Title	Biosynthetic engineering of polyene macrolides for generation of improved antifungal and antiparasitic agents
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Publication date	2008
Publication information	Current Topics in Medicinal Chemistry, 8 (8): 639-653
Publisher	Bentham Science Publishers
Item record/more information	http://hdl.handle.net/10197/8333
Publisher's version (DOI)	http://dx.doi.org/10.2174/156802608784221479

Biosynthetic engineering of polyene macrolides towards generation of improved antifungal and antiparasitic agents

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Abbreviations: ACP, acyl carrier protein; AmB, amphotericin B; AT, acyl transferase; DH, dehydratase; ER, enoyl reductase; ESMS, electrospray mass spectrometry; KR, ketoreductase; LAL, large ATP-binding regulator of the LuxR family; MFAME, N-methyl-N-D-fructosyl amphotericin B methyl ester; NMR, nuclear magnetic resonance; PI, pimaricin inducer; PKS, polyketide synthase; RT-PCR, reverse transcriptase-polymerase chain reaction; SARP, *Streptomyces* antibiotic regulatory protein; TE, thioesterase.

Abstract

Polyene macrolides are potent antifungal agents that are also active against parasites, enveloped viruses and prion diseases. They are medically important as antifungal antibiotics but their therapeutic use is limited by serious side effects. In recent years there has been considerable progress in genetic analysis and manipulation of the streptomycetes that produce nystatin, amphotericin B, candicidin, pimaricin and rimocidin/CE-108-related polyenes. This has led to engineered biosynthesis of several new polyenes that are not easily obtained as semi-synthetic derivatives. This review summarises recent advances made since the subject was last reviewed in 2003.

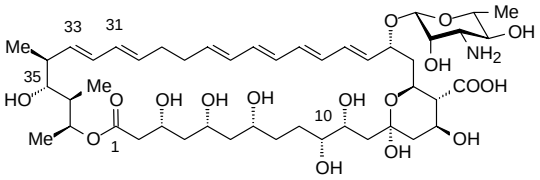
Polyene biosynthesis generally involves assembly and cyclisation of a polyketide chain, followed by oxidative modifications and glycosylation of the macrolactone ring. New derivatives have been obtained by engineering both early and late stages of polyene biosynthetic pathways. These compounds have allowed more detailed investigations of structure-activity relationships and some are likely to show improvements in therapeutic index. The biosynthetic approach is already yielding sufficient material for testing the toxicity and activity of new compounds, thus opening possibilities for discovery of leads for development of effective and safe antifungal and antiparasitic agents.

Keywords: Polyenes, polyketides, engineered biosynthesis, antifungals, antiparasitic drugs.

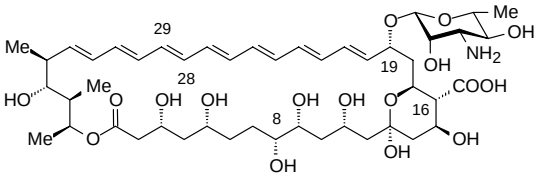
INTRODUCTION

Polyene macrolides are antifungal agents that are abundantly produced by streptomycetes and some other soil bacteria [1]. Polyenes generally consist of macrolactone cores with between three and eight conjugated double bonds. The macrolactone also incorporates a six-membered hemiketal ring with a methyl branch that is oxidized to a carboxyl group. Typically there is a single aminodeoxyhexose sugar residue, mycosamine, attached to the ring through a glycosidic bond [1]. A long-standing model for the mode of action proposes that polyene and sterol molecules interact to form a transmembrane channel that pierces the wall of the target cell [2]. Most polyenes are selectively toxic towards fungal cells because they have a high affinity for ergosterol, the predominant sterol in fungal membranes [3]. Polyenes interact to a lesser extent with cholesterol in mammalian membranes. These interactions are thought to cause toxic side effects when polyenes are used as antibiotics. Transmembrane channel formation may not account for all of the biological activities of polyenes, however. The conjugated double bonds in polyenes are susceptible to auto-oxidation and form radical intermediates that may damage cells [4]. In addition to being efficient antifungal antibiotics, polyenes are also active against enveloped viruses, parasites and prion diseases [5].

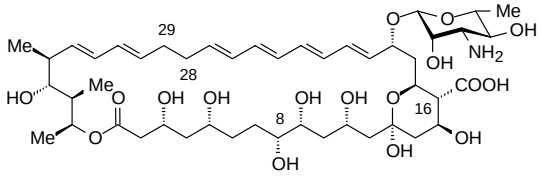
Amphotericin B (AmB) (**2**, Fig. 1) has been the leading antifungal drug for many years, and is still being used in clinical practice [6]. Several advantages of this drug attracted the attention of many laboratories in attempts for developing new derivatives: very potent antifungal activity, wide spectrum, and extremely low incidence of resistance development. On the other hand, there are several undesirable side effects associated with administration of AmB, such as nephrotoxicity. Due to these side effects, the clinical use of AmB is limited to those cases in which other therapies have failed.



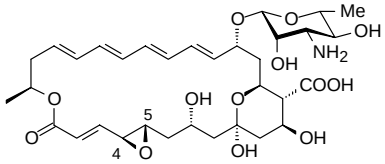
1 Nystatin A1



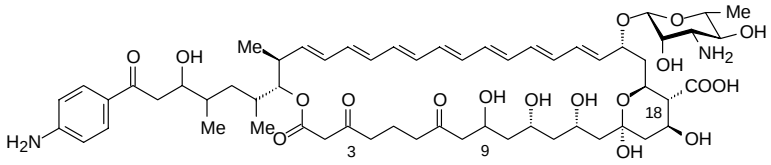
2 Amphotericin B



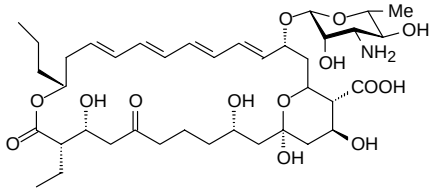
3 Amphotericin A



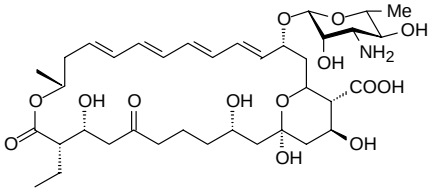
4 Pimaricin



5 Candicidin



6 Rimocidin



7 CE-108

Fig. 1 Structures of polyenes. Important ring atoms are numbered.

The toxicity of polyene antibiotics can be reduced by liposomal formulation or by chemical modification. New lipid formulations with much lower toxicity and better pharmacokinetic profile have been introduced for clinical use [6], nevertheless, these formulations remain very expensive for regular treatments. Some semi-synthetic derivatives (see below) have very low toxicity but their production is not economically favourable.

The continuous need for new antibiotics has intensified interest in genetic manipulation of natural product biosynthesis in streptomycetes. After the discovery of the modular nature of complex polyketide synthases (PKSs) [7], several groups initiated genetic studies on polyene producers with a view to generating improved polyene-based drugs by engineered biosynthesis. The initial work on the biosynthetic gene clusters for nystatin (**1**), amphotericin (**2**, **3**), pimaricin (**4**) and candicidin (**5**) (Fig. 1) was reviewed in 2003 [8]. Since then, Deng and co-workers have characterised the complete biosynthetic gene cluster for the aromatic heptaene FR008 and showed that it is identical to candicidin [9]. Malpartida's group characterized the cluster for the related tetraenes rimocidin (**6**) and CE108 (**7**) (Fig. 1) [10]. A biosynthetic gene cluster for the pentaene filipin was identified in the genome sequence of *Streptomyces avermitilis* [11]. Specialized reviews on pimaricin and nystatin biosynthesis have been contributed by Aparicio et al. [12] and by Fjaervik and Zotchev [13]. In general, polyene biosynthetic gene clusters encode modular PKS proteins that assemble the macrolactone cores, cytochrome P450 enzymes that catalyse exocyclic carboxyl group formation and hydroxylation/epoxidation, and enzymes for biosynthesis and attachment of mycosamine. Genes involved in export and regulation have also been identified. Since the initial characterization of these clusters, there have been significant advances in the analysis of the functions of these genes. Engineered biosynthesis has yielded several new polyene analogues that would not have been easily obtained by chemical synthesis. This review summarises recent progress with each of these polyenes. To assist the reader, the functions of the various polyene biosynthetic genes are summarized in Table 1. Figure 2 gives an overview of the biosynthesis of a typical polyene, pimaricin.

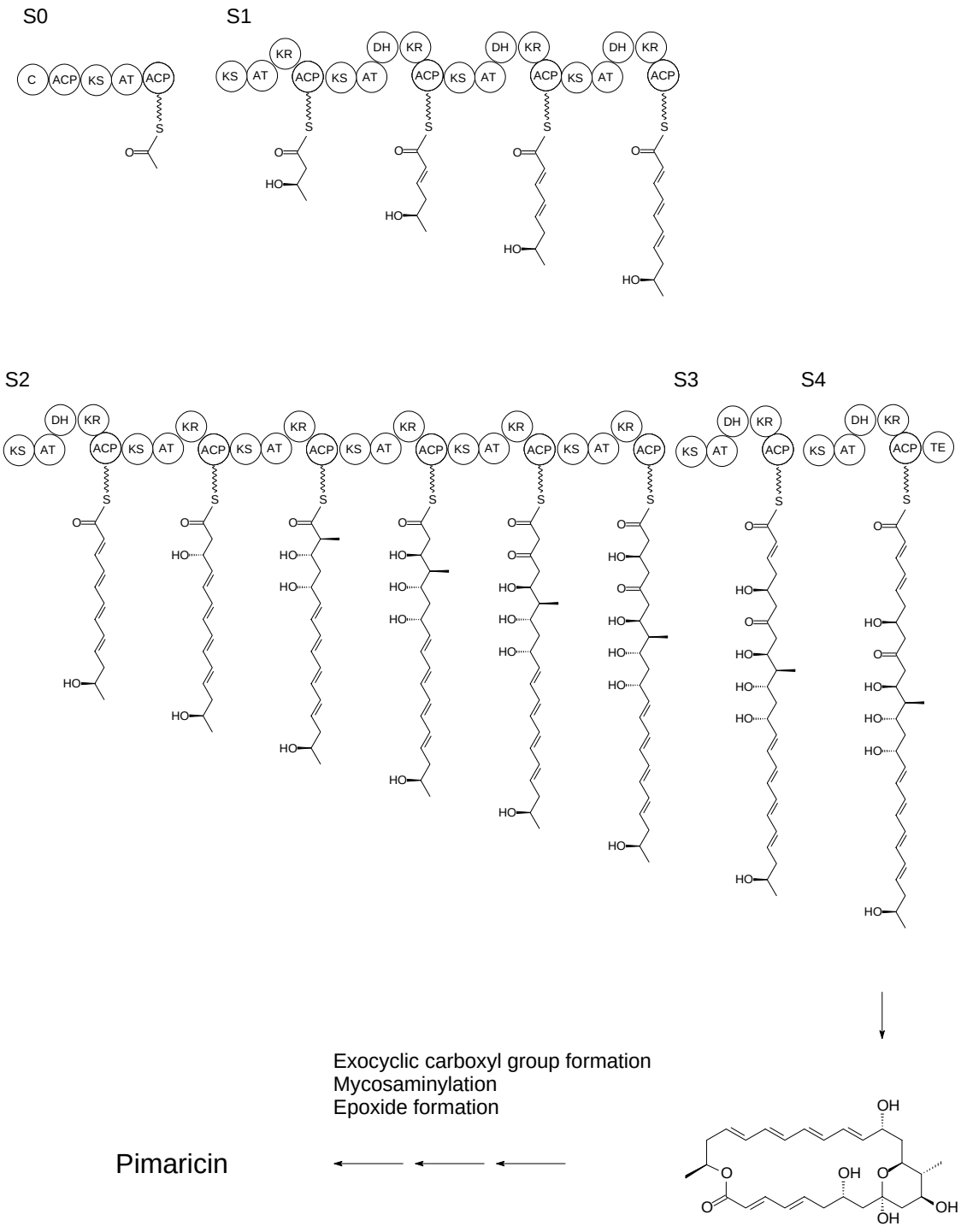


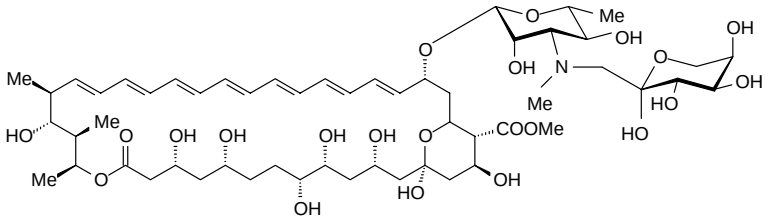
Fig. 2. Biosynthesis of pimaricin, a typical polyene. A PKS loading module S_0 activates an acetyl starter unit and four modular PKS proteins (S1 to S4) catalyse assembly of the polyketide precursor from one methylmalonyl and eleven malonyl extender units. The initial macrolactone product is converted to the final antibiotic in three steps.

Table 1. Functions of proteins encoded by polyene biosynthetic gene clusters. *Nys*, nystatin; *Amph*, amphotericin; *Pim*, pimaricin; *Rim*, rimocidin; *Can*, candicidin; *Fsc*, FR-008; *Pte*, filipin. (-) denotes an expected protein but not yet found.

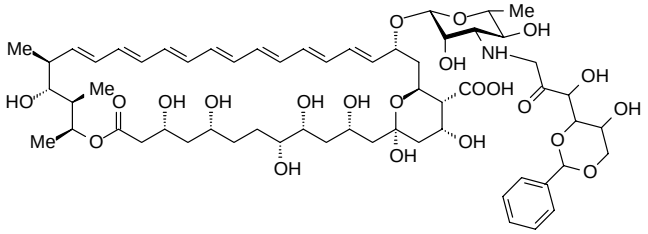
Function	Protein						
	Nys	*Amph*	*Pim*	*Rim*	*Can*	*Fsc*	*Pte*
Modular PKS proteins	A	A	S0	A	P1	A	A1
Acting order in the formation of the aglycones ↓	B	B	S1	-	P2	B	A2
	C	C	S2	-	P3	C	A3
	I	I	S3	-	PF	D	A4
	J	J	S4	-	-	E	A5
	K	K			-	F	
GDP-mannose 4,6-dehydratase	DIII	DIII	J	-	M	MIII	
GDP-ketosugar aminotransferase	DII	DII	C	F	A	MII	
Glycosyltransferase	DI	DI	K	E	G	MI	
Cytochrome P450 monooxygenase	L	L	D				C
	N	N	G	G	C	P	D
Ferredoxin	M	M	F	H	F	FE	E
Discrete Thioesterase	E	E	I	-	T	TE	H
ABC transporter	G	G	A		RA	TI	
	H	H	B		RB	TII	
Efflux pump			H				
p-amino benzoic acid synthase					pabAB	pabAB	
p-amino benzoic acid lyase		Orf2			-	pabC	
Crotonyl CoA-reductase				J			
Phosphopantetheine transferase	F						
Transcriptional regulation (LAL)	RI	RI			Orf1	RIV	
(LuxR)	RII	RII			Orf2	RIII	
(LAL)	RIII	RIII			Orf3	RII	
(PAS)	RIV	RIV	M			RI	F
(SARP-LAL)			R				R
Cholesterol oxidase			E	D			G
FAD-dependent monooxygenase						O	

GENERATING NOVEL POLYENES BY ENGINEERED BIOSYNTHESIS

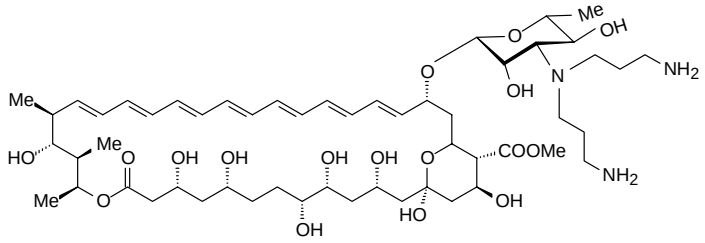
Over the past 40 years, chemists have undertaken chemical modification of polyene antibiotics with the aim of reducing toxicity, and most of such work has focused on AmB. These studies have shown that a positive charge on the amino group of mycosamine is important for antifungal activity, whereas suppression of charge on the exocyclic carboxyl group reduces toxicity [14]. The AmB analogues N-methyl-N-D-fructosyl amphotericin B methyl ester (MFAME) (**8**) [15] and MS8209 (**9**) [16] (Fig. 3) show dramatic improvements in terms of toxicity, but are extremely expensive to produce.



8 MFAME (*N*-methyl-*N*-D-fructosyl amphotericin B methyl ester)



9 MS8209



10 Bis(aminopropylene) amphotericin B methyl ester

Fig. 3. Semi-synthetic derivatives of Am B

AmB-arabinogalactan conjugates have improved water-solubility and reduced toxicity, while retaining antifungal activity [17]. Carreira and co-workers have synthesized bis(aminopropylene) AmB derivatives (**10**) with reduced toxicity and increased antifungal activity, as well as conjugates that are yielding important insights into the precise mode of action of polyene antibiotics [18]. These studies show that structural modification of polyene antibiotics can significantly improve their pharmacological properties. However, it has so far not been possible to develop a semi-synthetic polyene that is economically viable.

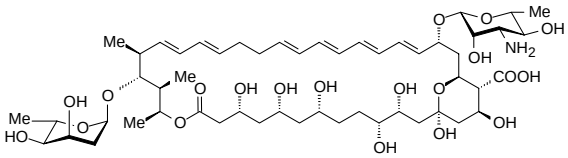
Although total synthesis of AmB has been achieved by two research groups [19, 20] most chemical manipulations use the natural product isolated from cultures of the producer organism, *Streptomyces nodosus*, as starting material. Engineered biosynthesis can generate analogues that are impossible to make by chemical modification, and it allows these compounds to be obtained as primary fermentation products. Previous reviews have discussed in detail the genes for polyene PKSs, the genes involved in biosynthesis of mycosamine from GDP-mannose, and genes for formation of exocyclic carboxyl groups and polyene hydroxylation [9, 12, 13]. Generally, engineered biosynthesis involves manipulation of cloned polyene biosynthetic genes, and introduction of the modified DNA into the producing organism to allow gene replacement by homologous recombination with the chromosome. Phage transduction, conjugation and protoplast transformation are standard methods for introduction of DNA into streptomycetes. These methods have been applied successfully to polyene producers, although progress has been delayed because these methods are time-consuming. However, once a genetically modified streptomycete has been obtained, it is a sustainable source of a novel compound. Heterologous expression and "recombineering" methods have been applied extensively to smaller biosynthetic gene clusters [21] and could be useful for exploiting polyene biosynthesis more fully.

Although a number of recombinant bacteria have been designed to synthesise potentially useful new analogues, in many cases the yields of the products were greatly diminished. Further work on regulation of polyene biosynthesis should lead to yield improvements. Studies on regulatory genes are currently most advanced with the pimaricin and nystatin clusters.

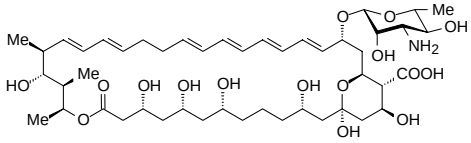
The remaining sections describe recent progress in engineered biosynthesis of derivatives of nystatin, amphotericin B, FR008/candicidin, pimaricin and rimocidin and CE-108.

NYSTATIN

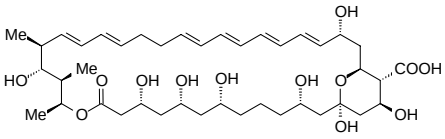
Streptomyces noursei ATCC 11455 is known to synthesise a complex mixture of polyenes that are structurally related to nystatin A_1 (**1**), the compound that is used as an antibiotic to treat superficial fungal infections [1]. Nystatins A_1, A_2 and A_3 are major components of this complex. Nystatin A_3 (**11**, Fig. 4) has an additional dideoxyhexosyl sugar residue (digitoxose) attached to the hydroxyl group at C-35. The structure of nystatin A_2 has not been determined. Bruheim and collaborators re-examined the nystatin complex and separated seven different polyenes from cultures of *S. noursei* ATCC 11455 [22]. Six of these compounds were identified as nystatin A_1 (**1**), nystatin A_3 (**11**), 10-deoxynystatin A_1 (**12**), 10-deoxynystatin aglycone (**13**), nystatin 1070 (**14**), nystatin 1053 (**15**) (=10-deoxynystatin 1070) (Fig. 4). NMR spectroscopy showed that nystatin 1070 (**14**) has mycarose at C-35 where nystatin A_3 (**11**) has digitoxose (mycarose is derived from digitoxose by C-methylation at C3″). The heptaene analogue of nystatin A_1 (**16**) was also found as a minor component. This analogue could result from skipping of the enoyl reduction step in cycle 5 of the polyketide chain extension. The amphotericin producer *Streptomyces nodosus* also produces a mixture of the tetraene amphotericin A (**3**) and the heptaene AmB (**2**) (Fig. 1).



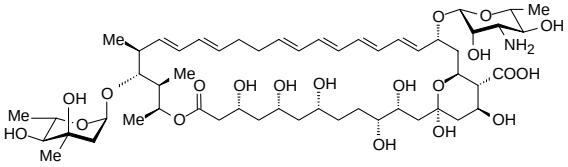
11 Nystatin A3



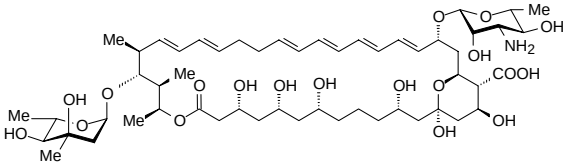
12 10-Deoxynystatin A1



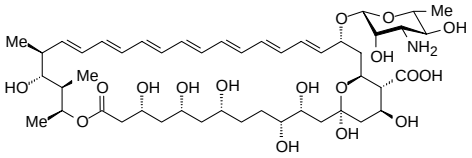
13 10-Deoxynystatin A1 aglycone



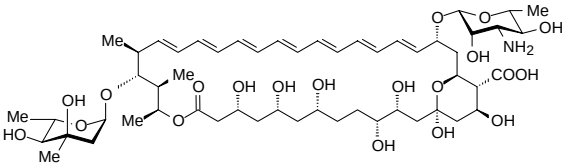
14 Nystatin 1070



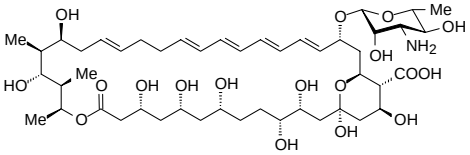
15 Nystatin 1053



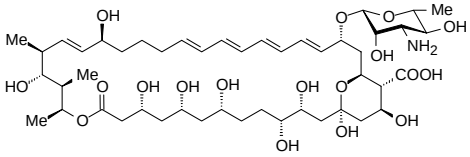
16 Nystatin A1 heptaene (S44HP)



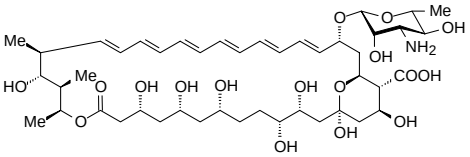
17 Nystatin 1068



18 33-hydroxy nystatin A1



19 31-hydroxy nystatin A1



20 Nystatin hexaene

Fig. 4. Nystatin derivatives produced by *S. noursei* ATCC 11455 and genetically engineered strains.

Studies on ER5 of the nystatin PKS

A targeted deletion of the ER5 domain of the nystatin PKS yielded mutant strain ERD44 that produces the S44HP heptaene (**16**), but not nystatin A_1 tetraene (**1**) (Fig. 1) [23]. This mutant also produced nystatin 1068 (**17**), the heptaene analogue of nystatin 1070 (**14**), the 10-deoxy analogue of 1068, and octaenes with molecular weights of 950 Da and 1094.6 Da [23]. The synthesis of the two latter analogues indicates that the NysC PKS protein with eliminated ER domain is capable of stuttering, a process in which a single module carries out two cycles of chain extension rather than one [24].

The ERD44 mutant has a sizeable deletion in the ER5 coding sequence [23]. The yields of the S44HP heptaene analogue were quite low, possibly because the large deletion had a negative effect on the PKS overall structure. Site-directed mutagenesis and gene replacement were used to introduce single amino acid residue changes into the ER5 domain [25]. Gly-5073 and Gly5074 of the NADPH-binding site were mutated to Ser and Pro, respectively. Identical changes have earlier been shown to efficiently inactivate ER4 domain of the erythromycin PKS [26]. The resulting GG→SP mutant of *S. noursei* produced the S44HP analogue at a relatively high level.

Chemical mutagenesis of *S. nodosus,* which normally produces a mixture of tetraene AmA (**3**) and heptaene AmB (**2**) (Fig. 1), resulted in a mutant that produced AmB exclusively [25]. The amphotericin PKS ER5 coding region from this mutant was amplified by PCR, sequenced, and found to contain a mutation that resulted in substitution of Ser4967 with Asn. A similar Ser→Asn substitution was introduced into the ER5 domain of the nystatin PKS, and the resulting mutant was shown to produce a mixture of heptaene (**16**) and tetraene (**1**) nystatins [25].

When *S. nodosus* is grown under conditions of glucose limitation, the proportion of heptaene increases from 37% to 63% of the total polyene produced. These conditions reduce the flow of glucose through the pentose phosphate pathway, thus limiting the availability of NADPH [25]. This suggests that ER5 of the amphotericin PKS has a low affinity for NADPH and that a low intracellular level of NADPH effectively inactivates the domain.

Mutagenesis of the loading module of the nystatin PKS

The loading module for the nystatin PKS has the domain composition KS_0-AT_0-DH_0-ACP_0. The amino acid sequence of the AT_0 domain suggested that it is specific for malonyl CoA. KS_0 was suggested to have a decarboxylase activity because it has a Ser residue at position 170 instead of the active site Cys residue found in C-C bond-forming domains [27]. Site-directed mutagenesis and domain exchange were used to investigate the functions of the loading module *in vivo* [28]. Changing Ser170 of KS_0 to Cys, Gln or Gly had no effect on nystatin biosynthesis whereas replacement of the malonyl specific AT_0 domain with the methylmalonyl-specific AT_1 resulted in no nystatin production. The malonyl-specific AT_3 domain could substitute for AT_0, indicating that malonyl groups can be loaded and decarboxylated to provide acetyl starter units. A fortuitous Ser413→Asn mutation in the KS_0 domain was found to abolish nystatin biosynthesis in a strain containing a KS_0-AT_3-DH_0-ACP_0 loading module. This indicates that a functional decarboxylase is essential when NysA loads malonyl groups. The Ser413→Asn mutation, however, did not abolish nystatin production in the wild-type NysA (KS_0S413N-AT_0-DH_0-ACP_0). This indicates that the decarboxylase activity is not absolutely essential for nystatin biosynthesis in a wild type strain and that AT_0 is capable of loading acetyl groups. These results indicate that the AT_0 domains of polyene PKS loading modules can use either acetyl CoA or malonyl CoA as a source of starter acyl units. Extensive modification of the AT_0 domain of NysA by means of both site-specific mutagenesis

and domain swapping aimed at changing the specificity of NysA towards methylmalonyl CoA has been attempted (Zotchev and Malpartida, unpublished results). However, none of the mutants was able to prime the nystatin biosynthesis with the propionyl starter, suggesting that the first extender module of nystatin PKS has strict specificity for the acetyl starter.

Engineered biosynthesis of hydroxynystatin analogues

Inactivation of DH3 and DH4 of the nystatin PKS resulted in production of 33-hydroxynystatin A_1 (**18**) and 31-hydroxynystatin A_1 (**19**), respectively (Fig. 4) [29]. These analogues were 2000 times more water-soluble than nystatin A_1 but had no antifungal activity. Zotchev and Lancelin suggest that a prerequisite for antifungal activity is that the polyene and polyol chains are of approximately equivalent length. Some reduction in activity was also observed with a hexaene derivative of nystatin (**20**), obtained by deletion of a module from the NysC PKS protein [23].

Analysis of the late steps in nystatin biosynthesis

Inactivation of *nysL* gene encoding P450 monooxygenase resulted in formation of 10-deoxynystatin A_1 (**12**) [30]. Interestingly, the same compound was produced when the *nysG* and *nysH* ABC transporters were inactivated in *S. noursei* [31]. This indicates that C-10 hydroxylation is coupled to export through NysG-NysH. The Zotchev group succeeded in overproducing the NysL P450 in *E. coli*. The recombinant protein was obtained in a soluble form, and its C-10 hydroxylating activity was demonstrated in an *in vitro* system with 10-deoxynystatin as a substrate [30].

Antifungal activities of nystatin analogues

The antifungal activities of the various nystatin analogues were found to be as follows: Nystatin heptaene S44HP (**16**) > octaene > mycarosyl nystatin heptaene 1068 (**17**) > nystatin A1 (**11**) > mycarosyl nystatin tetraene 1070 (**14**) [22]. The general picture is that heptaenes have a more potent antifungal activity than tetraenes. Interestingly, the diglycosylated nystatins had good antifungal activity. This would not have been predicted because the de Kruiff model [2] suggests that hydrophilic sugar residues would impair transmembrane channel formation.

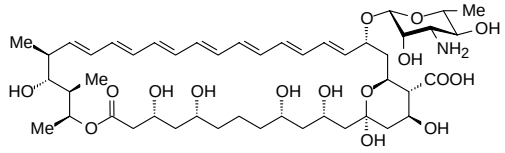
AMPHOTERICIN

The first amphotericin analogue produced by engineered biosynthesis was 8-deoxyamphotericin B (**21**) (Fig. 5), generated through insertional inactivation of the *amphL* cytochrome P450 gene [33]. Inactivation of *amphDIII* for GDP-mannose 4, 6 dehydratase resulted in formation of the aglycone 8-deoxyamphoteronolide B (**22**) (Fig. 5) [32]. The identification of these products indicated that the exocyclic carboxyl group formation can precede glycosylation and C-8 hydroxylation. The production of 8-deoxy amphotericin B indicates that glycosylation can proceed in the absence of C-8 hydroxylation.

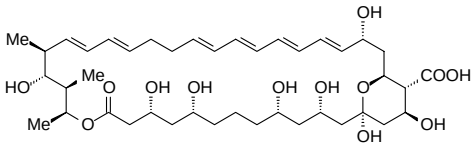
The *amphDIII* mutant produced several different polyene aglycones. Analysis of minor products by NMR spectroscopy indicated that the amphotericin PKS makes stereochemical mistakes during cycles one and two of polyketide chain assembly [32].

Most polyenes undergo a unique late modification that consists of oxidation of a methyl branch to a carboxyl group. The *amphN* gene was targeted because amphotericins lacking exocyclic carboxyl groups were predicted to be analogous to AmB methyl ester, a less toxic semi-synthetic analogue. Numerous initial attempts to inactivate the *amphN* gene were unsuccessful. The *amphNM* region was eventually deleted from the chromosome of *S. nodosus*, and the resulting mutant was shown to produce the heptaene 16-descarboxyl-16-

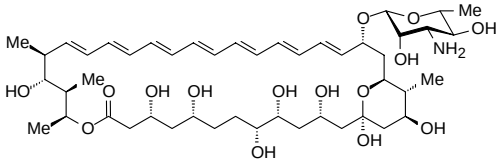
methyl amphotericin B (**23**) and the tetraene 8-deoxy-16-descarboxyl-16-methyl amphotericin A (**24**) (Fig. 5) [33]. This indicated that glycosylation with mycosamine proceeded normally even though the methyl branch attached to C-16 was not oxidized to a carboxyl group, implying that the AmphDI glycosyl transferase has some flexibility towards its aglycone substrate. The C-8 hydroxylation appears to proceed normally with the heptaene but does not occur with the tetraene. It was proposed that the AmphM ferredoxin normally co-operates with the C-8 hydroxylase AmphL. In the absence of AmphM, AmphL may interact with a different ferredoxin to form a complex that recognizes the heptaene but not the tetraene [33].



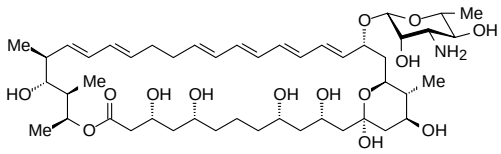
21 8-deoxyamphotericin B



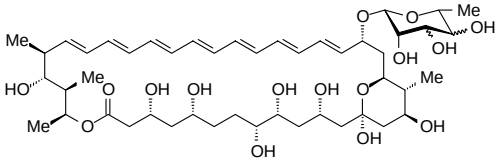
22 8-Deoxy-amphoteronolide A



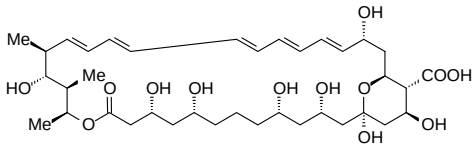
23 16-Descarboxyl-16-methyl-amphotericin B



24 8-Deoxy-16-descarboxyl-16-methyl-amphotericin A



25 16-Descarboxyl-16-methyl-19-*O*-desmycosaminyl-19-*O*-deoxyhexosyl-amphotericin B



26 Pentaene

Fig. 5. Amphotericin derivatives produced by genetically engineered strains of *S. nodosus*.

Another deletion mutant had lost the *amphNM* region and also the *amphDII* mycosamine synthase gene. This mutant produced a 16-descarboxyl-16-methyl-19-O-

desmycosaminyl-19-O-deoxyhexosyl amphotericin B (**25**) [33]. The sugar is expected to be rhamnose, and NMR analysis is currently in progress to confirm this (B. Rawlings, personal communication). An *amphDII* mutant would be expected to synthesise a ketosugar, but *S. nodosus* apparently has a ketoreductase that reduces the ketone group to an alcohol. These results suggest that AmphDI glycosyltransferase has some sugar-flexibility as well. This analogue was shown to have low antifungal activity.

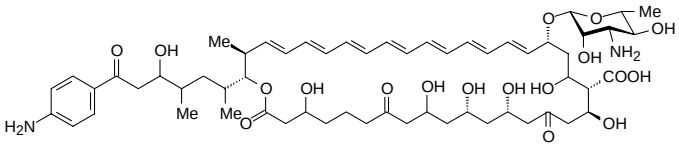
The first successful manipulation of the amphotericin PKS was the deletion of two modules from the AmphC protein, resulting in a mutant producing a pentaene (**26**) in good yield (Fig. 5) [34]. The latter pentaene was water-soluble, but had no antifungal activity. Recent ES-MS data indicate that while the exocyclic carboxyl group is formed on the pentaene, it is neither glycosylated nor C-8 hydroxylated (P. Caffrey, unpublished results).

The toxicity of amphotericin analogues was assessed by means of haemolytic assays. The 16-methyl-16-descarboxyl analogues **23**, **24** and **25** (Fig. 5) had lower haemolytic activities than AmB, while **23** and **24** retained antifungal activity. Thus, the properties of these new compounds appear to be comparable to those of AmB methyl ester.

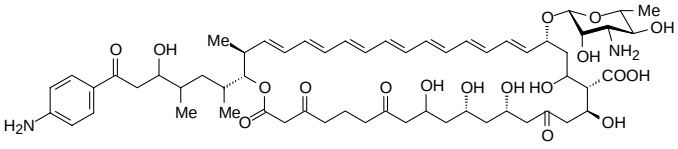
FR008 / CANDICIDIN

Deng and co-workers have investigated biosynthesis of the aromatic heptaene FR008, produced by *Streptomyces* sp. FR008 [9]. The organism produces a complex of four polyenes, FR008-I (**27**), FR008-II (**28**), FR008-III (**29**) and FR008-IV (**30**) (Fig. 6). Of these, only FR008-III had a hemiketal ring. FR008-I has a hydroxyl group at C-3 whereas the remaining three FR008-II, FR008-III and FR008-IV have a ketone group, indicating that the ketoreduction step may be omitted in the last cycle of polyketide chain extension during biosynthesis of the latter polyene. FR008-IV differs from the other three in having a methylene group at C-9 rather than a hydroxyl group, suggesting that both dehydration and

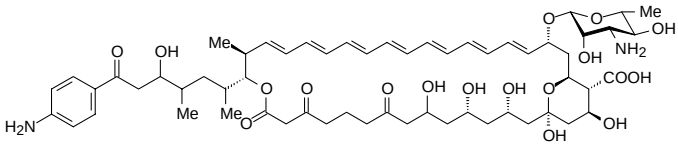
enoyl reduction occur in the extension cycle 18. The FR008 cluster has been completely sequenced. The four FR008 polyenes were shown to be identical to the candicidin complex produced by *Streptomyces griseus* IMRU3570. The candicidin biosynthetic gene cluster from *S. griseus* was partially characterised by Campelo and Gil [35, 36]. The characterised candicidin biosynthetic genes showed between 93 and 100 % sequence identity with their counterparts in the FR008 cluster, clearly indicating their common origin.



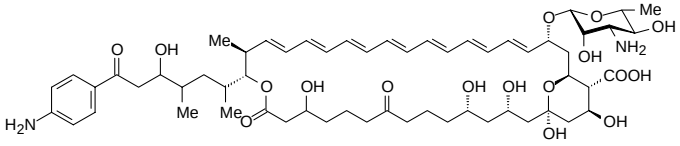
27 FR008-I



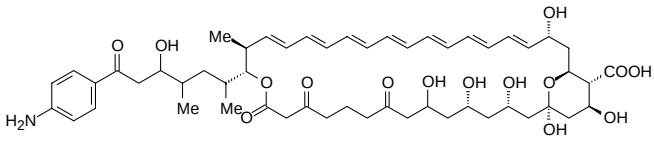
28 FR008-II



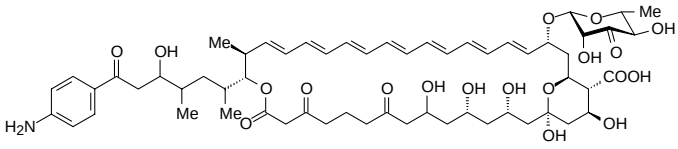
29 FR008-III



30 FR008-IV



31 FR008 VII



32 FR008-XI

Fig. 6. Polyenes produced by *Streptomyces* sp. FR008 and engineered strains.

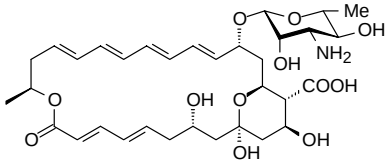
Deng's group found that deletion of the glycosyl transferase gene from *Streptomyces* FR008 resulted in production of the aglcyones of FR008 polyenes I to IV [9]. Figure 6 shows one example - FR008 VII (**31**) is the aglycone of FR008-III (**29**). Disruption of the aminotransferase gene involved in mycosamine biosynthesis resulted in production of mostly aglycones and small amounts of FR008 analogues glycosylated with a ketosugar. FR008-XI (**32**) is the ketosugar analogue of FR008-III (**29**). The low levels of these compounds indicates that polyene glycosyl transferases have a low affinity for GDP-ketosugar intermediates. Some aglycones were also detected in which the C-18 methyl branch was not oxidized to a carboxyl group.

PIMARICIN

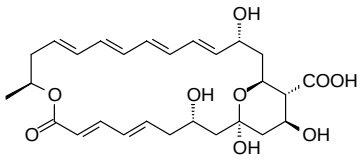
The pimaricin analogue 4, 5-de-epoxypimaricin (**33**) (Fig. 7), obtained by inactivation of the *pimD* gene in *Streptomyces natalensis* [37], was the first biosynthetically engineered polyene macrolide produced. Biosynthesis of this compound at high yield indicated that the formation of the epoxide group at C4-C5 is the last step in pimaricin biosynthesis. The *pimD* gene was overexpressed in *Escherichia coli* to obtain a functional recombinant protein with a hexahistidine affinity tag. This enabled its purification and kinetic analysis and the *in vitro* C4-C5 epoxidation of 4, 5-deepoxypimaricin into pimaricin [38].

The inactivation of the *pimK* glycosyltransferase gene in *S. natalensis* resulted in the formation of the aglycone 4, 5-de-epoxypimaricinolide (**34**) (Fig. 7) as a major product, indicating that epoxidation is impaired in the absence of glycosylation with mycosamine and suggesting that the PimD epoxidase has a strong preference for the glycosylated polyene

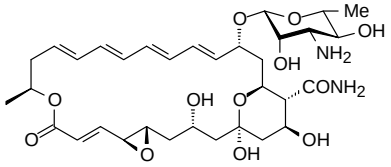
substrate. This aglycone product turned out to be extremely unstable and had no antifungal activity (J. F. Aparicio, unpublished results). This shows that the mycosamine sugar of pimaricin is essential for antifungal activity. This could not have been predicted because some smaller polyenes like filipin and fungichromin lack sugar moieties but display strong antifungal properties.



33 4, 5 De-epoxypimaricin



34 4, 5 De-epoxypimaricinolide



35 AB-400 (pimaricin amide)

Fig. 7. Structures of pimaricin derivatives.

The lack of an efficient gene delivery system for the pimaricin producing strain *S. natalensis* has hampered the development of novel pimaricin derivatives. The recent development of a gene transfer system based on intergeneric conjugation from *E. coli* [39] will enable easier manipulation of the genes responsible for pimaricin biosynthesis, and could prove valuable for the generation of new designer polyene macrolides with better antifungal activity and pharmacological properties.

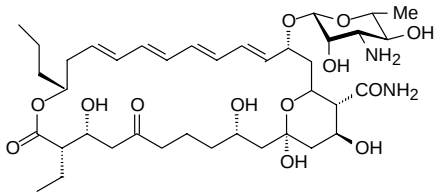
RIMOCIDIN / CE108

Malpartida's group isolated *Streptomyces diastaticus* var. 108, a streptomycete that produces two related tetraenes, rimocidin (**6**) and CE-108 (**7**), as well as oxytetracycline [40]. A genomic library of *S. diastaticus* was constructed in a phage vector, and its screening with the *eryAII* probe yielded two modular PKS gene clusters [10]. Targeted gene disruption revealed that one of these was responsible for the biosynthesis of both rimocidin and CE-108, and the disruption mutants were found to overproduce oxytetracyline. Disruption of the other cluster resulted in an increased yield of rimocidin/CE-108, probably because of increased supply of malonyl CoA to this pathway. These results indicate that disruption of competing pathways can be a useful approach towards increasing the yield of a specific metabolite.

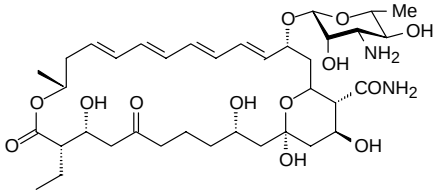
Sequencing of the rimocidin/CE-108 cluster revealed a modular PKS that apparently catalyses synthesis of both tetraenes. The Rim A loading module appears to be able to accept both acetyl CoA and butyryl CoA as the starter unit. The use of an acetyl starter results in production of CE-108, whereas the use of a butyryl starter results in production of rimocidin. How the starter units for rimocidin and/or CE-108 biosynthesis are selected is still unknown. Both compounds are simultaneously produced by *S. diastaticus* var. 108 and their relative proportions seem to be highly dependent on the fermentation conditions. The *rimJ* gene for a putative crotonyl-CoA reductase located within the *rim* cluster, plays an important role in providing butyryl units since production of CE-108 was shown to be substantially increased in the *rimJ* mutants. This suggests that in addition to the specificity of the acyltransferase domain within the RimA protein, the availability of butyryl units can be important for priming the rimocidin/CE-108 biosynthesis with either butyrate or acetate units.

The Malpartida group discovered an interesting late modification of polyenes in which the exocyclic carboxyl group of the macrolactone ring of rimocidin/CE-108 is converted to an amide by a genetically modified *S. diastaticus* var. 108 [41]. The engineered strain produces the parental CE-108 (**7**) and rimocidin (**6**), as well as the corresponding amide-derivatives CE-

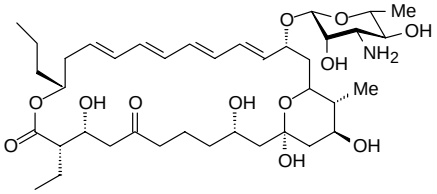
108B (**37**) and rimodicin B (**36**) (Fig. 8). The producer of polyene amide derivatives was unexpectedly generated when the parental strain was transformed with an SCP2*–based vector carrying the erythromycin resistance gene (*ermE*) for selection. There is no obvious explanation for the activation of this tailoring activity. A naturally-occurring strain catalyzing a similar chemical modification has also been isolated [42, 43]. This strain produces the tetraene pimaricin and its amide derivative AB-400 (**35**) (Fig. 7).



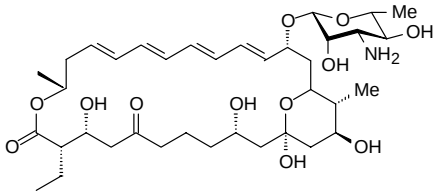
36 Rimocidin B



37 CE-108B



38 Rimocidin C



39 CE-108C

Fig. 8. Structures of rimocidin and CE-108 derivatives.

The rimocidin amides had several times the antifungal activity of their counterparts with exocyclic carboxyl groups, while their haemolytic activities were not increased [43]. The amide derivative (**37**) of CE-108 (**7**) is particularly interesting. While CE-108 (**7**) is weakly antifungal, its amide derivative CE-108B (**37**) has antifungal activity comparable to that of rimocidin (**6**). CE-108B (**37**) has a much lower haemolytic activity than rimodicin (**6**) and its amide (**36**). Thus, the amidated polyenes might be promising compounds for development of

safer antifungal drugs. Similarly, the amide form of pimaricin (AB-400) (**35**) (Fig. 7), obtained from *Streptomyces* sp. RGU5.3, was a more potent antifungal drug than the parent compound [43], while no significant differences in their haemolytic properties were detected. All these data suggest that modification of the exocyclic carboxy groups of polyene macrolides by amidation improves selective toxicity of these compounds towards fungal cells. This is also consistent with the data on semisynthetic polyene macrolide derivatives altered in exocyclic carboxyl groups [44, 45, 46]. The possibility of generating polyene amide derivatives *in vivo* is attractive because less toxic and highly active new polyenes could be generated by fermentation procedures.

Neither rimocidin nor CE-108 aglycones were converted to carboxamides in glycosyl transferase-deficient *rimE* mutants of *S. diastaticus* [43]. This shows that carboxamide group formation occurs after glycosylation; thus, the substrates for the amide formation are the fully decorated polyenes.

Interestingly, the tailoring amidotransferase from *S. diastaticus* var. 108 can catalyse not only conversion of rimocidin (**6**) and CE-108 (**7**) to amide forms (**36**, **37**) but also conversion of pimaricin to its amide form AB-400 (**35**) [43]. The putative amidotransferase from *S. diastaticus* var. 108 has a broader substrate range than that of the pimaricin amide producer *Streptomyces* sp. RGU5.3. However, conversion of AmB to its amide form could not be demonstrated. Work is in progress to identify the gene for the amidotransferase. The substrate range could then be increased by protein engineering.

The *rimG* cytochrome P450 monoxygenase gene is an additional post-PKS decorating activity in rimocidin biosynthesis. Inactivation of *rimG* yielded exclusive production of decarboxy-methyl polyenes, even in those conditions known to be adequate for formation of amide derivatives [43]. These results confirmed that the carboxamide group originates from modification of an exocyclic carboxyl group, and not from incorporation of a malonamyl

extender unit during macrolide ring assembly. The latter mechanism was suggested for the biosynthesis of oxytetracycline [47], which is also made by *S. diastaticus*. The non-carboxylated rimocidin C (**38**) and CE-108C (**39**) had lower haemolytic activities than the corresponding parental polyenes, with similar antifungal activity. These new compounds, as well as decarboxy-methyl polyenes obtained for amphotericin B, nystatin and pimaricin by genetic engineering may well be considered as promising new antifungal drug leads.

USE OF NEW POLYENES AS ANTI-PARASITIC DRUGS

Fungi and several parasites such as *Leishmania* and *Trypanosoma* have ergosterol-derived compounds as components of their membranes, making antifungal drugs targeting such membranes also potentially useful as antiparasitic agents [6]. Despite its toxicity, AmB and its semisynthetic derivatives have been used successfully against several parasitic infections such as leishmaniasis and trypanosomiasis [48, 49, 50]. Particularly promising were the new lipid formulations of AmB for clinical use against severe parasitosis; but the high cost of such formulations limits their use. The alternative drugs currently in use are being compromised because of the appearance of resistant strains, substantiating the need for new antiparasitic agents.

The polyenes CE-108B (**37**), CE108C (**39**), rimocidin B (**36**), rimocidin C (**38**), AB-400 (**35**), pimaricin (**4**), rimocidin (**6**) and AmB (**2**) have been tested *in vitro* against the epimastigote and amastigote forms of *Trypanosoma cruzi* [51]. The effectiveness of the antiparasitic activities against epimastigote form, measured by their IC_{50} values were: AmB > pimaricin = AB-400 = rimocidin B = rimocidin > CE-108B > rimocidin C > CE-108 > = CE-108C. Although AmB is more active against the epimastigote form, such differences are less significant compared to the activities against intracellular amastigote form. The cytotoxicity of each of these compounds towards mammalian macrophage cells was assessed. AmB and

rimocidin showed the highest levels of toxicity. Interestingly, almost no toxicity was detected with polyene amides at concentrations within the range of optimal antiparasitic activity.

REGULATION

The nystatin biosynthetic gene cluster was first shown to contain the *nysRI*, *nysRII* and *nysRIII* genes for LAL (large ATP-binding regulators of the LuxR family) proteins [28]. LAL proteins have an N-terminal ATP/GTP-binding domain and a C-terminal DNA-binding domain [52]. Another regulatory protein NysRIV has an N-terminal PAS domain and a C-terminal DNA-binding domain [28]. PAS domains monitor changes in light, redox potential, oxygen, overall energy level of a cell, and small ligands [53]. PAS domains are located in the cytosol and detect internal signals, but they can also sense environmental factors that cross the cell membrane. Homologues of NysRI, RII, RIII and RIV are present in the amphotericin and candicidin/FR-008 clusters [35, 36].

The pimaricin cluster contains two major regulatory proteins. PimR is the archetype of a new class of regulators [54]. The C-terminal half is homologous to LAL proteins and the N-terminal half has an OmpR-like DNA-binding domain found in many *Streptomyces* antibiotic regulatory proteins (SARPs) [55]. PimM, the second regulator of pimaricin biosynthesis, has been recently characterized [56]. It is a regulator of the LuxR type with a PAS sensory domain. The majority of prokaryotic PAS domains function as sensor modules of sensor kinases of two-component systems [53] but this is not the case with PimM.

Functional analysis of nystatin regulatory genes

The functions of the nystatin regulatory genes have been investigated in detail [57, 14]. The related *nysRI*, *nysRII*, *nysRIII* genes appear to form an operon whereas the *nysRIV* gene is

transcribed separately. An in-frame deletion was introduced into each gene so that inactivation of an upstream gene would not have a polar effect on downstream genes. Inactivation of any one of the *nysRI*, *nysRII*, *nysRIII* or *nysRIV* genes almost completely abolished nystatin production. This showed that the NysR proteins function as transcriptional activators. Complementation experiments were also carried out where each of the *nysR* genes was placed under the control of a strong *ermE* promoter and re-introduced into the various *nysR* mutants. Overproduced NysRII or NysRIII complemented the *nysRI* mutation. Overexpression of *nysRIV* complemented deficiencies in any of the four *nysR* genes. This indicates that NysRIV acts at a late stage in a regulatory cascade, probably as a direct transcriptional activator of biosynthetic genes.

Promoters from the nystatin cluster were also investigated [57]. The biosynthetic genes appear to be organized into at least seven transcription units. The seven likely promoter regions were fused to *xylE* reporter gene sequences and promoter activity was assessed in wild-type *S. noursei* and in the *nysR* mutants. The promoter region for the first three PKS genes was strong but was absolutely dependent on all four *nysR* genes. The promoter for the last three PKS genes was weaker but had slightly reduced activity in all four *nysR* mutants, indicating that expression of these genes is less dependent on the activators. Transcription from the promoter for the export genes required functional NysRII, NysRIII and NysRIV proteins whereas two promoters for glycosylation genes were constitutive. The *nysRI*, *nysRII* and *nysRIII* genes are transcribed from a single promoter that is autoregulated by NysRI and NysRII. The *nysRIV* gene has a separate promoter that is activated by all three NysRI, NysRII and NysRIII proteins.

The overall picture seems to be that NysRI responds to external signals and activates transcription of *nysRI-RII-RIII*. The protein products activate transcription of *nysRIV*. The NysRIV protein, to varying degrees, activates promoters for the different groups of nystatin

biosynthetic and export genes. The PAS domain of NysRIV suggests that its activity is further influenced by energy levels within the cell. The high degree of homology between the nystatin, amphotericin and candicidin/FR008 regulatory genes suggests that regulation of amphotericin biosynthesis is similar [57, 35, 36].

Functional analysis of pimaricin regulatory genes

Gene inactivation studies showed that PimR is a positive regulator of pimaricin biosynthesis [54]. Gene expression analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that *S. natalensis* Δ*pimR* shows little or no transcription of the *pim* genes except for the mutated *pimR* gene. This demonstrated that this regulator activates the transcription of all the genes of the pimaricin cluster but not its own transcription [54].

PimM is an ortholog of NysRIV [57], and its inactivation leads to impairment of pimaricin biosynthesis, suggesting that it constitutes a second activator. Gene expression analyses of the pimaricin gene cluster revealed that the genes responsible for initiation and first elongation cycles of polyketide chain extension (*pimS0* and *pimS1*) are among the main targets for regulation, and also that PimM plays its regulatory role independently of PimR [56]. Preliminary results indicate that both PimM and NysRIV regulators might follow a similar regulatory pattern for the expression of their respective polyenes [56], thus suggesting that this pattern could be shared by homologous regulatory genes found in the amphotericin [35], candicidin [36] or filipin [12] clusters.

Indirect transcriptional regulation

The biosynthesis of polyenes in *Streptomyces* is traditionally considered to be very sensitive to phosphate regulation. Concentrations of inorganic phosphate above 1mM drastically reduced pimaricin production. Recently it has been demonstrated that this negative regulation is exerted at the transcriptional level in *S. natalensis* [58]. At 10 mM phosphate,

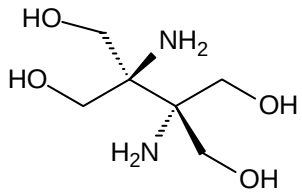
expression of all the pimaricin biosynthesis (*pim*) genes including the pathway-specific positive regulators *pimR* and *pimM* is fully repressed. Transcriptional control by phosphate of genes involved in antibiotic biosynthesis has been reported in a few other cases [59], including the polyene candicidin [60].

In *S. coelicolor* and *S. lividans*, the cellular response to phosphate limitation is controlled by the *phoU-phoR-phoP* genes that encode a two-component system [61]. PhoR is a membrane-bound sensor kinase and PhoP is a DNA-binding response regulator that controls transcription of *phoA* (for alkaline phosphatase) and other target genes. PhoU is a modulator of the phosphate response. The *phoU-phoR-phoP* region of *S. natalensis* has been characterised [58]. The PhoP protein from *S. coelicolor* binds to PHO box consensus sequences in the *S. natalensis phoU-phoRP* intergenic region, indicating that the system is autoregulated. Targeted gene disruptions yielded *phoP* and *phoR-phoP* deletion mutants of *S. natalensis*. In both cases, production of pimaricin increased up to 80 % in complex media and both showed reduced sensitivity to phosphate control. Four of the *pim* genes, including the *pimS1* and *pimS4* PKS genes, showed increased expression in the *phoP*-disrupted mutant [58]. However, no consensus PHO boxes were found in the promoter regions of any of the *pim* genes, suggesting that phosphate control of these genes is mediated indirectly by PhoR and PhoP via modifications of other regulators. Manipulation of this regulatory mechanism will improve yields of other polyene macrolides.

Signal networks

In bacterial cultures there is a mechanism called “quorum sensing” that allows the culture to detect a high density of population and react by different mechanisms of adaptation [62]. Some cells produce signals of intercellular communication that transmit the message of nutrient limitation or high density population to the remaining cells of the culture, which in

turn respond to this stimulus by the transcription (or repression) of given genes. Although this mechanism is general, the chemical molecules that act as signals are different depending on the species. *Streptomyces* species use butyrolactones; *Vibrio*, *Xanthomonas* and other Gram-negative bacteria use homoserine-lactones, and *Bacillus* use oligopeptides. In *S. natalensis* Martín and collaborators have identified, purified and characterized, a novel quorum-sensing inducer PI factor (**40**) [2,3-diamino -2,3-bis (hydroxymethyl) -1,4-butanediol] (Fig. 9) which elicits pimaricin production at nanomolar concentrations [63]. The presence of similar quorum-sensing inducers in other polyene-producing strains has not been reported to date, but it seems conceivable that this kind of signal could play a fundamental role on the production of these compounds.



40 PI factor

Fig. 9. Structure of pimaricin –inducing factor

This group has also discovered that glycerol, ethylene glycol, 1,2-propanediol or 1,3-propanediol can elicit the production of the tetraene pimaricin in its producer strain *S. natalensis*, and also increase the production of other polyenes by their respective producer strains, including *S. noursei* (nystatin), *S. rimosus* (rimocidin) or *S. griseus* (candicidin), among others [64]. Although the precise mechanism by which glycerol elicits polyene production remains to be established, the action of glycerol seems to be independent of the PI-factor inducing effect [64].

Cholesterol oxidases as new modulators of small-ring polyene macrolide biosynthesis

The pimaricin cluster includes the *pimE* gene for a cholesterol oxidase that had no immediately obvious role in biosynthesis of the antibiotic. Cholesterol oxidase (EC 1.1.3.6) is a flavoprotein that catalyzes both, the oxidation of cholesterol to 5-cholesten-3-one with the reduction of molecular oxygen to hydrogen peroxide, and the isomerization of the Δ^5-bond to yield 4-cholesten-3-one as the final product. This enzyme participates in the first step of a metabolic pathway for utilizing cholesterol (or other sterols with a 3-β-hydroxyl group) as a carbon and energy source for primary metabolism [65]. Gene inactivation and complementation studies led to the surprising finding that PimE is essential for the biosynthesis of pimaricin. This extracellular enzyme was purified from *S. natalensis* culture broths to homogeneity, and shown to restore pimaricin production when added to the mutant culture broths or "resting cells". Other cholesterol oxidases also triggered pimaricin production suggesting that these enzymes could act as signaling proteins for polyene biosynthesis [66].

Cholesterol oxidase genes are present in two other known biosynthetic gene clusters of small-size polyenes. These are the *pte* gene cluster of *S. avermitilis* [12], and the gene cluster responsible for the biosynthesis of the 28-membered tetraenes rimocidin and CE-108 in *S. diastaticus* var. 108 [43]. It is therefore plausible that the genes for cholesterol oxidases might have been acquired in the process of evolution by the polyene antibiotic gene clusters in order to provide a prompt response to the presence of fungi (whose cell envelopes contain ergosterol) in the environment via expression of the polyene antibiotic biosynthesis genes. This modulation of polyene macrolide biosynthesis by extracellular proteins is totally new in bacteria and will constitute a novel working area that will aid in understanding the molecular mechanisms of signal transduction.

So far, the presence of common regulatory circuits controlled by cholesterol oxidase enzymes cannot be extended to larger polyenes since no cholesterol oxidase encoding genes

have been found in the gene clusters for the nystatin, amphotericin or candicidin [28, 67, 35, 36, 37, 38].

CONCLUSIONS

Over the past four years, engineered biosynthesis has yielded over twenty new polyene derivatives. Some of these compounds appear to have reduced toxicity and to retain antifungal and antiparasitic activity. Further manipulation of PKS and polyene modification enzymes will allow production of libraries of new compounds and complete investigation of structure-activity relationships. Recent developments in genetic manipulation of streptomycetes should accelerate this work. The most promising new polyenes will be required in large quantities for testing as potential drug candidates. Considerable progress has been made in understanding the regulation of polyene biosynthesis. This knowledge will be invaluable for maximizing yields of new polyenes.

ACKNOWLEGEMENTS

F.M. acknowledges the grant BIO2005-02785 from Spanish MEC.

J. F. A. acknowledges the grant BIO2004-00066 from Spanish MEC.

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