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REVIEW

Biosynthesis and pathway engineering of antifungal polyene macrolides in actinomycetes

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Abstract Polyene macrolides are a large family of natural products typically produced by soil actinomycetes. Polyene macrolides are usually biosynthesized by modular and large type I polyketide synthases (PKSs), followed by several steps of sequential post-PKS modifications such as region-specific oxidations and glycosylations. Although known as powerful antibiotics containing potent antifungal activities (along with additional activities against parasites, enveloped viruses and prion diseases), their high toxicity toward mammalian cells and poor distribution in tissues have led to the continuous identification and structural modification of polyene macrolides to expand their general uses. Advances in in-depth investigations of the biosynthetic mechanism of polyene macrolides and the genetic manipulations of the polyene biosynthetic pathways provide great opportunities to generate new analogues. Recently, a novel class of polyene antibiotics was discovered (a disaccharide-containing NPP) that displays better pharmacological properties such as improved water-solubility and reduced hemolysis. In this review, we summarize the recent advances in the biosynthesis, pathway engineering, and regulation of polyene antibiotics in actinomycetes.

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Introduction

Polyene macrolides are a large family of polyketides with potent antifungal activities. They include antibiotics such as nystatin A1 (**1**), amphotericin A and B (**2** and **3**), pimaricin (**4**), candicidin/FR-008 (**5**), and CE-108/rimocidin (**6** and **7**) (Fig. 1). Polyene antibiotics are structurally characterized by polyhydroxylated macrocyclic lactones comprised of 20–40 carbons with three to eight conjugated double bonds [34, 87]. The macrolactone typically contains a six-membered hemiketal ring with a carboxyl group derived from a methyl branch via an oxidation. Another structural characteristic of polyene antibiotics is a deoxyaminosugar residue attached to the macrolactone core through a glycoside bond. The primary antifungal mechanism of the polyene antibiotics involves the interaction of polyenes with ergosterol in the fungus to form transmembrane channels [7, 8, 76]. These channels cause the leakage of cellular K^+ and Mg^{2+}, eventually leading to the death of the fungal cells. The interactions between polyene molecules and ergosterol seem to occur through the conjugated double bonds of the polyene molecules. Interestingly, recent studies also show that natamycin (also named pimaricin) inhibits the growth of fungi via the immediate inhibition of amino acid and glucose transport across the plasma membrane [75]. This may be attributed to a reversible and ergosterol-specific inhibition of membrane transport proteins. The ergosterol-dependent inhibition of membrane proteins is a general action of all polyene antibiotics and some have been shown to permeabilize the fungal plasma membrane as well.

In contrast to many other antifungal agents, resistance to polyene antibiotics is considered an exceptionally rare event [30, 64]. The polyene antibiotics are the only group of antifungal antibiotics that directly target the plasma membrane via a specific interaction with the main fungal sterol, ergosterol [10].

Unfortunately, however, the relatively high toxicity of polyene antibiotics on mammalian cells and the poor distributions of these compounds in tissues, due to low water solubility, limit their wide use in the clinic [27, 46]. In order to improve the therapeutic efficacy and reduce the toxicity of polyenes, several strategies have been applied, including combination therapy, modification of the polyene molecule, modification of the physical state of polyene, or changes in the drug delivery system [31, 62, 77]. Notably, liposomal formulation and chemical modifications of polyene molecules reduces toxicity, but the processes are expensive and not economically favorable [46, 59, 60, 77]. Therefore, attempts to screen and isolate new classes of polyene antibiotics with minimal side-effects have not ceased. Recently, a solubility-improved and less-hemolytic nystatin-like polyene compound named NPP, which harbors a unique disaccharide moiety (**8**, Fig. 1) was identified through genome screening of actinomycetes and characterizing its biosynthetic gene cluster [45]. Another new antifungal polyene JBIR-13 (**9**, Fig. 1) whose aglycone is similar to pimaricin, (but bears a different deoxyaminosugar moiety) was identified from *Streptomyces bicolor* NBRC 12746 [43].

During the last decade, polyene biosynthetic gene clusters for nystatin, amphotericin, pimaricin, candicidin/FR-008, and rimocidin/CE-108 have been fully cloned and characterized [4, 5, 14, 19, 20, 25, 29, 70]. Several reviews summarize the advances in the biosynthesis of these polyene macrolides [3, 32, 33, 50, 86]. In general, modular type I polyketide synthases assemble the polyene macrolactone aglycones. Subsequently, a cytochrome P450 enzyme catalyzes an oxidation of the exocyclic methyl group via triple hydroxylations, to form a carboxyl group that seems to be related to compound toxicity [87]. After an amino sugar moiety (typically mycosamine) is attached to the macrocyclic aglycone by a glycosyltransferase, another cytochrome P450 enzyme catalyzes a hydroxylation or epoxidation to complete the assembly of polyene antibiotics. This process is described for the biosynthesis of nystatin A1 (Fig. 2) [14]. With advances in understanding the biosynthetic pathways of polyene macrolides, a number of less toxic or more water-soluble derivatives of

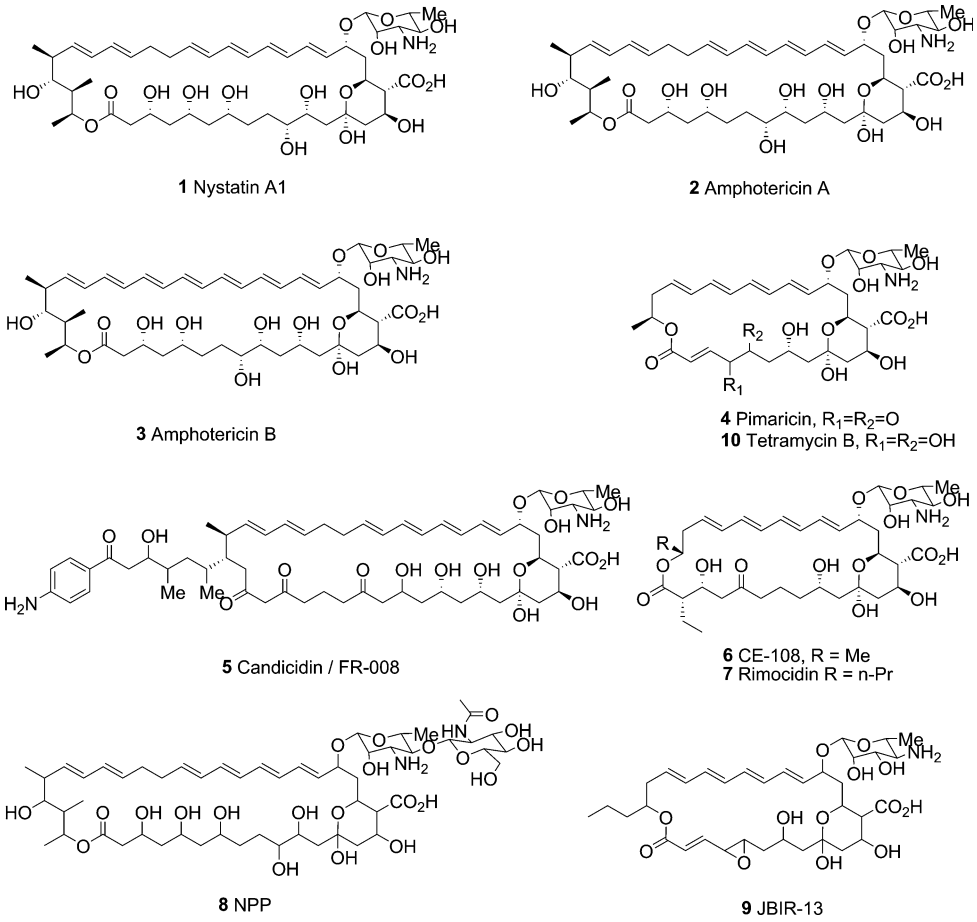

Fig. 1 The structure of typical polyene macrolides

antifungal polyene macrolides have been generated by genetic manipulation of their biosynthetic pathways [18]. Several reviews about this topic discuss the genetic approaches that generate new polyene analogues [18]. This review will summarize the recent progress, especially since the year 2008.

Overview of biosynthesis and PKS pathway of polyene macrolides

Since the biosynthetic gene cluster for pimaricin was reported in 1999, the gene clusters for polyene biosyntheses including nystatin, amphotericin, and candicidin/FR-008, have been cloned and characterized by genetic and biochemical approaches. Although they follow a general biosynthetic process, including assembly of a polyene macrocyclic ring followed by one or two steps of oxidation and glycosylation, certain exceptions still exist such as the incorporation of a second sugar to nystatin A3 [85]. Recently, our group used a polyene cytochrome P450 hydroxylase-specific genome screening strategy and discovered a biosynthetic gene cluster that shows high homology to that of nystatin, from *Pseudonocardia autotrophica* KCTC9441 [41]. The structure of this polyene compound named NPP was eventually elucidated as an analogue of nystatin A1 with an N-acetyl-2-aminoglucose attached to the mycosamine via an α(1-4) link (**9**, Fig. 1). Although the exact mechanism of the post-PKS biosynthesis of this polyene is not understood, additional glycosyltransferases were found upstream of the NPP biosynthetic gene cluster, offering the chance to elucidate the entire biosynthetic machinery of NPP (Fig. 2, unpublished data). You and co-workers also identified the biosynthetic gene cluster for tetramycin (**10**, Fig. 1), an analogue of pimaricin, by genome scanning [21]. The only

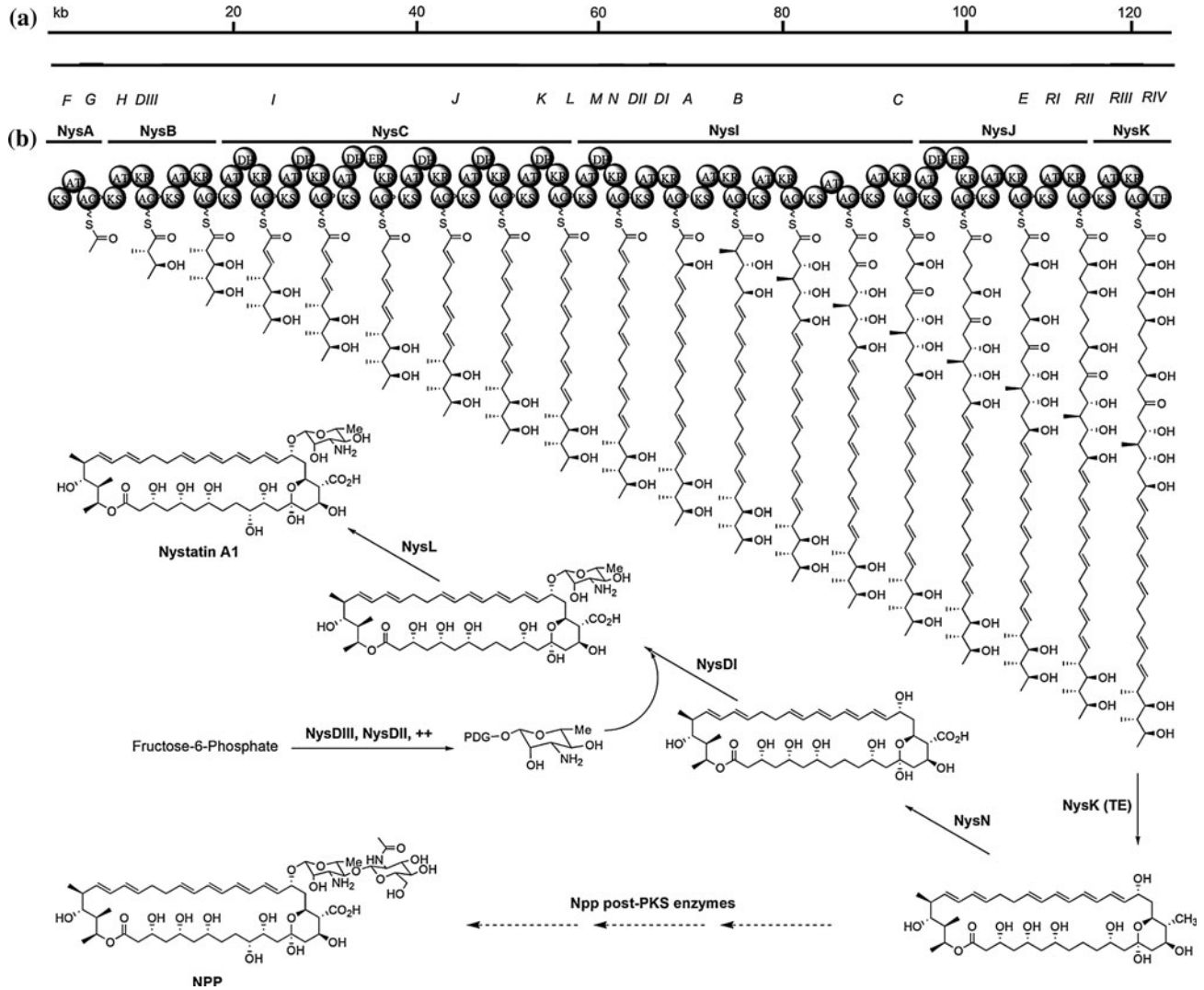

Fig. 2 The biosynthetic model of nystatin and its close relative, NPP

difference is that a cytochrome P450 enzyme catalyzes a hydroxylation in tetramycin biosynthesis (not an epoxidation as in pimaricin).

The backbones of polyene macrolides, macrocyclic rings, are assembled by type I modular PKSs. The acyltransferase domains (ATs) select starter units and extender units, and attach them to acyl carrier proteins (ACPs) via a phosphopentatheine arm. The substrate specificity of ATs is an important source of structural diversity. The other resource to produce the structural diversity is the modifications of the β-keto group, which is formed by catalysis of ketosynthase (KS). The reductive status of the β-keto is totally dependent on the presence or absence of functional reducing domains as affected by ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). Therefore, the engineering of polyene macrolide PKS pathways is mainly focused on the AT and β-keto reducing domains.

Engineering of the loading modules of polyene PKS

The loading module selects a starter unit for initiating the assembly line. If this module selects different starter units, diversified polyketides can be formed (for example rimocidin and CE-108 [70]). The loading module RimA can accept both acetyl-CoA and butyryl-CoA as starter units. Activation of an acetyl starter unit by RimA results in the production of CE-108, while selection of butyryl-CoA as a starter unit by RimA results in the formation of rimocidin [70]. Changes in the substrate selectivity of the loading module will facilitate the diversification of polyene antibiotics. This strategy was successfully used to generate other macrolide antibiotics, such as erythromycin and avermectin derivatives [47, 66]. Zotchev's group first carried out studies on the loading module NysA of the nystatin PKS, which contains a KS-like domain with a Ser instead of Cys residue in its active site [13]. This study revealed that NysA can accept both acetyl-CoA and malonyl-CoA as the starter unit for initiating nystatin biosynthesis.

Subsequently, in-depth studies on the loading modules of several polyene biosynthetic pathways were performed [36]. The modifications of the AT_0 domain of NysA, by site-specific mutagenesis or domain swapping aimed at changing the substrate specificity towards methylmalonyl-CoA, did not generate the mutants capable of initiating nystatin biosynthesis with a propionate as starter. Furthermore, they used RimA, the loading module of rimocidin/CE-108 PKS (able to use both acetyl-CoA and butyryl-CoA as starter units), to complement the *nysA*-deficient strain *S. noursei* NDA59. The RimA was able to restore the nystatin production, but no nystatin analogue originating from butyryl-CoA was formed, even though the culture medium was supplemented with crotonic or butyric acid. In contrast, NysA could not restore the production of either CE-108 or rimocidin by introducing *nysA* to the *rimA*-deficient mutant. These results suggest that NysB has a more flexible docking domain than RimB, able to interact with both NysA and RimA [36].

$PimS_0$ is a loading module of pimaricin PKS and has a domain arrangement identical to RimA, and a high homology (70 % identity). The $PimS_0$ was only able to restore the production of CE-108, but not rimocidin, indicating that the AT domain of $PimS_0$ has a strict substrate specificity towards either acetyl-CoA or malonyl-CoA. Based on the information obtained, four hybrids between NysA and RimA were constructed under the control of the nysAp promoter (Fig. 3). The C-terminus of the hybrids was always represented by the NysA counterpart to ensure proper interaction with NysB during polyketide biosynthesis. When these hybrids were introduced to the *nysA*-deficient NDA59 strain, along with *rimJ* encoding a putative crononyl-CoA reductase to ensure the supply of butyryl-CoA, only hybrid A was able to restore the nystatin production, no new nystatin analogues were produced, and other hybrids (B, C, and D) failed to restore the nystatin production. However, hybrids C and D were able to restore the production of rimocidin and CE-108 in the *rimA*-deficient mutant [36].

Different from other polyenes, candicidin/FR-008 (**5**) has an aromatic ring as a starter unit. Deng and coworkers

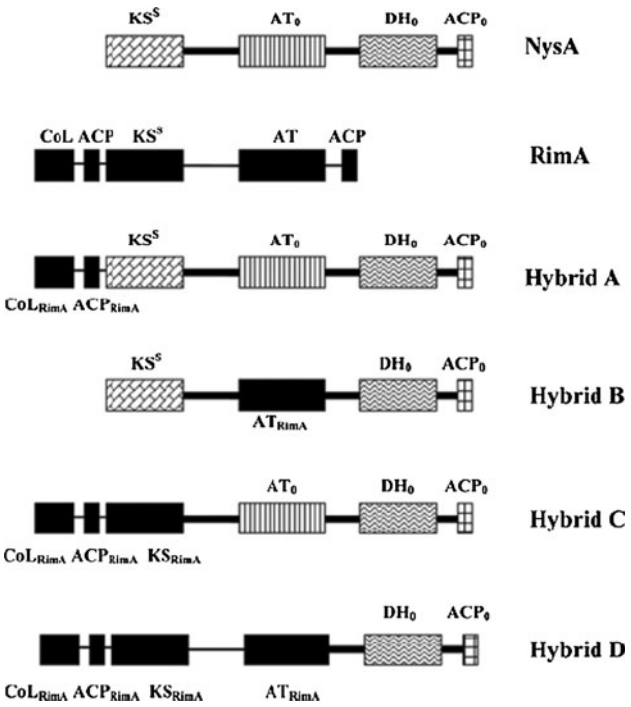

Fig. 3 Schematic representations of the NysA and RimA loading modules and their hybrids [36]. *Solid boxes* and *thin lines* represent RimA enzymatic domains and interdomain linkers, respectively

have cloned the biosynthetic gene cluster of FR-008, and have found that the PKS machinery takes *p*-aminobenzoic acid as the starter unit to initiate polyketide assembly. They confirmed that *pabC-1* and *pabC-2* encoding 4-amino-4-deoxy-chorismate (ADC) lyase are related to the biosynthesis of *p*-aminobenzoic acid in vivo and in vitro. Inactivation of *pabC-1* reduced the production of FR-008 to about 20 % of that of the wide-type strain, while inactivation of *pabC-2* resulted in about a 50 % decrease in productivity of FR-008. Both inactivation mutants can be fully complemented with the *Escherichia coli pabC* gene [84]. However, it is not clear whether this starter unit is able to be substituted with other aromatic compounds to generate FR-008 analogues.

Engineering of the extension modules of polyene PKS

The polyene antibiotic molecules interact with the ergosterol of fungal cell walls through hydrophobic conjugated double bonds reported to provide stability of the interaction [11, 80]. One of the notorious drawbacks of polyene antibiotics is poor solubility. To improve water-solubility and reduce toxicity of polyenes, one of the most effective and commonly adopted approaches is to modify the structure of the aglycone by changing the reductive status of the β-keto form through engineering of the KR, DH, or ER domain of PKS.

In the process of engineering the nystatin biosynthetic PKS, the first target was the inactivation of the ER5 domain of the NysC. This yielded mutant strain ERD44 producing the expected heptaene S44HP (**11**, Fig. 3) [16]. The S44HP is structurally similar to amphotericin B, but approximately ten times more soluble than amphotericin B. The S44HP also seems to have a wider therapeutic window than amphotericin B, in spite of having a lower maximal tolerated dose [78]. In the polyol region of the nystatin A1 molecule, only C-9 is saturated by the action of DH and ER in module 15 of the nystatin PKS NysJ (Fig. 2) [15]. To obtain an analogue bearing a hydroxyl group at C-9, the inactivation of DH15 (by site-specific mutagenesis of the active site His) led to the accumulation of BSG002, 9-hydroxy-10-deoxynystatin (**12**, Fig. 4). The production of BSG002 implied that the subsequent PKS modules 16-18 were able to tolerate the modifications of this polyol region. However, 10-deoxygenation indicated that the NysL cytochrome P450 hydroxylase might not accept the alteration of C9-C11 region. The production of BSG002 was reduced to 30 % of the wild-type level under the same conditions (0.88 ± 0.04 g/l). This analogue was assayed to be at least 2-fold less hemolytic than nystatin A1, while its antifungal activity was also reduced about 4-fold (Table 1). Subsequently, the ER5 mutation GG5073SP was introduced into the DH15-deficient mutant and the resultant recombinant mutant BSM3 produced 9-hydroxy-10-deoxy-S44HP designated BSG003 (**13**, Fig. 4). The BSG003 showed about a 60-fold decrease in antifungal activity compared to S44HP and much lower hemolytic activity (Table 1) [12]. The inactivation of the KR16 domain of module 16 and the KR17 domain of module 17 was performed by introducing the double mutation YA3404FE and TA5145FE, respectively in the nystatin PKS NysJ. The KR16 and KR17 mutations were individually introduced into the S44HP-producing mutant strain GG5073SP, generating mutants BSM4 and BSM2. The KR16 inactivation mutant produced 7-oxo-7-deoxy-28,29-didehydro nystatin designated BSG017 (**14**, Fig. 4), and the KR17 inactivation mutant correspondingly produced 5-oxo-5-deoxy-28,29-didehydro nystatin denoted as BSG013 (**15**, Fig. 4). Both analogues showed reduced antifungal activity and increased hemolytic activity (Table 1).

Amphotericins, medically important antifungal antibiotics, include amphotericin A (a tetraene) and amphotericin B (a heptaene), both of which are produced by *S. nodosus* [19]. The biosynthetic pathway of amphotericin is nearly the same as nystatin. The early work on amphotericin engineering was focused on the post-PKS part. For example, inactivation of cytochrome P450 enzymes encoding *amphL* and *amphN* (or *amphMN*) resulted in the generation of 8-deoxyamphotericin B and 16-decarboxyl-16-methyl-amphotericin B, respectively. Inactivation of *amphDIII*, a gene encoding a sugar biosynthetic enzyme for GDP-mannose-4,6-dehydratase, gave aglycone 8-deoxyamphoteronlide B. The mutant derived from the inactivation of both *amphNM* and *amphDII* produced a 16-descarboxyl-16-methyl-19-O-desmycosaminyl-19-O-deoxyhexosyl amphotericin [17, 23].

The first successful genetic manipulation of the amphotericin PKS was the deletion of two modules, module 5 and 6, from the AmphC protein, resulting in a mutant producing a pentaene in good yield [22]. Recent efforts have been made to alter the structure of the polyol region of amphotericin by engineering KR domains in amphotericin PKS AmphI and AmphJ. Previous studies on modular PKSs indicated that deletion of PKS domains could result in low yields, probably due to incorrect folding of the multi-domain polypeptide. Similar to the engineering of nystatin PKS domains, point mutations were introduced to the targeted domain to construct mutants. The inactivation of KR12 in the hexamodular AmphI seemed to be difficult in the wild-type strain, so this inactivation was achieved in the Δ*amphNM* strain, which lacks the *amphN* cytochrome P450 gene and the *amphM* ferredoxin gene, producing 8-deoxy-16-decarboxyl-16-methyl-amphotericin A (**22A**, Fig. 5) and 16-decarboxyl-16-methyl-amphotericin B (**22B**, Fig. 5) [23]. The resulting Δ*amphNM-KR12* double mutants

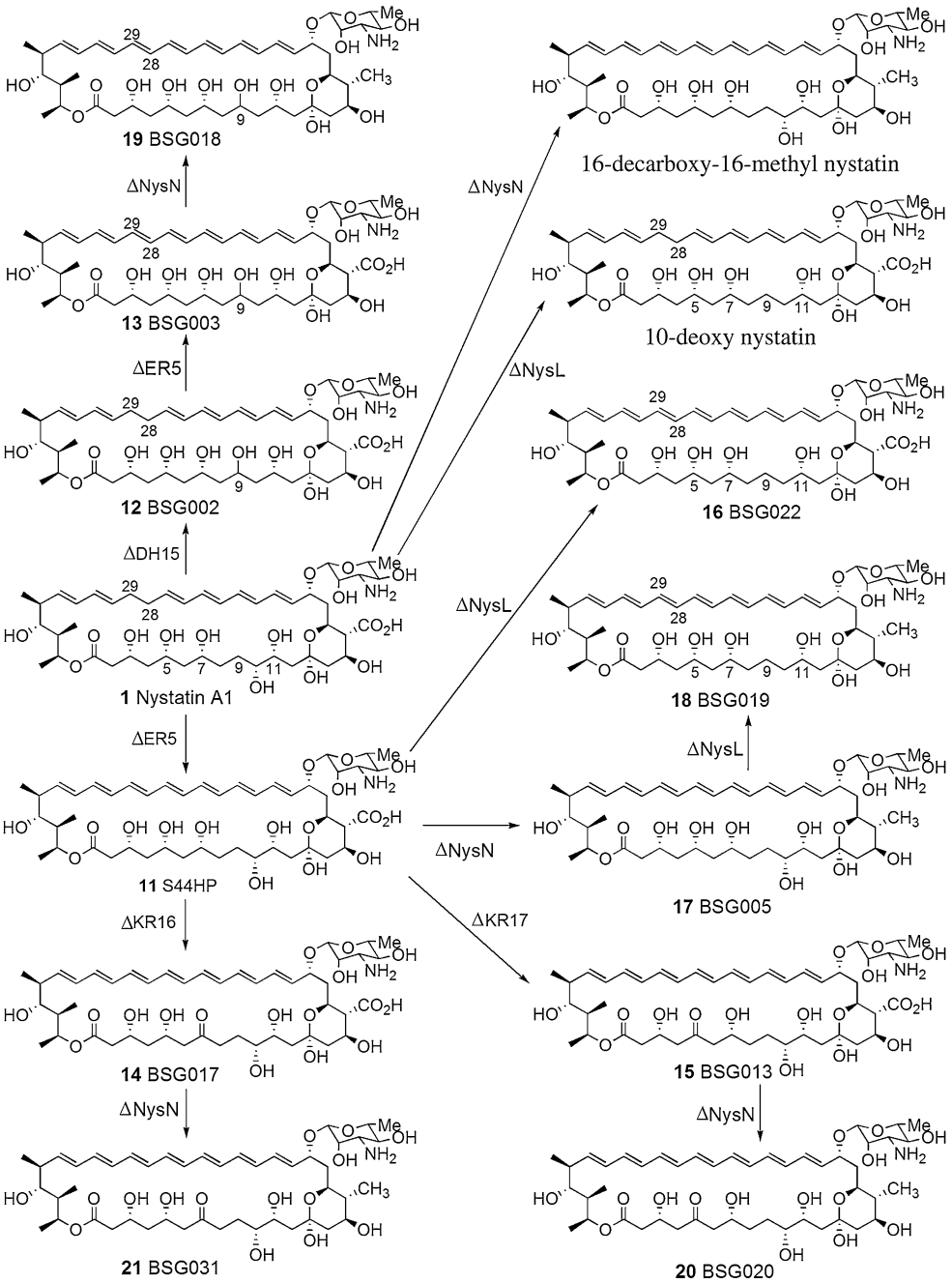

Fig. 4 Nystatin derivatives produced by *S. noursei* ATCC 11455 and genetically engineered strains through pathway engineering

produced trace amounts of 16-decarboxyl-8,15-dideoxy-16-methyl-15-oxoamphotericin A (**23A**, Fig. 5) and its 8-hydroxylated version (**24A**, Fig. 5), as well as 6-decarboxyl-8,15-dideoxy- 16-methyl-15-oxo-amphotericin B (**23B**, Fig. 5) and its 8-hydroxylated version (**24B**, Fig. 5). This mutant did not accumulate aglycone, indicating that the change of the hydroxyl group to a keto group at C-15 does not affect the activity of the glycosyltanferase AmphDI. Inactivation of the KR16 domain in AmphJ successfully generated the Δ*KR16* mutant by replacing the tyrosine in the KR16 active site with phenylalanine in the amphotericin-producing wild-type strain. The fermentation of the Δ*KR16* mutant strain gave 7-oxoamphotericin A and B (**25A** and **25B**, Fig. 5) in comparative yields to the wild-type strain. This result suggested that a keto group at C-7 does not affect the functions of all post-modification enzymes. Compared to amphotericin B, 7-oxo-amphotericin B had a lower antifungal activity but an approximately 10-fold decrease in hemolytic activity. Surprisingly, neither antifungal activity nor hemolytic activity was detected from 7-oxo-amphotericin A. The bioassays also showed that a keto group at C-15 caused some loss in antifungal activity [56, 67]. Furthermore, to obtain 19-deoxy-19-oxoamphoteronolides that can easily be used to construct a library of analogues,

Table 1 Reported antifungal and hemolytic activities of nystatin A1 analogues generated by the engineered biosynthetic pathway

Compound	Mutation (s)	MIC_{90} (μg/ml)[a]	HC_{50} (μg/ml)[b]	References
Nystatin A1		2.0 ± 0.3	85	[15]
16-DecNys	NysN	1.8 ± 0.5	175	[15]
S44HP(**11**)	ER5	0.20 ± 0.03	2.5	[15]
BSG002(**12**)	DH15	10.5 ± 3.5	180	[15]
BSG003(**13**)	ER5, DH15	12.0 ± 0.6	>600	[12]
BSG017(**14**)	ER5, KR16	0.92 ± 0.03	3.3	[15]
BSG013(**15**)	ER5, KR17	0.43 ± 0.07	3.0	[15]
BSG022(**16**)	ER5, NysL	0.20 ± 0.02	1.4 ± 0.15	[12]
BSG005(**17**)	ER5, NysN	0.11 ± 0.03	4.0 ± 0.3	[12]
BSG019(**18**)	ER5, NysN, NysL	0.12 ± 0.03	4.5 ± 0.3	[12]
BSG018(**19**)	ER5, DH15, NysN	9.0 ± 0.45	>200	[12]
BSG020(**20**)	ER5, KR17, NysN	0.19 ± 0.03	9.0	[15]
BSG031(**21**)	ER5, KR16, NysN	0.37 ± 0.07	3.8	[15]
Nystatin A1[c]		0.43 ± 0.02[c]	33 ± 0.54[c]	[45]
NPP[c]		1.08 ± 0.06[c]	403.7 ± 0.97[c]	[45]

[a] Tested using *C. albicans* ATCC10231 as a test organism

[b] Tested by using horse blood cells

[c] MIC_{50} using *C. albicans* ATCC10231 and HC_{50} using horse blood cells (both Nystatin A1 and NPP were tested together under the same condition)

inactivation of the KR10 domain in the AmphI was performed by an approach similar to that used for the inactivation of KR16. However, the resulting mutant, KR10-1, did not produce the expected 19-deoxy-19-oxoamphoteronolides, but alternatively accumulated a polyene pyrone decaketide (**26**, Fig. 5). The production of pyrone decaketide suggested that the assembly line skipped two modules of AmphC. This compound also suggested that the shortened polyketide intermediate **28** with a keto group at the β-position normally reduced by KR10 can be efficiently extended by module 11 to generate a 3,5-diketo intermediate **27**. However, KR11 cannot reduce this 3,5-diketo intermediate, so it is easily cyclized to form pyrone. However, another mutant, KR10-2 (also derived from the inactivation of KR10), produced a mixture of heptaene and tetraene dodecaketides that were released from the module 11 as pyrones **29A** and **29B** (Fig. 5). They were derived from the intermediate **31** by a mechanism similar to that predicted in Fig. 5. In the Δ*KR10* mutants, module 11 can extend the β-keto intermediate, but KR11 cannot function as usual, so 3,5-diketo intermediates were released from module 11 and cyclized to form pyrones [56].

Engineering of cytochrome P450 enzymes in polyene post-PKS

In general, the biosynthetic gene cluster for polyene macrolides contains two genes encoding cytochrome P450 enzymes: one catalyzing the last step modification, hydroxylation or epoxidation (NysL, AmphL, and PimD) [3, 40, 79]; the other catalyzing the first modification where the exocyclic methyl group is oxidized to carboxyl group (NysN, AmphN, RimG, and FscP) [3]. Recently, characterizations of these P450 monooxygenases catalyzing the hydroxylation or epoxidation of the polyol region have been completed. Deletion of *pimD* in the pimaricin producer *S. natalensis* resulted in the accumulation of 4,5-de-epoxy pimaricin. Later, PimD was characterized biochemically for its epoxidation activity during the biosynthesis of pimaricin [55]. Inactivation of *amphL* during amphotericin production by gene replacement, led to the production of 8-deoxyamphotericin A and B [17]. Similarly, inactivation of *nysL* in the nystatin producer *S. noursei* by gene replacement generated a mutant that produced 10-deoxynystatin, and NysL was characterized in vitro for its ability to catalyze the hydroxylation at C-10 during the biosynthesis of nystatin [79]. These cytochrome P450 enzymes were able to tolerate alterations to the structures of their polyene region, alterations of their exocyclic carboxyl group to a methyl group, and were even partially active when the structure of their polyol region was changed; thus the combinatorial engineering of this gene and other genes generated larger structural diversities. For example, the double mutations of ER5 and NysL generated a mutant strain (BSM11) which provided 10-deoxy-28,29-didohydro nystatin named BSG022 (**16**, Fig. 4) which has a similar antifungal activity as S44HP (Table 1). The hemolytic activity of BSG022 shows a 2-fold increase compared to S44HP (Table 1) [12].

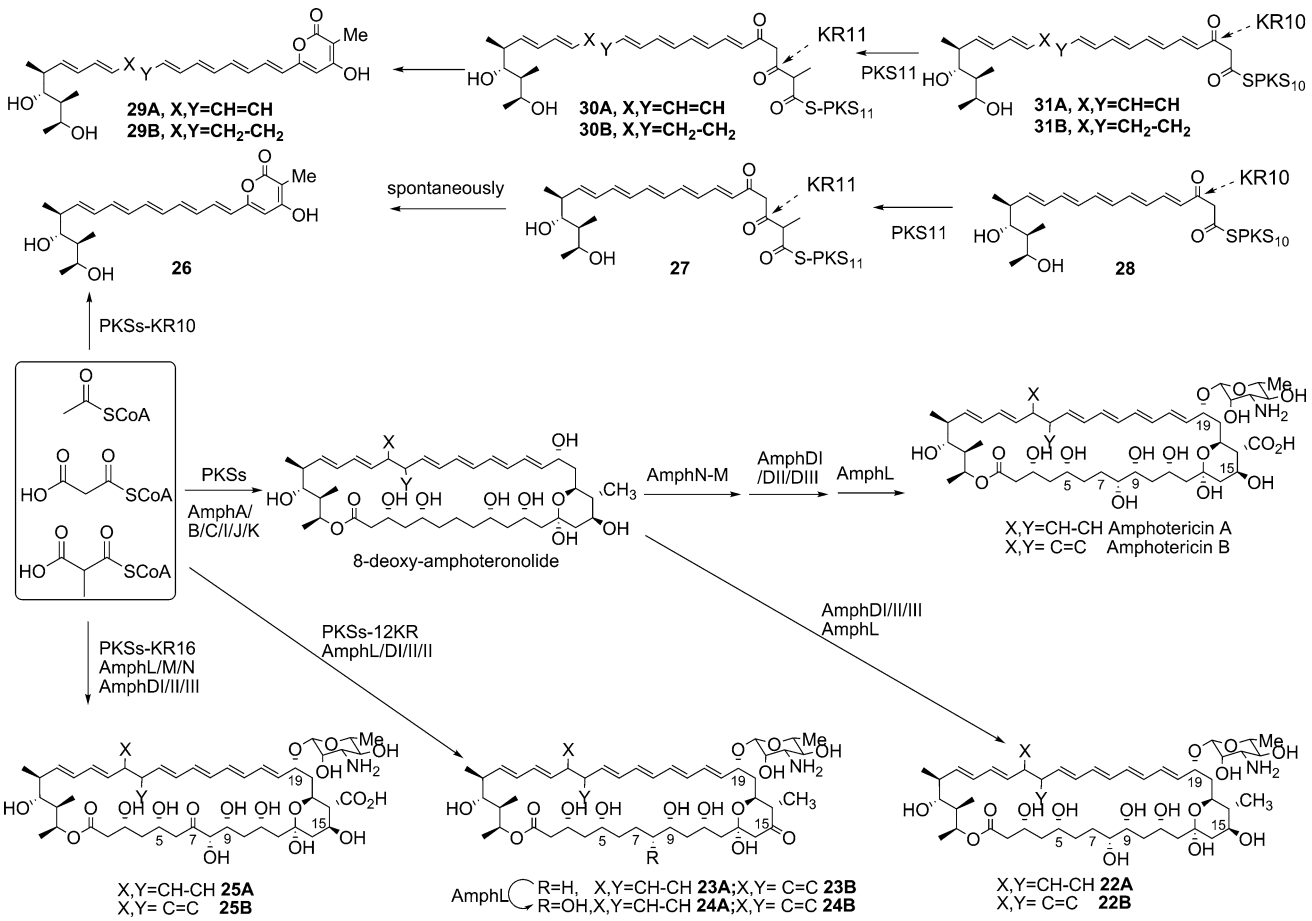

Fig. 5 Amphotericin A and B derivatives produced by *S. nodosus* and genetically engineered strains through pathway engineering

However, these hydroxylation reactions are significantly influenced by glycosylation. If the glycosylation of the polyene aglycones was blocked, deoxygenated analogues were usually generated [17, 45].

The other cytochrome P450 enzyme catalyzes the conversion of a methyl group to an exocyclic carboxyl group, proposed as the first step of the post-modifications. The exocyclic carboxyl group seems to be associated with the toxicity of the polyene antibiotics, so it became the target of engineering to obtain polyene analogues with low toxicity [53]. Several groups carried out the inactivation of this methyl oxidase such as AmphN, RimG, FscP, and NysN. The resulting mutants yielded polyene analogues with a methyl group instead of a carboxyl group. All these analogues showed lower toxicity but retained antifungal activities [23, 24, 70]. The production of these decarboxyl polyenes indicated that the inactivation of these type of genes had no effect on the assembly of polyketide lactones or subsequent modification steps. Thus, combining of the inactivation of this gene with other genes encoding PKS and post-modification enzymes will generate a large chemical diversity of the polyene macrolides.

Combinatorial inactivation has been performed in the nystatin biosynthetic pathway. Previously, inactivation of ER5 resulted in a mutant that produced the heptene nystatin analogue S44HP [16]. The active site of the *nysN* Cys was replaced with Ala to construct a mutation CL346AS in the *nysN* gene that affected the heme binding. This mutation was introduced into the S44HP-producing mutant strain *S. noursei* GG5073SP [11], generating the mutant BSM1 that carried double mutations in *NysN* and *ER5*. The fermentation of BSM1 accumulated the expected analogue BSG005, 16-decarboxy-16-methyl-28,29-didehydro nystatin (**17**, Fig. 4). This analogue had an about 2-fold and 20-fold increased antifungal activity (Table 1), compared to S44HP and nystatin, respectively. Compared to S44HP, its hemolytic activity exhibited about a 1.5-fold decrease (Table 1). Subsequently, the inactivation of *nysL* in the BSG005-producing strain carrying the *nysN* CL346AS mutation, yielded the mutant strain BSM12 that produced the expected analogue BSG019, 10-deoxyl-16-decarboxy-16-methyl-28, 29-didehydro nystatin (**18**, Fig. 4). If the *nysN* CL346AS mutation was introduced into the mutant BSM13 containing double mutations in *ER5* and *DH15*,

and producing 9-hydroxy-10-deoxy-28,29-didehydronystatin BSG003 (**13**, Fig. 4), the resulting mutant BSM14 generated BSG018, 9-hydroxy-10-deoxy-16-decarboxy-16-methyl-28, 29-didehydronystatin (**19**, Fig. 4). If the *nysN* CL346AS mutation was introduced into the BSG013- and BSG017-producing mutant strains (carrying the KR17 and KR16 mutation, respectively), the recombinant mutant strains yielded 5-oxo-5-deoxy-16-decarboxy-16-methyl-28,29-didehydro nystatin, named DSG020 and 7-oxo-7-deoxy-16-decarboxy-16-methyl-28, 29-didehydro nystatin, designated BSG031 (**20** and **21**, Fig. 4). These analogues had improved antifungal activities and decreased hemolytic activities (Table 1).

Engineering of the sugar moiety in polyene post-PKS

Sugar moieties are crucial for the biological activity of many antibiotics [37, 59, 61, 73, 74, 82, 83]. There is considerable interest in manipulating sugar moieties of these antibiotics to improve their pharmacological properties. The majority of polyene macrolide antibiotics (such as amphotericin B, nystatin A1, candicidin/FR-008, pimaricin, and rimocidin/CE-108) contain one single deoxyaminosugar, mycosamine, linked to the aglycone by a glycosidic bond. However, perimycin is an unusual polyene in that the sugar is perosamine, an isomer of mycosamine [65]. The aminosugar moiety is important for the biological activity of the polyene antibiotics [58].

All polyene clusters contain genes encoding GDP-mannose-4, 6-dehydratase (AmphDIII, CanM/FscMIII, NysDIII, and PimJ), GDP-ketosugar aminotransferase (AmphDII, CanA/FscMII, NysDII, PimC, and RimF), and glycosyltransferase (AmphDI, CanG/FscMI, NysDI, PimK, and RimE). Previously, the inactivation of AmphDIII in the amphotericin-producing strain *S. nodosus* resulted in a mutant that accumulated 8-deoxyamphoteronolide and some mannosyl-amphotericin, indicating that the mycosamine moiety is biosynthesized from GDP-Mannose [17]. In the proposed biosynthetic pathway of the polyene macrolides depicted by Fig. 6, GDP-mannose is first converted to GDP-4-keto-6-deoxymannose by GDP-mannose 4,6-dehydratases. GDP-4-keto-6-deoxymannose should undergo a 3,4-isomerization to give GDP-3-keto-6-deoxymannose, the substrate of the aminotransferase for biosynthesis of GDP-3-amino-3,6-dideoxymannose (GDP-mycosamine). However, no genes putatively encoding GDP-4-keto-6-deoxymannose 3,4-isomerase were found in the polyene macrolide biosynthetic gene cluster, so it is assumed that this isomerization is a spontaneous reaction or catalyzed by enzymes located outside of the polyene macrolide gene cluster. After the isomerization, GDP-ketosugar aminotransferases catalyze the conversion of GDP-3-keto-6-deoxymannose to GDP-3-amino-3,6-dideoxymannose (GDP-mycosamine). If GDP-ketosugar aminotransferase (for example PerDII involved in perimycin biosynthesis) directly acts on the GDP-4-keto-6-deoxymannose, GDP-4-amino-4,6-dideoxymannose (GDP-perosamine) is generated instead of GDP-mycosamine. Finally, the glycosyltransferases catalyze the attachment of deoxyaminosugar to polyene macrolactones [37].

The initial sugar donor in the biosynthetic pathways of polyenes, GDP-mannose was proposed to be synthesized from fructose-6-phosphate. The genes putatively involved in the biosynthesis of GDP-mannose from fructose-6-phosphate, included *manA-C* encoding phospho-mannose isomerase (PMI), phospho-mannose mutase (PMM), and GDP-mannose pyrophosphorylase (GMPP), respectively [61]. The inactivations of *manA* and of both *manA* and *B* genes resulted in the production of 8-deoxyamphoteronolide, supporting the idea that GDP-mannose is derived from fructose-6-phosphate, as shown in Fig. 6 [61]. The biosynthetic pathway of GDP-mannose from fructose-6-phosphate will be a target of engineering for the biosynthesis of natural products containing a sugar moiety derived from mannose.

The function of GDP-mannose 4, 6-dehydratase was demonstrated in vitro by using NysDIII to catalyze the conversion of GDP-mannose to GDP-4-keto-6- deoxymannose [58]. The function of NysDII in a coupled reaction with NysDIII was also characterized, but the expected product was not detected, supporting the idea that the substrate of the GDP-ketosugar aminotransferases is GDP-3-keto-6-deoxymannose instead of GDP-4-keto-6-deoxymannose. Therefore, the *nysDI* and *NysDII* genes were inactivated individually by in-frame deletions. The resulting mutants produced nystatinolide and 10-deoxynystatinolide as major products. The function of GDP-ketosugar aminiotransferase was initially characterized by Deng and co-workers. The inactivation of *FcsMII* encoding GDP-ketosugar aminotransferase resulted in the generation of aglycones as major components and small amounts of FR-008 analogues with a ketosugar instead of mycosamine [25]. Later, the functions of NysDI and AmphDI glycosyltransferase were characterized via their reversibility. The substrate specificities of NysDI and AmphDI were probed for aglycones and nucleotide sugars [83]. Recently, the Caffrey group performed a rationally engineered biosynthesis of 19-perosaminyl-amphotericin B in the amphotericins-producing strain *S. nodosus* [37]. Perimycin is an unusual polyene macrolide that contains a perosamine (4-amino-4,6-dioxymannose) instead of mycosamine (3-amino-3, 6-dioxymannose). The perosamine is also biosynthesized from GDP-mannose via dehydration and transamination catalyzed by GDP-mannose-4, 6-dehydratase (PerDIII) and GDP-ketosugar aminotransferase

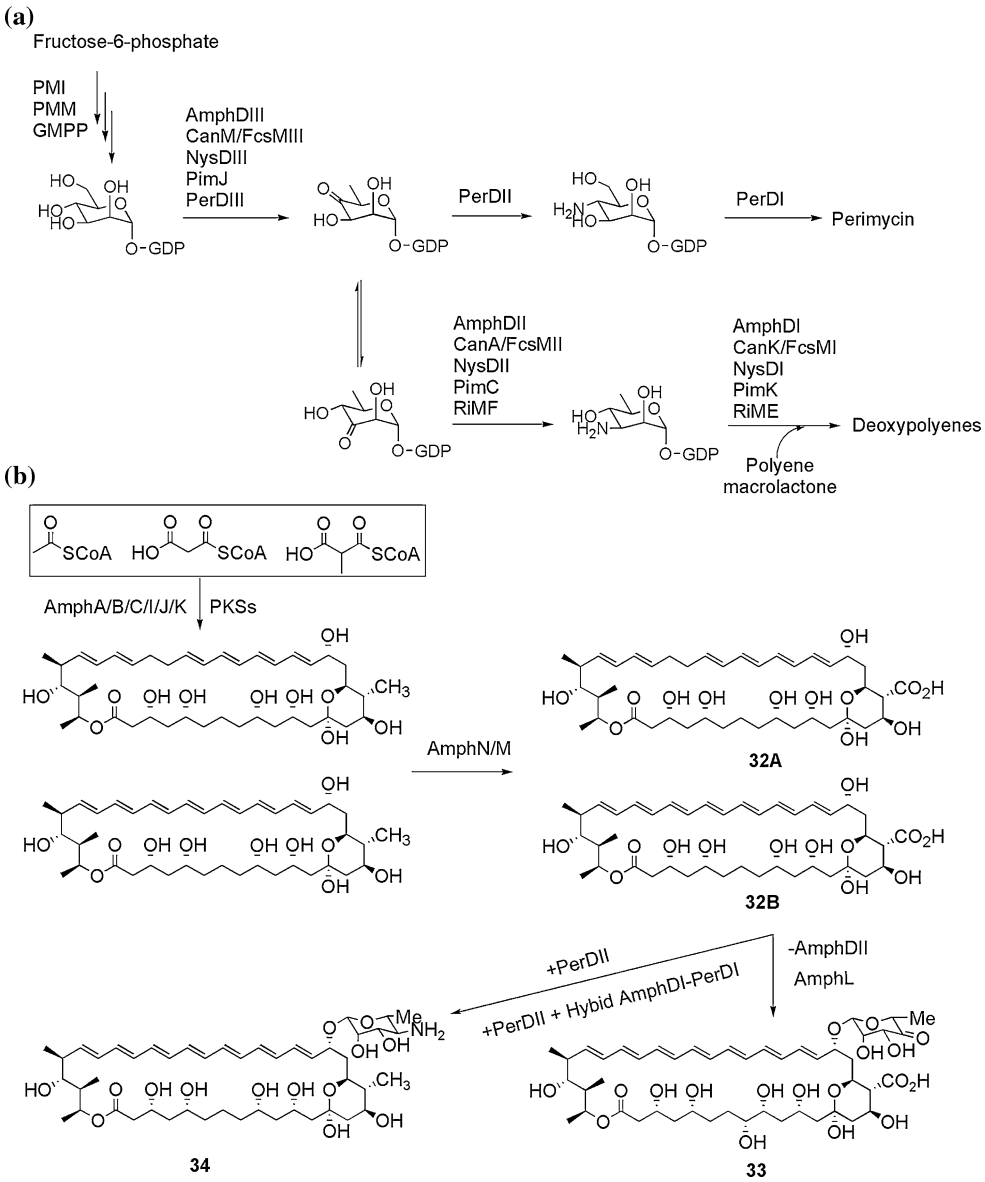

Fig. 6 The biosynthetic pathway of the sugar moiety of polyenes and its engineering

(PerDII, a homologue of AmphDII), respectively (Fig. 6). Although the Δ*amphDII-NM* mutant was constructed and produced 16-descarboxyl-16-methyl-19-*O*-deoxyhexosyl amphoteronolide A and B [23], the Δ*amphDII* mutant was constructed to get perosaminyl-amphotericins. The Δ*amphDII* mutant produced 8-deoxyamphoteronilides A and B (**32A** and **32B**, Fig. 6), and 6-deoxyhexosyl-amphoteronolide B (**33**). The *perDII* gene in the vector pIAGO was introduced into the Δ*amphDII* mutant. The resulting strain accumulated not only **32B** and **33**, but also trace amounts of amphotericin B analogue glycosylated with perosamine (**34**), implying that the glycosyltransferase AmphDI has low affinity to GDP-perosamine. Thereupon, a hybrid glycosyltransferase was constructed with the N-terminal region (1-247 residues) of AmphDI for aglycone recognition and the C-terminal region (248-458 residues) of PerDI for GDP-perosamine binding and introduced to the above Δ*amphDII* mutant. The resultant transformants significantly increased the production of **34**, and decreased **32B** production. Compound **34** showed different antifungal and hemolytic activities from amphotericin B. This work will promote the engineering of the biosynthetic pathway of GDP-sugar to generate new polyene analogues [37].

Most polyene antibiotics contain a single deoxyaminosugar attached to the macrolactones. Some nystatin analogues contain two sugars attached to different positions. Most recently, our groups identified nystatin-like polyene (NPP) containing a disaccharide, mycosamine (α1-4)-N-acetyl-2-aminoglucose [45]. Another nystatin analogue with a disaccharide mycosamine-glucose was proposed by MS–MS analysis and a biosynthetic gene cluster identified

in the *Pseudonocardia* P1 strain collected from *Apterostigmaden tigerum* garden worker ants [9]. Interestingly, NPP harboring a disaccharide moiety had more than 300-fold higher solubility and 10-fold lower hemolytic activity than nystatin, which contains only mycosamine (table 1). The additional *N*-acetyl-glucosamine increased the solubility of the polyene compound. In some instances, the sugar moieties may mainly serve to solubilize the natural product by enabling the hydrophobic aglycone portions to partition into aqueous phases, thereby allowing for higher intracellular and extracellular concentrations. Lentinan-mimic branched with *N*-acetyl-D-glucosamine exhibited remarkable solubility in both organic solvents and water [44]. Glucosamine hydrochloride has been used as a carrier in solid dispersion formulations to enhance the dissolution rate of poorly water-soluble drugs [1]. All *N*-alkylated chitosan derivatives with monosaccharides were insoluble in aqueous solution (pH 7), while *N*-alkylated chitosan derivatives with disaccharides were readily soluble in distilled water [1]. The novel glycosyltransfer system in NPP has ensured a promising future for the glycodiversification of natural products in the search for new and improved polyene antibiotics.

Regulation of the polyene biosynthetic pathway

Like the biosynthesis of most secondary metabolites produced in actinomycetes, polyene macrolides are synthesized through tight regulatory networks in the cell, and thus, low levels are typically observed in wild-type strains. Low levels are typically controlled by pathway-specific transcriptional regulators encoded within the respective biosynthetic gene cluster. A family of genes whose products contain an OmpR-like DNA-binding domain was named as SARP (*Streptomyces* antibiotic regulatory proteins) and are typically found in non-macrolide polyketide biosynthetic gene clusters [6, 48, 57]. Unlike the SARP family, a different transcriptional family of regulators was identified and contains a relatively large protein with an N-terminal ATP-binding domain represented by discernable Walker A and B motifs, and a C-terminal LuxR type DNA-binding domain, such as *nysRI-III* in nystatin, *orf1-3* in candicidin and *fscRI-III* in FR-008 biosynthetic clusters [25, 72]. Regulators belonging to this so-called LAL (large ATP-binding regulators of the LuxR) family, whose prototype member is the *Escherichia coli* MalT involved in the uptake and catabolism of maltodextrins [26], have been identified and characterized in several macrolide antibiotic pathways [35, 42, 81]. Another type of pathway-specific regulator with an odd architecture, PimR combines an N-terminal SARP domain with a C-terminal half homologous to guanylate cyclases and LAL regulators. Members of this class include the regulator PteR from *S. avermitilis*, located in the biosynthetic gene cluster for the pentaene filipin [38], Moreover, combining an N-terminal PAS sensory domain with a C-terminal HTH motif of the LuxR type for DNA binding, was also identified in a polyene macrolide pathway cluster such as NysRIV for nystatin, PimM for pimaricin, AmphRIV for amphotericin, PteF for filipin, and NppRIV for NPP was identified, respectively [2, 22, 39, 63, 71].

J. Santos-Aberturas et al., provided evidence that PAS/LuxR regulators (such as PimM, AmphRIV, NysRIV, or PteF) devoted to the biosynthesis of polyene antibiotics are functionally equivalent, to the extent that the production of a given compound can be boosted by the introduction of a heterologous positive regulator of this class into the producing strain, proving the functional conservation of this regulatory pattern followed by PimM in other polyene gene clusters [68]. Moreover, they also proved that PimR exerts its positive effect on pimaricin production by controlling PimM expression levels. PimM is a regulator which activates transcription from eight different promoters of pimaricin structural genes directly [69].

Phosphate control for the biosynthesis of many secondary metabolites that belong to different biosynthetic groups is a well-known phenomenon, indicating that phosphate control is a general mechanism governing secondary metabolism [28, 51]. All *Streptomyces* and related actinobacteria sequenced so far have molecular mechanisms of regulation mediated by the two-component system PhoR-PhoP and involve a third component, PhoU, that modulates the signal transduction cascade for the primary metabolism and the biosynthesis of antibiotics [49, 52]. In the biosynthesis of pimaricin, *S. natalensis* is very sensitive to phosphate regulation. Mendes et al., showed that phosphate control of pimaricin biosynthesis is mediated by the PhoR-PhoP system, using a *phoP*-disrupted mutant and a *phoR-phoP* deletion mutant, and suggested that manipulation of this regulatory mechanism will improve the production of other polyene macrolides [54].

It has been reported that six putative NPP pathway-specific regulatory genes are present in a biosynthetic gene cluster in *P. autotrophica*. Three pathway-specific regulatory genes, *nppRI*, *RIII*, and *RVI* are predicted to belong to a typical LAL-type transcriptional family. While all the *P. autotrophica* exconjugants containing extra copies of pathway-specific regulatory genes exhibited improved NPP productivities, the one containing *nppRIII* showed the highest NPP productivity improvement in the late exponential growth stage [39]. Moreover, the *P. autotrophica nppRIII*-disruption mutant failed to produce NPP with significantly reduced transcription levels of most *npp* biosynthetic genes, suggesting that overexpression of the key pathway-specific regulatory gene, *nppRIII* is critical for

maximum productivity of an intrinsically low-level metabolite like NPP [39].

Concluding remarks

Polyene antibiotics such as nystatin, amphotericin, pimaricin, candicidin/FR008, CE-108, and rimocidin are a large family of very valuable antifungal polyketide compounds bearing macrolactone rings with 20- to 40- carbon atoms including 3–8 conjugated double bonds. The antifungal mechanism of these polyene antibiotics include the formation of channels that mediate the leakage of cellular K^+ and $Mg^{2+,}$ leading to the death of the fungal cell. Over the past four years, in order to improve the therapeutic efficacy and reduce the toxicity of polyenes at high doses, several strategies, including the use of combination therapy, manipulation of PKS or post-PKS for structural modification, and altering the physical state of the therapeutic agent in the drug delivery system have been employed.

The biosynthetic gene clusters and the complete structures of a number of polyenes have been investigated, including amphotericin, nystatin, pimaricin, and candicidin/FR-008. Using this genetic information, a number of approaches have been used to address the problems associated with their medical use, including pathway-engineered polyene analogues. Since the backbones of polyene macrolides are assembled by programmed type I modular PKSs, the structural diversity of polyene macrolide is achievable through the engineering of polyene PKS domains; AT-modification for specificity of starter or extender units as well as KR-DH-ER manipulation for controlling the reduction processes of β-ketos. Accordingly, the engineering of polyene macrolide PKS pathways, focused on the AT and β-keto processing domains have been successfully applied in several polyene macrolides. Subsequently, polyene macrolide post-PKS enzymes such as cytochrome P450 hydroxylase and glycosyltransferase, are also attractive targets for generating engineered polyene analogues. Cytochrome P450 enzymes, which catalyze an oxidation of the exocyclic methyl group to form a carboxyl group and a regio-specific hydroxylation have been manipulated. Moreover, polyene-specific glycosyltransferase which attaches an amino sugar moiety (typically mycosamine) has been inactivated to generate various polyene aglycone compounds.

Thanks to the advances in understanding of the biosynthetic pathways of polyene macrolides, a number of side-effects of antifungal polyene macrolides have been minimized through polyene pathway engineering. In addition, attempts to screen and isolate new classes of polyene antibiotics with minimal side-effects have been tried. Recently, a solubility-improved and less-hemolytic nystatin-like novel polyene compound named NPP, which harbors a unique disaccharide moiety, was identified through actinomycetes genome screening. Along with previous reviews on the polyene biosynthetic pathway, this review has further proved the feasibility of genetic approaches for generating novel polyene analogues, and set the stage for the biotechnological applications of polyene structural diversification.

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