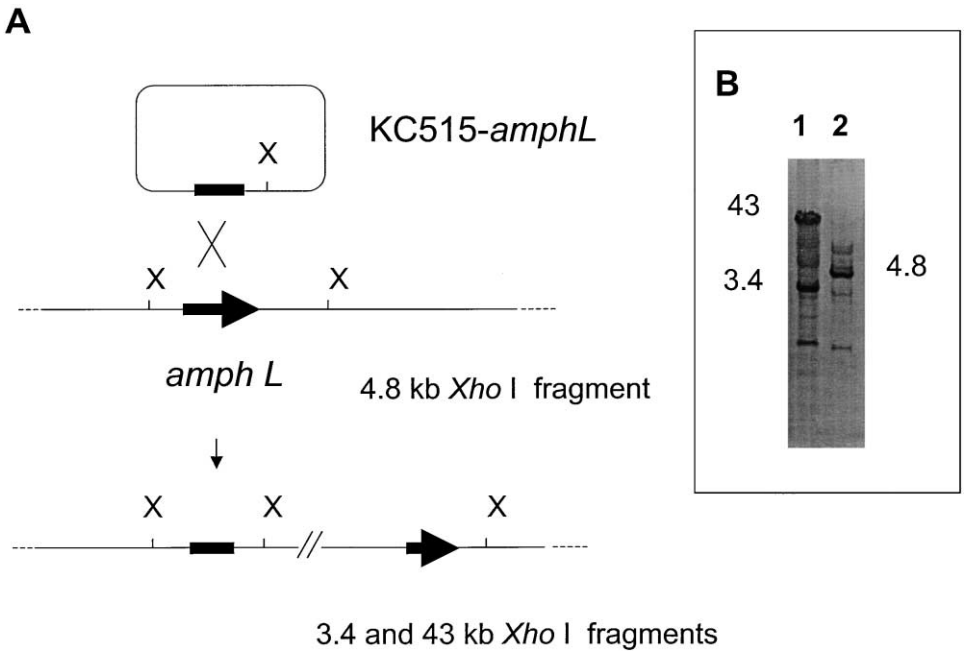


Figure 6. Targeted Disruption of the *amphL* Gene

(A) Schematic diagram showing integration of the KC515 *amphL* phage into the chromosomal *amphL* gene. This results in loss of the 4.3 kb XhoI fragment containing the *amphL* gene and formation of 3.4 and 43 kb fragments containing the 5′ and 3′ regions.
(B) An *amphL* gene probe was used to analyze the XhoI digests of genomic DNA from an *amphL* mutant (lane 1) and *S. nodosus* ATCC14899 (lane 2) by Southern hybridization.

extraction with alcoholic solvents. The deoxyamphotericins that were extracted were contaminated with overwhelming amounts of membrane lipids and other nonpolyenic material. Despite considerable effort, it has not so far been possible to obtain material pure enough for structural characterization by NMR. Therefore, the designation of these compounds as 8-deoxyamphotericins rather than 16-descarboxyl-16-formylamphotericins is based on the homology between AmphL and PimD (vide supra). The presence of methyl homologs of these deoxyamphotericins may be due to the use of propionate as a starter unit; however, as the corresponding homologs were not detected in the *amphDIII* mutant, an alternative explanation is that the 8-deoxyamphotericins undergo *O*-methylation on the sugar residue, possibly as a detoxification procedure. Methyl homologs of amphotericins A and B were not reported in cultures of wild-type *S. nodosus*.

The 8-deoxyamphotericins retained antifungal activity in agar diffusion tests. The inhibition zone diameters were comparable to those of amphotericin B at equivalent total heptaene concentrations (data not shown). A partially purified fraction containing 8-deoxyamphotericin B and its methyl homolog was tested against liquid cultures of *S. cerevisiae*. With this material, a total heptaene concentration of 1.25 μg per ml was sufficient to inhibit growth of the yeast cells. The minimum inhibitory concentration for purified amphotericin B was estimated as 0.3125 μg per ml. These results indicate that 8-deoxyamphotericins have a slightly lower activity than amphotericin B.

Discussion

Manipulation of biosynthetic genes is yielding novel polyene macrolides. Aparicio and coworkers have produced deepoxypimaricin [17] and Zotchev et al. [18] have produced a hexaene derivative of nystatin as well as trace levels of a heptaene analog. This study describes the first amphotericin analogs that have been generated by engineered biosynthesis and provides the first insights into the order in which the late stages of amphotericin biosynthesis occur.

The *amphDIII* mutant produced 8-deoxyamphoteronolides as major products, indicating that C-8 hydroxylation is impaired in the absence of glycosylation. The failure of the AmphL P450 to hydroxylate the 8-deoxyamphoteronolides may result from their reduced solubility or from strict molecular recognition of glycosylated polyene substrates.

The C-16 carboxyl group was present in all the polyenes isolated from the *amphDIII* mutant, with no evidence for the formation of 16-descarboxyl-16-methylamphoteronolides. This indicates that oxidation of the C-16 methyl branch occurs efficiently in the absence of the other two modifications. Thus, it seems likely that 8-deoxyamphoteronolides are normal biosynthetic intermediates that are substrates for glycosylation. Taken together, these results indicate that the late stages of amphotericin biosynthesis can occur in the following order: exocyclic carboxyl group formation, glycosylation with mycosamine, and finally C-8 hydroxylation.

It is important to mention preliminary data that suggests that this sequence of events may not be obligatory

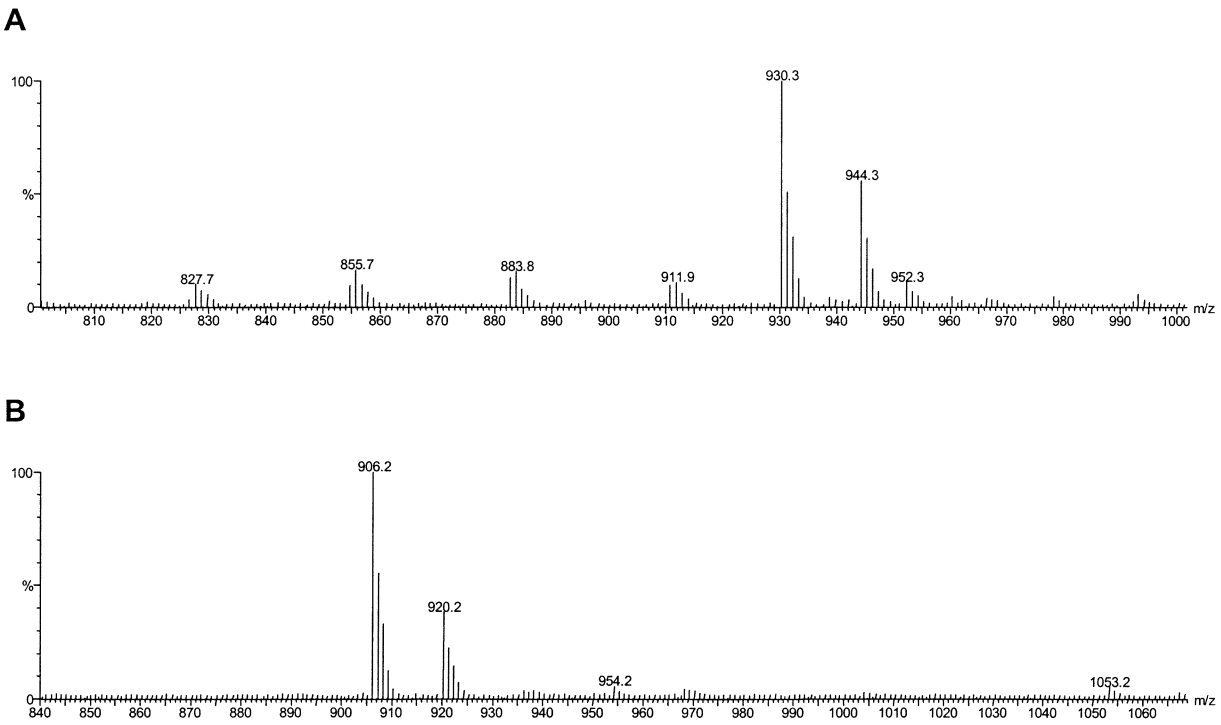


Figure 7. Analysis of Polyenes Produced by the *amphL* Mutant by Mass Spectrometry

Analysis by ESMS in positive ion mode (A) and negative ion mode (B) showing the presence of 8-deoxyamphotericins B **1c** and **1d**. As explained in the text, each of these compounds is represented by three peaks due to isotopic natural abundance. The third of the peaks corresponding to **1c** overlap with the first of the **1d** peaks.

in all circumstances. We have recently disrupted the *amphN* gene after numerous unsuccessful attempts (M.C. and P.C., unpublished data). Although purification and chemical analyses have not yet been carried out, the *amphN* mutant produces low yields of heptaenes and tetraenes that retain antifungal activity. This raises the possibility that glycosylation can occur in the absence of exocyclic carboxyl group formation. Gene disruption studies may not be sufficient for determining whether these two steps occur in a preferred order. On the other hand, this approach promises to yield several amphotericin analogs rather than the few that could be generated from a single linear pathway.

The 8-deoxyamphoteronolides had reduced water solubility and diminished antifungal activity. However, the *amphDIII* mutant could be useful in glycosylation engineering experiments aimed at altering the sugar residue of amphotericin B. A wide range of aminodeoxysugars are derived from dTDP-glucose, and several groups are constructing gene cassettes for their in vivo synthesis in streptomycetes. Similar cassettes can be introduced into *S. nodosus*. In addition, the *amphDI* gene could be subjected to error-prone PCR or DNA shuffling to develop glycosyltransferases that can recognize the polyene aglycone and other dTDP-glucose-derived amino sugars. Successfully redesigned glycosyltransferases could be detected by screening for restored antifungal activity.

The detection of epimeric 8-deoxyamphoteronolides is interesting and suggests that the amphotericin system may be useful for determining how PKS modules determine alkyl stereochemistry in nascent chains.

The *amphL* mutant produced 8-deoxyamphotericins although the yields were significantly lower than the yields of 8-deoxyamphoteronolides from the *amphDIII* mutant. This may reflect regulatory phenomena or feedback mechanisms that are not yet understood. The 8-deoxyamphotericins were less soluble than amphotericin B. However, they retained activity and could be tested as antifungal drugs. Polyenes are also active against *Leishmania* parasites, enveloped viruses, prion proteins [6], and hepatocellular tumors [19]. These additional biological activities appear to result from interactions of the polyene with sterol-rich membranes. Some of the amphotericin analogs could be useful for some or all of these potential medical applications.

Significance

These results show that formation of the exocyclic carboxyl group of amphotericin B can occur prior to glycosylation with mycosamine. The second hydroxylation, at C-8, appears to be inefficient in the absence of prior glycosylation. One possible order of the macrolactone tailoring steps is exocyclic carboxyl group formation, mycosaminylation, and C-8 hydroxylation.

The *amphDIII* mutant produced 8-deoxyamphoteronolides that had little antifungal activity. However, this mutant strain can now be used as a host in engineering experiments to glycoslate the amphoteronolide macrolactone with a wide range of (amino)deoxysugars, any of which may significantly improve the therapeutic properties over those of amphotericin B.

Disruption of the *amphL* gene gave novel deoxyamphotericins that retained good antifungal activity in preliminary tests. These are the first amphotericin analogs that have been produced by engineered biosynthesis. Further work should reveal whether these compounds or their derivatives have any clinical significance as antifungal antibiotics or as antiviral, antiparasitic, or antiprion agents.

Experimental Procedures

Genetic Procedures

A Perkin Elmer GeneAmp 2400 thermocycler was used to carry out PCRs with *Taq* or Vent DNA polymerases, as described [20]. The oligonucleotide primers were synthesized by GenoSys, Cambridge, UK. The oligonucleotides used to amplify the *amphDIII* region were 5′ CCGAGGATCCGCACCAGATGCAAAACGAC 3′ and 5′ TAAACTG CAGGACAGCACGCTGCCGGTGTTG 3′. The oligonucleotides used to detect the *amphL* phage were 5′ GCGGGGATCCTAGCTGAAG CAGCTGCTGCAC 3′ and 5′ CAGGTCGACATGGGTGGCAAC 3′.

Methods for DNA manipulation were as described by [20]. *Escherichia coli* strain XL1-Blue MR was used as a host for propagation of plasmids. Plasmid DNA was isolated using the alkaline-SDS procedure. Restriction enzyme digestions and ligations were carried out using standard procedures. Competent *E. coli* cells for transformation were prepared by the calcium chloride method.

Methods for preparation of streptomycete spores and protoplasts were those described by Hopwood et al. [21]. *Streptomyces lividans* 66 (John Innes strain 1326) was used for propagation of phage KC515 [22]. Phage KC515 DNA was isolated using the small-scale method [21]. DNA fragments ligated to KC515 DNA were introduced into *S. lividans* 66 protoplasts by transfection [21]. Recombinant phage were identified by PCR with primers specific for the cloned inserts.

S. nodosus ATCC 14899 was used as the parent strain for gene disruptions. To obtain lysogens, recombinant phage were plated on *S. nodosus* spores to give near confluent lysis. After overnight incubation, plates were overlayed with soft nutrient agar containing thiostrepton (50 μg/mL). Thiostrepton-resistant lysogens resulting from integration of the phage were streaked on tryptone soya (TS) agar containing thiostrepton. Lysogens were cultured in the absence of thiostrepton then protoplasts were allowed to regenerate. Regenerated protoplasts were toothpicked onto TS agar and TS agar containing thiostrepton to identify thiostrepton-sensitive revertants.

Labeling of probes with digoxigenin-dUTP and detection of hybridizing DNA were carried out using a Boehringer Mannheim DIG DNA labeling and detection kit. For Southern hybridization, DNA fragments were transferred to nylon membranes by capillary transfer [20].

Production and Analysis of Polyenes

For polyene production, *S. nodosus* was grown on fructose-dextrin-soybean medium [7]. Samples of culture (100 μl; containing suspended mycelia) were mixed with volumes of butanol (900 μl) and sonicated for 20 min. The extract was centrifuged, and the supernatant was diluted with methanol. Amphotericin B gives four specific UV absorption peaks at 346, 364, 382, and 405 nm with $\epsilon = 1.7 \times 10^5\ M^{-1}cm^{-1}$ at 405 nm [23]. Amphotericin A absorbs at 280, 292, 305, and 320 nm, with $\epsilon = 0.78 \times 10^5\ M^{-1}cm^{-1}$ at 318 nm. Amphotericin B standard was obtained from Sigma.

Bioassays were carried out with *S. cerevisiae* NCYC 1006 as an indicator organism. A 100 ml volume of molten cooled yeast agar medium (3 g yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, 15 g/L agar) was mixed with approximately 10^6 yeast cells and poured into petri dishes. Test samples were pipetted into wells punched in the solidified agar. Plates were incubated at 30°C for 20 hr and inhibition zones were measured. Alternatively, 1 ml volumes of liquid yeast medium containing varying concentrations of polyenes were each inoculated with 10^3 yeast cells. Tubes were incubated with shaking at 30°C for 20 hr, and growth was assessed by measuring the absorbance (turbidity) at 540 nm.

Chemical Methods

UV spectrophotometry was performed using a Hewlett Packard 8452A diode array spectrophotometer with 1 cm path length. Electrospray mass spectrometry was performed on a Micromass Quattro LC. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker DRX 400 spectrometer. HPLC was performed on a Shimadzu LC-4A with a UV detector.

For preparative scale production and purification of polyenes, a glycerol deep (1 mL) was added to GYE media (100 mL; 10 g/L yeast extract; 10 g/L glucose [pH 7]) and shaken in an orbital incubator shaker (New Brunswick Series 25) at 28°C for 48 hr at 160 rpm. Portions (10 mL) of the resulting growth were each transferred to eight 500 ml production media (fructose, 20 g/L; dextrin, 60 g/L; soybean flour, 30 g/L; $CaCO_3$, 10 g/L) and shaken at 28°C for 72 hr at 160 rpm. The growth was centrifuged (Sorvall RC-5B) and the finely divided mycelial fraction added to methanol (4 L) with occasional shaking for 48 hr. Yields varied from 50 mg/L to 500 mg/L of tetraenes by UV assay with smaller amounts of heptaenes. The supernatant was collected and reduced in volume in vacuo to ca. 200 ml (largely aqueous). Centrifugation yielded a yellow precipitate that was shaken in methanol (80 mL) and the solid removed by centrifugation to yield a clear yellow solution whose polyene content was mainly tetraene (>90%) but still contained gram quantities of nonpolyene material (membrane components) and an unknown yellow metabolite. Addition of excess ethereal diazomethane resulted in ca. 50% of the polyene material present now having an Rf of 0.45 to 0.5 with the yellow impurity at Rf 0.7 and some polyene material remaining on the baseline (CH_2Cl_2:MeOH, 9:1). Purification by flash chromatography (CH_2Cl_2:MeOH, 9:1) gave a pure mixture of tetraenes, which were further purified by normal phase HPLC (MeOH:EtOAc, 1:19), to yield 8-deoxyamphoteronolide A, an epimer, and a third peak containing a mixture of compounds whose RMM corresponded to 8-deoxy- and amphoteronolide A. Similar procedures were used for the growth of the *amphL* mutant, with extraction of the mycelia using methanol or butanol followed by selective precipitation and column chromatography.

Acknowledgments

This work was supported by a grant from the European Union (GENOVA QLRT-1999-00095; to P.C.). P.C. wishes to thank Joseph O'Neill and Terence Union for technical assistance. B.R. and E.G. would like to thank Dr. Graham Eaton for assistance with electrospray mass spectrometry; Dr. Gerry Griffith for assistance with NMR spectroscopy; and Derek Messenger for technical assistance.

Received: July 24, 2003
Revised: September 22, 2003
Accepted: September 23, 2003
Published: December 19, 2003

References

1. Bolard, J. (1986). How do polyene macrolide antibiotics affect cellular membrane properties? Biochim. Biophys. Acta *864*, 257–304.
2. Abu-Salah, K.M. (1996). Amphotericin B: an update. Br. J. Biomed. Sci. *53*, 122–133.
3. Schaffner, C.P. (1984). Polyene macrolides in clinical practice: pharmacology and adverse and other effects. In Macrolide Antibiotics, Chemistry, Biology and Practice, S. Omura. ed. (New York: Academic Press Inc.), pp. 457–507.
4. Georgopapakadou, N.H., and Walsh, T.J. (1996). Antifungal agents: chemotherapeutic targets and immunologic strategies. Antimicrob. Agents Chemother. *40*, 279–291.
5. Cheron, M., Cybulska, B., Mazerski, J., Gryzbowska, J., Czerwinski, A., and Borowski, E. (1988). Quantitative structure-activity relationships in amphotericin B derivatives. Biochem. Pharmacol. *37*, 827–836.
6. Hartsel, S., and Bolard, J. (1996). Amphotericin B: new life for an old drug. Trends Pharmacol. Sci. *17*, 445–449.
7. McNamara, C.M., Box, S., Crawforth, J.M., Hickman, B.S., Nor-

wood, T.J., and Rawlings, B.J. (1998). Biosynthesis of amphotericin B. J. Chem. Soc. [Perkin 1] *1*, 83–87.

8. Caffrey, P., Lynch, S., Flood, E., Finnan, S., and Oliynyk, M. (2001). Amphotericin biosynthesis in *Streptomyces nodosus*: deductions from analysis of polyketide synthase and late genes. Chem. Biol. *8*, 713–723.
9. Nicolaou, K.C., Chakraborty, T.K., Ogawa, Y., Daines, R.A., Simpkins, N.S., and Furst, G.T. (1988). Chemistry of Amphotericin B. Degradation studies and preparation of amphoteronolide B. J. Am. Chem. Soc. *110*, 4660–4672.
10. Weissman, K.J., Timoney, M., Bycroft, M., Grice, P., Hanefeld, U., Staunton, J., and Leadlay, P.F. (1997). The molecular basis of Celmer's rules: the stereochemistry of the condensation step in chain extension on the erythromycin polyketide synthase. Biochemistry *36*, 13849–13855.
11. Staunton, J., and Weissman, K.J. (2001). Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. *18*, 380–416.
12. Doumith, M., Legrand, R., Lang, C., Salas, J.A., and Raynal, M.-C. (1999). Interspecies complementation in *Saccharopolyspora erythraea*: elucidation of the function of *oleP1*, *oleG1*, and *oleG2* from the oleandomycin biosynthetic gene cluster of *Streptomyces antibioticus* and generation of new erythromycin derivatives. Mol. Microbiol. *34*, 1039–1048.
13. Rawlings, B.J. (2001). Type 1 polyketide biosynthesis in bacteria (Part B). Nat. Prod. Rep. *18*, 231–281.
14. Omura, S., and Tanaka, H. (1986). Production, structure and antifungal activity of polyene macrolides. In Macrolide Antibiotics, Chemistry, Biology and Practice, S. Omura, ed. (New York: Academic Press Inc.), pp. 351–404.
15. Brautaset, T., Sekurova, O.N., Sletta, H., Ellingsen, T.E., Strom, A.R., Valla, S., and Zotchev, S.B. (2000). Biosynthesis of the polyene antibiotic nystatin in *Streptomyces noursei* ATCC11455: analysis of the gene cluster and deduction of the biosynthetic pathway. Chem. Biol. *7*, 395–403.
16. Aparicio, J.F., Caffrey, P., Gil, J.A., and Zotchev, S.B. (2003). Polyene antibiotic biosynthesis gene clusters. Appl. Microbiol. Biotechnol. *61*, 179–188.
17. Mendes, M.V., Recio, E., Fouces, R., Luiten, R., Martin, J.F., and Aparicio, J.F. (2001). Engineered biosynthesis of novel polyenes: a pimaricin derivative produced by targeted gene disruption in *Streptomyces natalensis*. Chem. Biol. *8*, 635–644.
18. Brautaset, T., Bruheim, P., Sletta, H., Hagen, L., Ellingsen, T.E., Strom, A.R., Valla, S., and Zotchev, S.B. (2002). Hexaene derivatives of nystatin produced as a result of an induced rearrangement within the *nysC* polyketide synthase gene in *S. noursei* ATCC 11455. Chem. Biol. *9*, 367–373.
19. Charbonneau, C., Fournier, I., Dufresne, S., Barwicz, J., and Tancrede, P. (2001). The interactions of amphotericin B with various sterols in relation to its possible use in anticancer therapy. Biophys. Chem. *91*, 125–133.
20. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
21. Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M., and Schrempf, H. (1985). Genetic Manipulation of Streptomyces: A Laboratory Manual (Norwich, UK: John Innes Foundation).
22. Rodicio, M.R., Bruton, C.J., and Chater, K.F. (1985). New derivatives of the *Streptomyces* temperate phage ϕC31 useful for the cloning and functional analysis of *Streptomyces* DNA. Gene *34*, 283–292.
23. Kawabata, M., Onda, M., and Mita, T. (2001). Effect of aggregation of amphotericin B on lysophosphatidylcholine micelles as related to its complex formation with cholesterol or ergosterol. J. Biochem. *129*, 725–732.