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Recent advances in antifungal drug discovery based on polyene macrolide antibiotics

Olga A. Omelchuk,[a, b] Anna N. Tevyashova,[a, b] Andrei E. Shchekotikhin [a, b]

[a] *Gause Institute of New Antibiotics*
ul. Bol'shaya Pirogovskaya 11, 119021 Moscow, Russia Federation
[b] *D.Mendeleev University of Chemical Technology of Russia*
Miusskaya pl. 9, 125047 Moscow, Russian Federation

The review summarizes recent advances in the synthesis of new derivatives of polyene macrolide antibiotics by chemical modification and genetic engineering methods, results of structure – activity relationship studies and progress in the development of effective and safe drug formulations of this class of antibiotics. Attention is focused on structural changes of polyenes, resulting in a significant decrease in toxicity with retention or even enhancement of antifungal activity. Current concepts on the mechanisms of biological action of these antiobiotics in terms of their therapeutic activity and toxicity are considered.
The bibliography includes 77 references.

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1. Introduction

The incidence rate of hospital-related fungal infections (nosocomial mycoses) has increased over the past decades.[1] Systemic mycoses are particularly dangerous since they are characterized by severe symptoms and cause high rates of morbidity and mortality (50% – 70%).[1] Immunocompromised patients and patients with diseases or states accompanied by neutropenia (haemoblastoses, hematopoietic stem cell transplantation and solid tumours), as well as patients in critical and intensive care units and in organ transplant clinics, are at high risk of developing mycoses.[1] Yeast-like fungi of the genus *Candida* are the fourth most common cause of nosocomial (hospital-acquired) bloodstream infections in the United States.[2]

Clinically used antifungal agents are divided into several classes according to the chemical structure and putative mechanism of action. The commonly used first-line agents include drugs based on azoles (fluconazole, itraconazole, voriconazole, *etc.*) and echinocandins (micafungin, caspofungin, anidulafungin, *etc.*) (Fig. 1).

The mechanism of action of imidazole and triazole antifungal agents is associated with the impairment of the biosynthesis of ergosterol, which is a major component of fungal cell walls. These compounds inhibit the fungal cytochrome P450-dependent enzyme lanosterol 14α-deme-

O.A.Omelchuk. Junior Researcher at the Laboratory of Chemical Transformation of Antibiotics at the GINA, PhD student at the Department of Organic Chemistry of the MUCTR.
E-mail: omelchuk.93@mail.ru
Current research interests: organic synthesis, chemical modification methods of macrocyclic lactam antibiotics, structure – activity relationship studies.
A.N.Tevyashova. Doctor of Chemical Sciences, Senior Researcher at the Laboratory of Chemical Transformation of Antibiotics at the GINA, Assistant Professor at the Department of Organic Chemistry of the MUCTR.
E-mail: chulis@mail.ru
Current research interests: chemical modification of glycoside antibiotics, studies of structure – activity relationship and mechanisms of action of antibiotics.
A.E.Shchekotikhin. Doctor of Chemical Sciences, Professor of the Russian Academy of Sciences, Acting Director of the GINA, Head of the Laboratory of Chemical Transformation of Antibiotics at the same Institute, Head of the Department of Organic Chemistry of the MUCTR.
Telephone: +7(499)245 – 0295, e-mail: shchekotikhin@mail.ru
Current research interests: organic synthesis methodology, synthesis and study of biologically active compounds, development of new chemotherapeutic agents.

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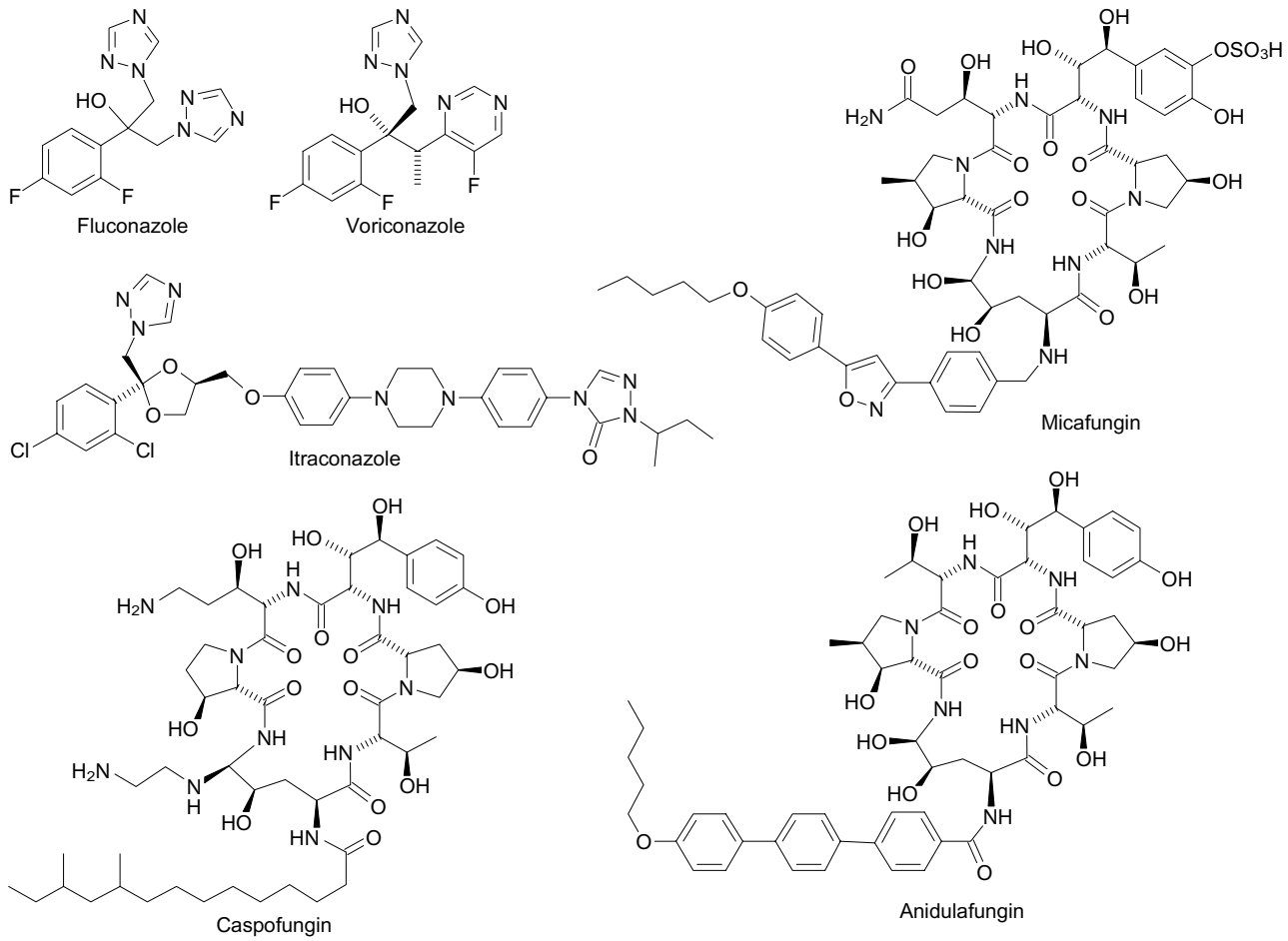


Figure 1. Structures of selected antifungal agents.

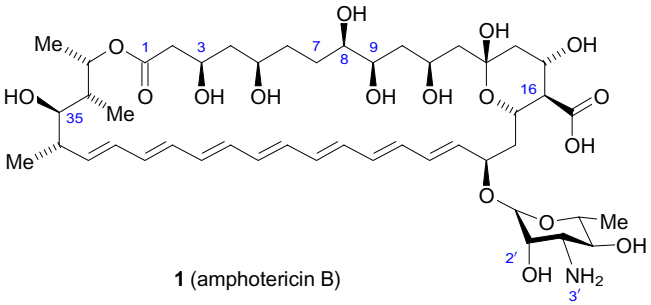
thylase, thereby blocking ergosterol synthesis. Echinocandins are cyclic peptide antibiotics bearing a hydrophobic side chain. They are noncompetitive inhibitors of (1,3)-β-D-glucan synthase responsible for the synthesis of 1,3-β-D-glucan. The latter is an essential component of fungal cell walls, while it is absent in mammalian cells. The blocking of 1,3-β-D-glucan synthesis leads to osmotic instability and fungal cell wall lysis.

Azole and echinocandin agents are characterized by low toxicity and a broad-spectrum antifungal activity.[3] However, the widespread use and long-term application of azoles and echinocandins in therapy of mycoses led to a serious threat of emergence and spread of resistant pathogenic fungal strains.[4–7] Therefore, polyene macrolide antibiotics, primarily amphotericin B (AmB, **1**), are still of great clinical importance. Amphotericin B remains the gold standard for antifungal therapy since it has evaded resistance for more than half a century (clinically significant resistance to AmB is very rare). Other significant polyene macrolides are nystatin A_1 (**2**) and natamycin (**3**) (Fig. 2). Common structural features shared by AmB, natamycin and nystatin include a conjugated double-bond system and the carbohydrate residue mycosamine.

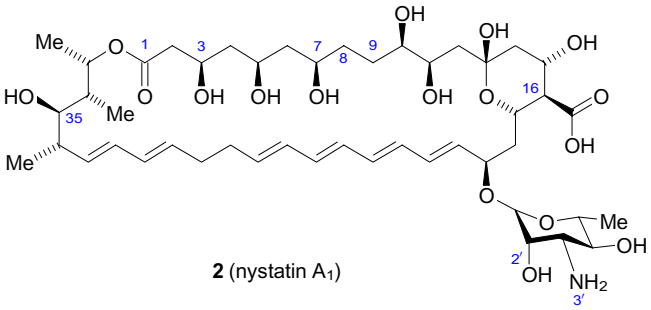
The mechanism of antifungal action of polyenes involves the inhibition of cell membrane function. It was found that amphotericin B can form channels in fungal cell membranes, thereby causing leakage of ions and small molecules from the cell. The exact nature of interactions of polyenes with cell membrane components has been extensively intvestigated. In this review, we summarize the most important results of these studies.

The use of polyene antibiotics as agents of choice is limited by high nephrotoxicity and hemolytic toxicity, low bioavailability and adverse effects associated with their parenteral use. The targeted structural modification by chemical or engineered biosynthetic approaches is an important way of reducing toxicity of natural antibiotics. Another approach for improving the toxicological profile of polyene antibiotics is based on the development of new drug formulations and targeted drug delivery systems. Despite a considerable amount of research on this topic, advances in the drug design based on macrolide polyenes have not been systematically analyzed. This review summarizes for the first time the progress achieved in the last five years in the improvement of pharmacological properties of polyene antibiotics by the above-mentioned methods and in studies of the mechanism of their action.

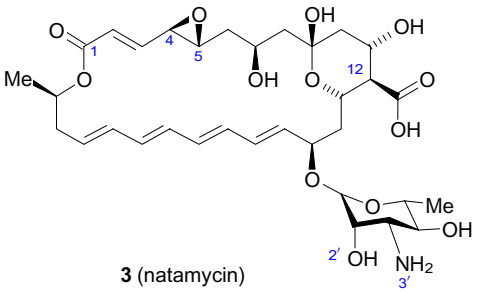
The following abbreviations are used in the review:
Alloc — allyloxycarbonyl,
AmB — amphotericin B,
Boc — *tert*-butoxycarbonyl,
CSA — camphorsulfonic acid,
DDQ — 2,3-dichloro-5,6-dicyano-1,4-benzoquinone,
DEIPS — diethylisopropylsilyl,



1 (amphotericin B)



2 (nystatin A_1)



3 (natamycin)

Figure 2. Structures of the polyene macrolides amphotericin B, nystatin A_1 and natamycin.

DIPEA — *N*,*N*-diisopropylethylamine,
DMAE — *N*,*N*-dimethylethylenediamine,
DMPU — 1,3-dimethyl-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one,
DPPA — diphenylphosphoryl azide,
dppf — 1,1′-bis(diphenylphosphino)ferrocene,
Fmoc — 9-fluorenylmethyloxycarbonyl,
Im — imidazole,
m-CPBA — *m*-chloroperoxybenzoic acid,
ME — microemulsion,
ME-AmB — microemulsion formulation of amphotericin B,
MIC — minimum inhibitory concentration,
MIDA — *N*-methyliminodiacetylboronate building blocks,
MNBA — 2-methyl-6-nitrobenzoic acid,
Ms — methanesulfonyl (mesyl),
MTT — 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,
NaHDMS — sodium bis(trimethylsilyl)amide,
NE-AmB — nanoemulsion formulation of amphotericin B,
PG — polyglucose,
PGGA — poly-γ-glutamic acid,
PI — propidium iodide (3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide),
Pip — piperidine,
PLGA-PEG — poly(lactide-co-glycolide)-block-poly(ethylene glycol),
PMB — *p*-methoxybenzyl,
PMP — *p*-methoxyphenyl,
POPC — palmitoyloleoylphosphatidylcholine,
PrG — protective group,
Py — pyridine,
PyBOP — (benzotriazol-1-yloxy)tri(pyrrolidino)phosphonium hexafluorophosphate,
ROS — reactive oxygen species,
SDS — sodium 3α,12α-dihydroxy-5β-cholane-24-*O*-sulfate,
Su — succinimide,
TBAF — tetra-n-butylammonium fluoride,
TBS — *tert*-butyl(dimethyl)silyl,
TDBTU — *O*-(4-oxo-3,4-dihydro-1,2,3-benzotriazin-3-yloxy)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate,
TES — triethylsilyl,
Tf — triflyl (trifluoromethanesulfonyl),
TFA — trifluoroacetic acid,
TIPS — triisopropylsilyl,
TMS — trimethylsilyl,
TMSE — (trimethylsilyl)ethyl,
XPhos — 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl.

2. Investigation of the mechanism of action of polyene antibiotics

Despite more than five decades of research, the mechanism of action of AmB and other polyene antibiotics is not fully elucidated. Experimental studies showed that polyene macrolides have higher affinity for ergosterol than for other sterol components of cell membranes. For polyene macrolides, the mechanism of action was most studied for amphotericin B. It has long been assumed that the mechanism of antifungal action of polyenes is primarily associated with the formation of transmembrane channels and subsequent leakage of ions from the fungal cells. Alternative models of biological action of these antibiotics are also available. Thus, it was suggested that the antifungal effect of amphotericin B is based on extraction of sterols, which reside on the outer surface of the membrane lipid bilayer, resulting in the disruption of sterol-dependent intracellular biochemical processes. There is experimental evidence that amphotericin B causes induction of oxidative stress both in fungal and mammalian cells.

The advances in studies of interactions of AmB with sterols in the lipid bilayer are summarized in the review.[8] Besides, some aspects of the mechanism of antifungal action of polyenes are considered in studies of 2010 dealing with amphotericin B (Ref. 9) and chemical modification of polyene macrolides.[10] As opposed to the published reviews, the

present section considers key issues of interactions of polyene antibiotics with the cell membrane in terms of their effect on therapeutic activity and toxicity of these compounds. Emphasis is given to the analysis of new studies of secondary mechanisms of biological activity of polyenes and factors responsible for the rare occurrence of resistance to amphotericin B, which have not been mentioned in earlier studies.

2.1. Interactions of polyenes with lipids in the cell membrane

Welscher *et al.*[11] demonstrated that the tetraene antibiotic natamycin (**3**) does not form pores in fungal cell membranes, and its antifungal effect is based only on specific binding to ergosterol. The results of studies of Arima *et al.* (see, for example, Ref. 12) provide evidence that the penetration of natamycin into Langmuir monolayers of cholesterol and disruption of their integrity are observed only at a cholesterol concentration of 50%, which is much higher than the cholesterol concentration in the mammalian cell membrane. The effect of natamycin on a monolayer, which mimics a real cell membrane, *i.e.*, on the monolayer containing 25% cholesterol, proved to be insignificant. High affinity for ergosterol, the absence of channel-forming activity and weak interactions with cell membranes in mammals may account for its low toxicity in the case of oral or topical administration for the treatment of superficial fungal infections.

Interactions of amphotericin B with lipid bilayers of different composition [palmitoyloleoylphosphatidylcholine (POPC), POPC – cholesterol, POPC – ergosterol], which mimic fungal and mammalian cell membranes, were studied by Foglia *et al.*[13] They found that AmB causes a more pronounced change in the surface pressure in the POPC – ergosterol membrane; a similar pattern was observed for the rate of antibiotic interactions with lipid monolayers. Amphotericin B induces the formation of small domains in the POPC monolayer, which shrink and disappear within a few minutes. In cholesterol-containing layers, AmB also causes the formation of smaller domains, but they are formed much more slowly than in POPC layers, whereas the formation of domains in the ergosterol-containing layer occurs quickly and these domains are more heterogeneous in size. The results of this study confirm the selective effect of AmB on ergosterol-containing fungal membranes.

Neumann *et al.*[14] studied the effect of sterols on amphotericin B self-aggregation in a lipid bilayer by molecular dynamics simulation. It was found that head-to-head dimers are formed in all the lipid layers under study (ergosterol-containing, cholesterol-containing and sterol-free). Electrostatic interactions of the polyol components of the AmB molecule and hydrogen bonding between polyols are the driving force for dimerization. Head-to-head dimers are thermodynamically least favourable and kinetically least stable in the ergosterol-containing lipids bilayer. Consequently, at therapeutic concentrations AmB exists in ergosterol-containing membranes mainly in the monomeric form. The observed tendency of AmB to form dimers in lipids of different composition is consistent with different affinity of this antibiotic for sterols. The authors suggested that channels are more likely to be formed by amphiphilic AmB species (monomers or AmB – sterol complexes) than by stable dimers with a hydrophobic outer surface. Strong interactions of AmB with cell membrane components prevent the formation of stable dimers, thereby increasing the channel-forming activity of the antibiotic.

Kang *et al.*[15] evaluated vacuole-targeting fungicidal activity of AmB against the *Saccharomyces cerevisiae* strain BY4741 and its AmB-resistant mutant, in the cell wall of which ergosterol was replaced by episterol. At therapeutic concentrations, AmB was found to be easily incorporated into vacuoles of the wild-type strain and is accumulated there, whereas its concentration in the cellular cytoplasm of the mutant strain is insignificant. The results of this experiment suggest that ergosterol is essential for the penetration of amphotericin B into the cell.

Burke's research team[16] proposed a new sterol sponge model and demonstrated that the ability of AmB to form extramembranous aggregates is a necessary condition for manifestation of antifungal activity. These aggregates extract ergosterol from the fungal cell wall, thereby breaking its integrity. According to this model, the formation of transmembrane channels plays a minor role in antifungal activity of AmB. This discovery contributes to understanding of the factors responsible for selectivity of AmB towards fungi and provides an explanation for the absence of resistance to this antibiotic. The removal of ergosterol from the cell membrane leads to impairment of a number of vitally important ergosterol-dependent intracellular metabolic processes, and the simultaneous mutation of all genes encoding all proteins that are involved in these processes is unlikely. The separation of the channel-forming and sterol-extracting activities of amphotericin B is a new promising approach for reducing its toxicity for humans.[16]

2.2. Study of secondary mechanisms of action of polyenes

Ample experimental data suggest the existence of other biological targets for polyene antibiotics in fungal cells. Chudzik *et al.*[17] studied the effect of amphotericin B on the plasmic membrane of *Candida albicans* protoplasts. At concentrations of 0.05 – 0.1 μmol litre^{-1}, AmB causes a decrease in the membrane electrical resistance, which correlates with a decrease in membrane tightness. The treatment of the protoplasts with AmB at therapeutic concentrations does not change the percentage of living and dead cells (PI-positive, PI is propidium iodide) but causes a significant decrease in the population of replicating cells as well as morphological and structural pathological changes, including cytoplasm shrinking, alteration of the morphology of the nucleus and mitochondria, a sudden decrease in the reduction level of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and oxidative stress. The results of this study show that the induction of *C. albicans* cell death by AmB is a multistage and long-term process involving intracellular signal pathways.[17]

Ferreira *et al.*[18] considered the possibility of induction of endogenous reactive oxygen species (ROS) and peroxynitrite by AmB in cells of the *Cryptococcus gattii* reference strain ATCC 32068 and *C. gattii* clinical isolates. The authors showed that AmB causes lipid peroxidation in *C. gattii* cells, thereby stimulating the production of reactive O- and N-containing radicals that enhance peroxidase activity. Besides, the synergistic enhancement of antifungal activity of AmB was observed in the presence of catalase/superoxide dismutase inhibitors and, on the contrary, this activity was found to be reduced in the presence of peroxynitrite scavengers.[18]

More recently, Mesa-Arango *et al.*[19] demonstrated that the production of reactive oxygen species is a key component of the mechanism of antifungal action of amphotericin B. It was found that AmB induces the generation of ROS in all 44 tested strains of pathogenic isolates (*Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Cryptococcus neoformans* and *Cryptococcus gattii*). The formation of ROS in AmB-resistant cultures was studied. The inhibition of the respiratory chain complex I in cells of AmB-susceptible strains by the specific inhibitor rotenone at concentrations which do not cause cell death blocks the induction of ROS by AmB, thereby protecting cells from death. However, this effect was not observed in cells of amphotericin B-resistant strains. The investigation of the characteristic features of AmB-resistant fungal strains demonstrated that the basal cell respiration in these strains proceeds through an alternative route inherent in all *Candida* species. Besides, the AmB-resistant strains showed an alteration in the mitochondrial membrane potential and had higher catalase activity compared to the AMB-susceptible strains. It was found that AmB does not induce protein carbonylation in the resistant strains.[19] Therefore, the induction of reactive oxygen species by amphotericin B is a general phenomenon, and the oxidative/nitrosative stress plays a key role in the antifungal action of this antibiotic, which can be considered as another possible explanation for the low level of development of resistance to AmB.

Recently, high affinity of AmB towards DNA was revealed by isothermal titration calorimetry, UV-Vis spectroscopy and circular dichroism spectroscopy. Molecular docking studies showed that the binding of amphotericin B to DNA is energetically favourable. The treatment of human embryonic kidney cells (HEK-293) by this antibiotic led to an increase in the induction of DNA repair proteins. It was demonstrated that AmB can intercalate into DNA strands, thereby causing their damage.[20] When using AmB for the therapy of mycoses, one should take into account not only oxidative stress but also genotoxicity of the antibiotic against human cells.

Baek *et al.*[21] described the biological effect of nystatin, which has been previously unknown for polyene antibiotics. This antibiotic was found to induce the production of the proinflammatory MIP-1 chemokine in a dose-dependent fashion, which causes macrophage chemotaxis towards the site of infection and accelerates health recovery.

To study the factors responsible for the lack of resistance to AmB and other polyene antibiotics, Vincent *et al.*[22] sequenced genomes of AmB-resistant strains of the pathogenic fungi *Candida* sp. They demonstrated that the resistance of the *C. albicans* ATCC 200955 clinical isolate to polyenes is associated with chromosomal transposon mutation in the ERG2 locus, thereby causing induction of the stress state in the cells. A high level of expression of the heat shock protein Hsp90 is required for survival of the mutant strain, due to which this strain is hypersensitive to oxidative stress and febrile temperatures and it can easily be attacked by the immune system of the infected organism. Moreover, AmB-resistant strains have defects in filamentation and tissue and organ invasion. These fungi are avirulent in *in vivo* assays in mice. Antibiotics that cause fungal avirulence are highly promising as new-generation agents.

3. Chemical modification of natural polyene antibiotics

Advances in modifications of polyene antibiotics achieved at the end of the 20th century–first decade of the 21th century are summarized in a review.[10] Derivatives of AmB, the most important representative of polyene macrolides, which were prepared since the discovery of AmB and up to 2010, were described by Volmer *et al.*[9] The modifications at the carboxyl group attached to the C(16) atom and at the amino group of the mycosamine residue of AmB were studied in most detail. The carboxyl group at position 16 is easily accessible to chemical modification, for example, to esterification or amidation. The amino group of mycosamine can be chemically modified by condensation with carboxylic acids or by means of alkylation. It was demonstrated that the presence of a partially or fully charged nitrogen atom is essential for biological activity of amphotericin B, while the negatively charged carboxyl group at the C(16) atom has no significant effect on the biological properties of the antibiotic. A number of derivatives with reduced haemotoxicity and enhanced antifungal activity were prepared by modification of this position. Therefore, this chemical modification of polyenes holds promise for the design of highly effective and safe agents for the treatment of mycoses.

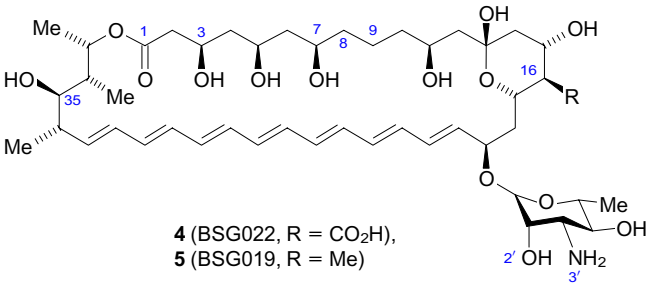
3.1. Local structural changes of polyenes: modification of hydroxy groups and substituents at the C(16) and N(3′) atoms

Preobrazhenskaya's research team[23,24] performed systematic structure–activity relationship analysis of a series of polyene antibiotics. New derivatives of amphotericin B (**1**) and biosynthetic analogues of nystatin, namely, BSG022 (**4**), BSG003 (**6**) and BSG005 (**8**), modified at the C(16)-carboxyl group (see below compounds **10**–**15**, **17** and **18**) or at the amino group of mycosamine (compounds **16** and **19**–**21**) were prepared using methods developed earlier[23] for the transformation of the polyene antibiotic S44HP (**9**) (Fig. 3). Besides, the authors evaluated activity of the previously uncharacterized C(16)-methyl derivatives BSG019 (**5**) and BSG018 (**7**) (see Fig. 3).

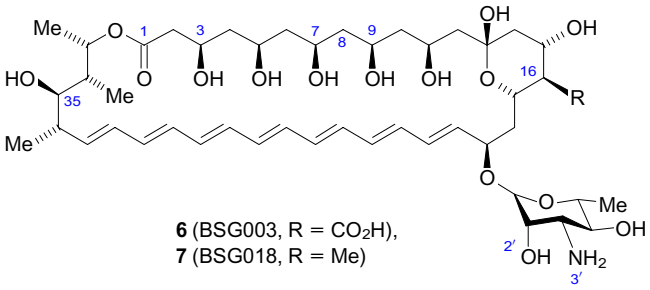
Four modification pathways were tested: amidation of the carboxyl group in position 16; glycosylation at the 3′-amino group followed by the Amadori rearrangement of N-glycosyl derivatives; reductive alkylation of the amino group in position 3′ of mycosamine; and acylation of the amino group in position 3′. The first route was used to prepare a series of AmB derivatives, carboxamide **10**–**15**; the second modification gave Amadori rearrangement product **16**. The yields of the target compounds were not reported in the cited study (Scheme 1).

To study the effect of the polyol chain on biological activity of polyenes, water-soluble *N*-(2-dimethylaminoethyl)amides **17** and **18** were prepared by carboxamidation of the compounds BSG003 and BSG022 with *N*,*N*-dimethylethylenediamine (DMAE) in the presence of a condensing agent (Scheme 2).[24]

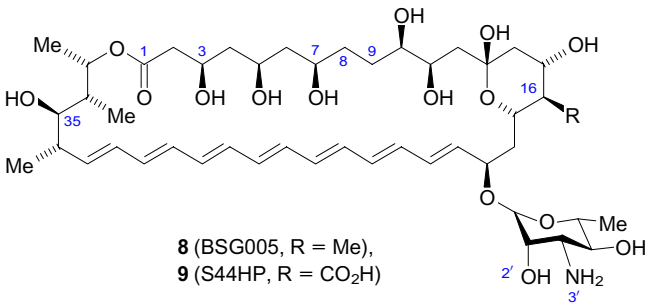
N(3′)-Aminoacyl derivative **19** and N(3′)-alkyl derivatives **20** and **21** were prepared by modification of the amino group of the mycosamine moiety of the polyene BSG005 (Scheme 3).[24]



4 (BSG022, R = CO_2H),
5 (BSG019, R = Me)



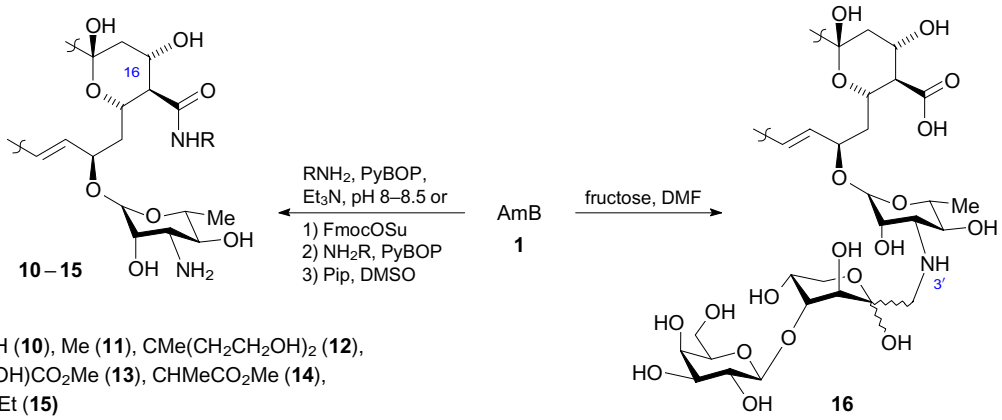
6 (BSG003, R = CO_2H),
7 (BSG018, R = Me)



8 (BSG005, R = Me),
9 (S44HP, R = CO_2H)

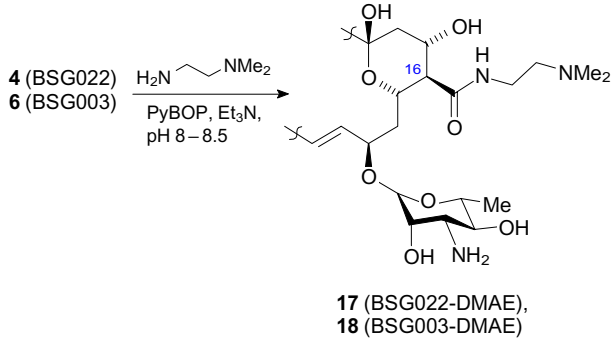
Figure 3. Structures of the polyene macrolides S44HP, BSG005, BSG022, BSG003, BSG019 and BSG018 prepared using modified genetically engineered producer strains.[23, 24]

Scheme 1



R = $(CH_2)_2OH$ (**10**), Me (**11**), $CMe(CH_2CH_2OH)_2$ (**12**), $CH(CH_2OH)CO_2Me$ (**13**), $CHMeCO_2Me$ (**14**), CH_2CO_2Et (**15**)

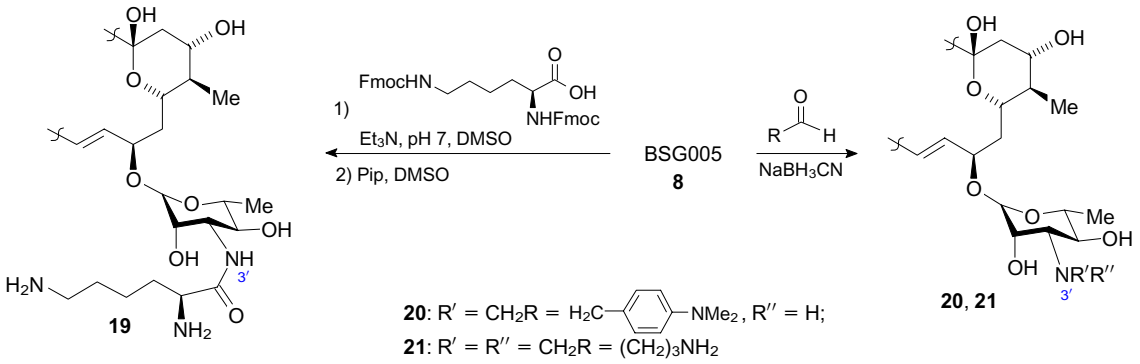
Scheme 2



17 (BSG022-DMAE),
18 (BSG003-DMAE)

New compounds were tested for antifungal activity as compared with the related derivatives of the antibiotic S44HP (Ref. 23) and the starting polyenes. This study revealed the effect of the polyol chain C(7) – C(10) and substituents at the C(16) and N(3′) positions. A comparative analysis of *in vitro* antifungal activity of the corresponding derivatives with reference to the arrangement of hydroxy groups showed that the most active polyenes contain OH groups in positions 8 and 9 or in positions 7 and 10 [AmB (**1**), BSG005 (**8**), S44HP (**9**), compounds **10** – **15** and related S44HP derivatives], whereas the derivatives containing OH groups in positions 7 and 9 (polyenes **4** – **7**) have the lowest activity. Besides, the introduction of the *N*,*N*-dimethylethylenediamine moiety (compounds **17** and **18**) did not lead to an increase in activity, whereas the activity of S44HP-DMAE (see Ref. 23) was the same as that of AmB. In the N-unsubstituted analogues, the replacement of the carboxyl group [S44HP (**9**)] by methyl [BSG005 (**8**)] in position 16 has no significant effect on antifungal activity. On the contrary, biological properties of polyene BSG005 derivatives **19** – **21** modified at the amino group and related S44HP derivatives[23] depend both on the nature of the substituent in position 16 and the nature of the substituent in the mycosamine moiety.[24] The screening of the library of the synthesized derivatives for antifungal activity revealed the most promising compounds for *in vivo* studies using a murine Candida sepsis model with leukopenia. Based on the results of these studies, semisynthetic polyene antibiotics [*N*-(L-lysyl)-BSG005 (**19**) and 2-(*N*,*N*-dimethylamino)ethylamide of acid S44HP (Ref. 23)] were found to exhibit *in vitro* and *in vivo* activity comparable with that of AmB and have low toxicity and they were selected for thorough preclinical trials.[24]

Scheme 3

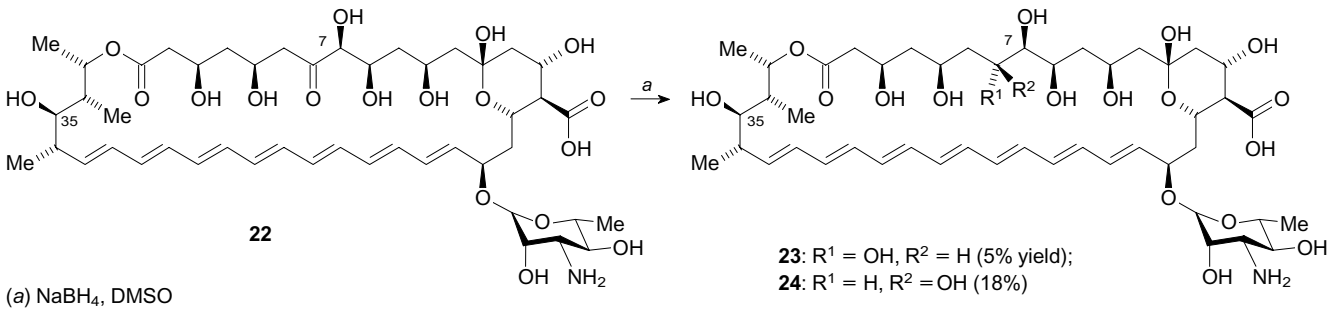


Yamamoto *et al.*[25] studied the effect of the polyol moiety of AmB on its biological properties.[25] The evaluation of biological properties of 7-oxoamphotericin B (**22**), which has been synthesized earlier by a microbiological method, showed that the introduction of the oxo group at position 7 of the polyol unit of AmB leads to an eightfold decrease in antifungal activity and loss of haemolytic activity. The reduction of the C(7)-keto group to the hydroxy group by treatment of 7-oxoamphotericin B (**22**) with sodium borohydride affords a mixture of diastereomers **23** and **24** (Scheme 4). In general, 7-hydroxyamphotericins **23** and **24** proved to be less active than AmB; however, as opposed to derivative **22**, compounds **23** and **24** have haemolytic activity. The observed effects are apparently attributed to the impairment of the optimal conformational rigidity of the macrocycle.[25]

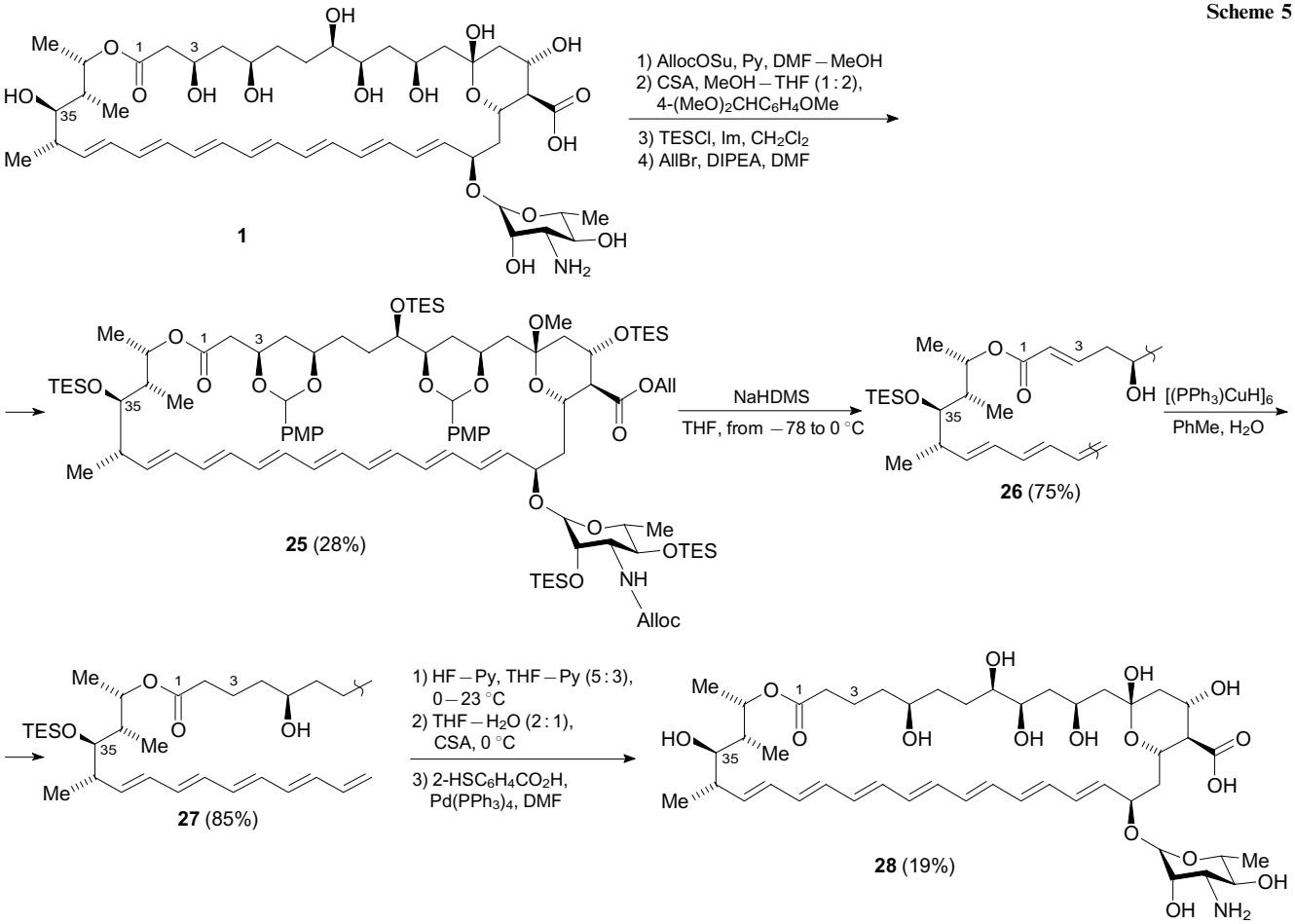
Recently, it was shown that AmB, due to its tendency to form ion channels, can replace transporter proteins missing in yeast mutant strains, thereby restoring a normal physiological state of the cells.[26] Davis *et al.*[27] performed molecular modelling studies and suggested that AmB molecules self-assemble in a lipid membrane layer, with polyol regions lining a channel interior and polyene moieties of AmB pointing outside. In the proposed model, the narrowest region of the channel is near the C(3)-hydroxy group. Hence, the modification of this hydroxy group would alter conductance of the transmembrane channel of amphotericin B. To test this hypothesis, the authors synthesized the 3-deoxy derivative of AmB. Since the amphotericin B molecule contains 10 hydroxy group, the selective removal of one OH group is a challenging issue. However, the unique β positioning of the C(3)-OH group with respect to the carboxyl carbon atom C(1) of lactone provides an opportunity for selective elimination of a water molecule to form an α,β-unsaturated lactone, and the subsequent chemoselective reduction of the latter gives target product **28**. This transformation was accomplished in nine steps. In the first four steps, all free OH groups and the amino group of amphotericin were protected to form compound **25**. The treatment of derivative **25** with sodium bis(trimethylsilyl)-amide (NaHMDS) at low temperature led to selective elimination of *p*-methoxyphenyl acetal at position 3 to form intermediate **26**. The α,β-unsaturated C–C bond of the macrolactone was subjected to regioselective reduction with Stryker's reagent. The sequential removal of all protective groups in intermediate **27** yielded targeted 3-deoxyamphotericin B (**28**) (Scheme 5). The new derivative was characterized by multidimensional NMR spectroscopy and the single ion channel electrophysiology method. It was shown that 3-deoxyamphotericin B possesses the same macrocycle conformation as the native antibiotic. The new derivative is also able to form transmembrane ion channels; however, the ion conductance of these pores is three times lower compared to the channels formed by AmB. Therefore, the C(3)-hydroxy group of amphotericin B plays a key role in the conductance of the transmembrane channel and, consequently, modifications of this position provide an opportunity for further tuning of functions of the resulting channels.[27]

The modification of position 2′ of mycosamine in AmB is of particular interest because the 2′-hydroxy group is presumably involved in binding of polyene to sterols.[8] Croatt and Carreira[28] described the synthesis of three new 2′-derivatives of AmB and evaluated their biological activity compared to AmB. The derivatives were synthesized by glycosylation of aglycone of 16-methoxycarbonylamphotericin B (**33**), which was prepared by a method developed earlier, with a mycosamine analogue **32**. Intermediates **32** and **33** were not isolated and purified because of their instability. Mycosamine **32** was synthesized by epoxidation of 3-hydroxy-6-(4-methoxybenzyloxy)-2-methyl-3,6-dihydropyran (**29**) followed by epoxide ring opening of com-

Scheme 4



Scheme 5



pound **30** with sodium azide, acetylation of the 3-hydroxy group, removal of the methoxybenzyl protection in derivative **31** and condensation of the 2-OH group with trichloroacetonitrile (Scheme 6). Glycosylation with mycosamine **32** and subsequent removal of all protective groups gave 2′-*epi*-16-methoxycarbonylamphotericin B (**35**). Its isomer **37** was prepared by a similar reaction of aglycone **33** with (±)-mycosamine **32**, deprotection and separation of the mixture of products **35** and **37** by reversed-phase HPLC. The alkylation of the 2′-OH group with methyl iodide after the removal of acyl protection afforded 2′-*epi*-2′-methoxy-16-methoxycarbonylamphotericin B (**36**) (see Scheme 6).[28] The authors did not report the total yield of the target compounds.

The activity evaluation of these derivatives demonstrated that the configuration of the 2′-hydroxy group has no effect on biological activity of antibiotic **35**. 2′-Methoxy derivative **36** exhibited much lower activity than AmB (**1**), and anomer **37** of AmB was fully inactive. These results suggest the important role of the mycosamine moiety in antifungal properties of AmB.[28]

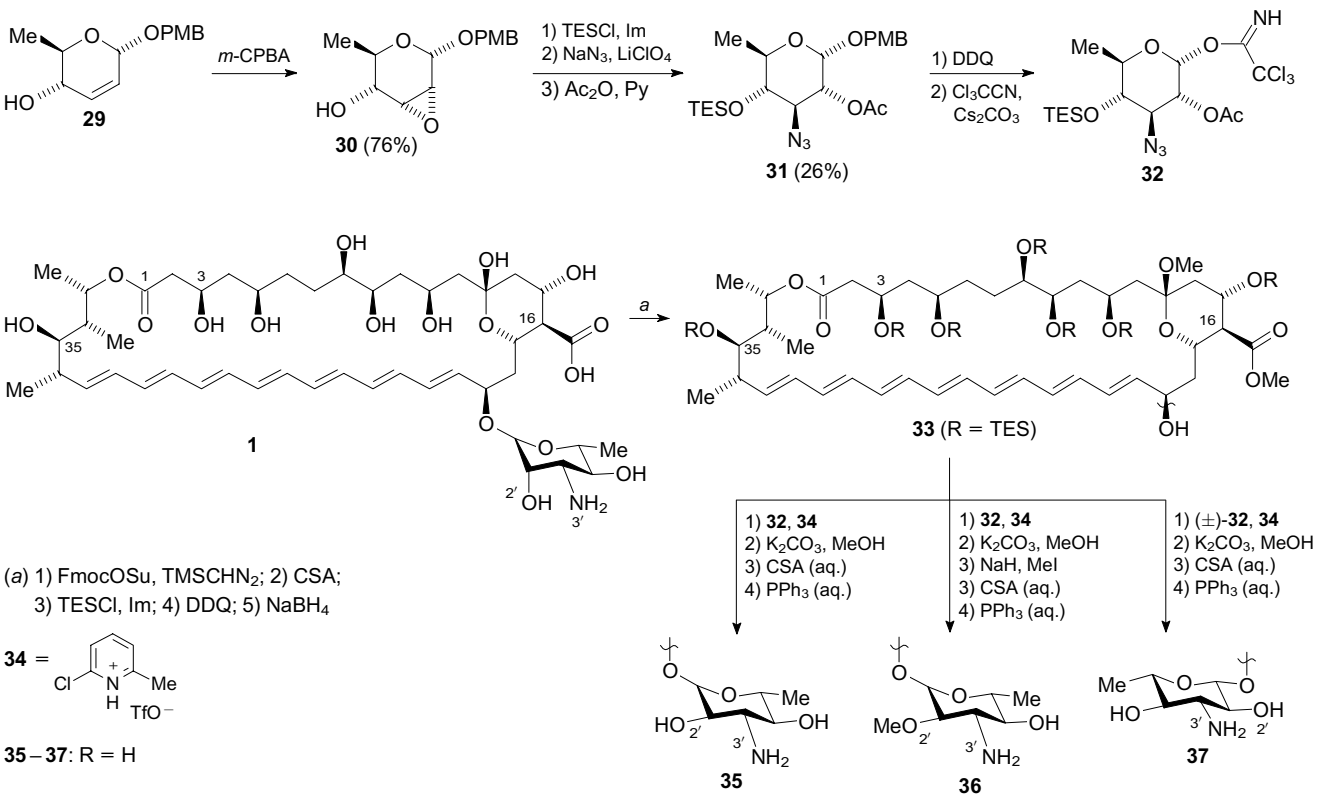
Burke's research team[29] developed a procedure for regioselective acylation of the 2′-hydroxy group of AmB mycosamine. 2′-Acyl derivative **39** prepared from partially protected substrate **38** is a convenient intermediate for the synthesis of 2′-substituted AmB derivatives. More recently, these authors[30] made an attempt to synthesize 2′-deoxyamphotericin B (**41**) from 2′-acyl derivative **39**; however, after the removal of the 2′-hydroxy group (compound **40**), the molecule became more labile and the yield of the target compound was unacceptably low (Scheme 7).

Target 2′-deoxy-AmB was synthesized by glycosylation of AmB aglycone **44** (prepared from substrate **43**) with 4-azido-5-(*tert*-butyldimethylsilyloxy)-2-hydroxy-5-methyltetrahydropyran (**42**) (Scheme 8).[30] After deprotection of compound **45**, the product was isolated in 12% yield.

Unexpected results were obtained in the study of the binding of AmB derivative **41** to sterols. Thus, 2′-deoxy-AmB (**41**) binds ergosterol but does not bind cholesterol. Moreover, *in vitro* antifungal activity of derivative **41** is similar to that of the natural antibiotic, whereas it is nontoxic to human cells. This is apparently due to the fact that the 2′-hydroxy group of mycosamine stabilizes the antibiotic conformation favourable for binding sterols *via* hydrogen bonding with the C(13) position of the aglycone through a bridging water molecule. The removal of the 2′-OH group should alter the native conformation of AmB and facilitate more selective binding of ergosterol, as confirmed experimentally. Therefore, derivatives devoid of the 2′-OH groups hold great promise for the development of new antifungal agents.[30]

Later, these authors suggested that the salt bridge between the carboxyl group at the C(16) atom and the 3′-amino group stabilizes the macrocycle conformation favourable for binding of both ergosterol and cholesterol. Based on this assumption, Burke's research team[31] made efforts to break the intramolecular C(16) – N(3′) bridge. They found that 16-azidocarbonyl-AmB, which was pre-

Scheme 6

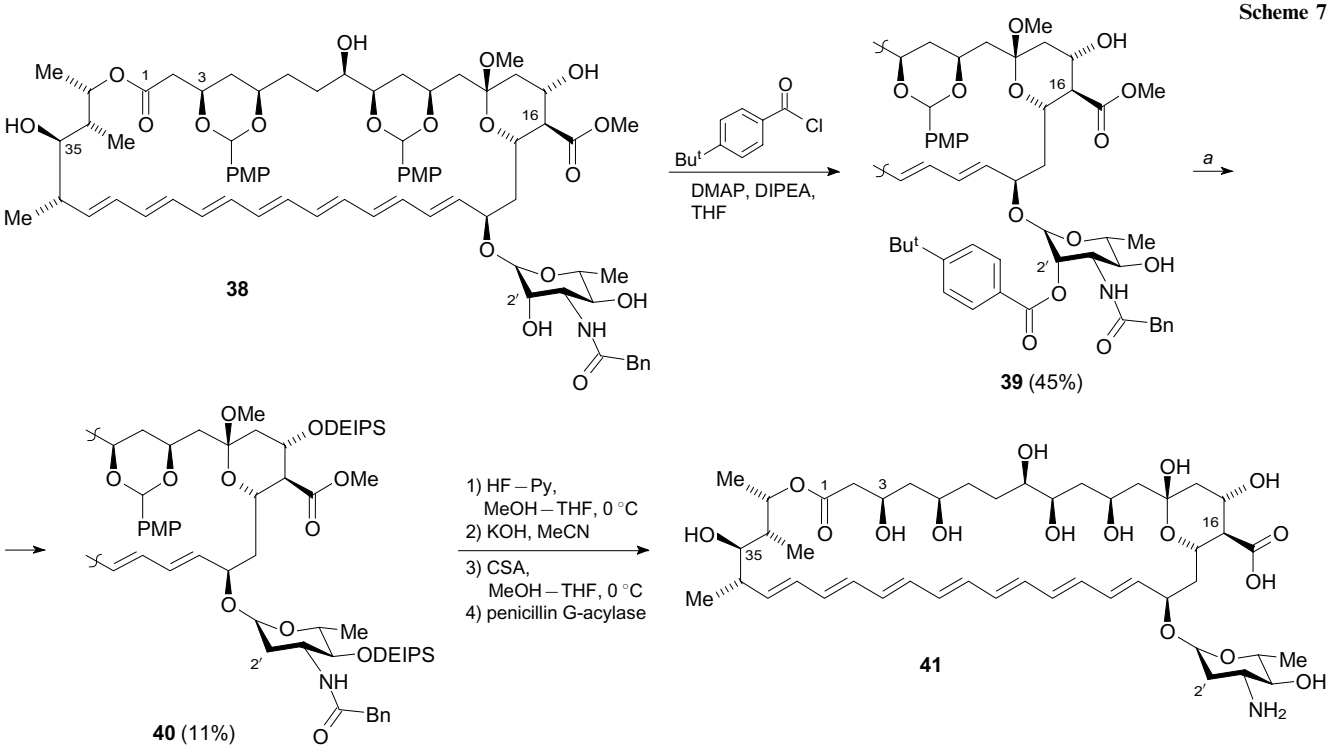


(*a*) 1) FmocOSu, $TMSCHN_2$; 2) CSA; 3) TESCl, Im; 4) DDQ; 5) $NaBH_4$

34 =

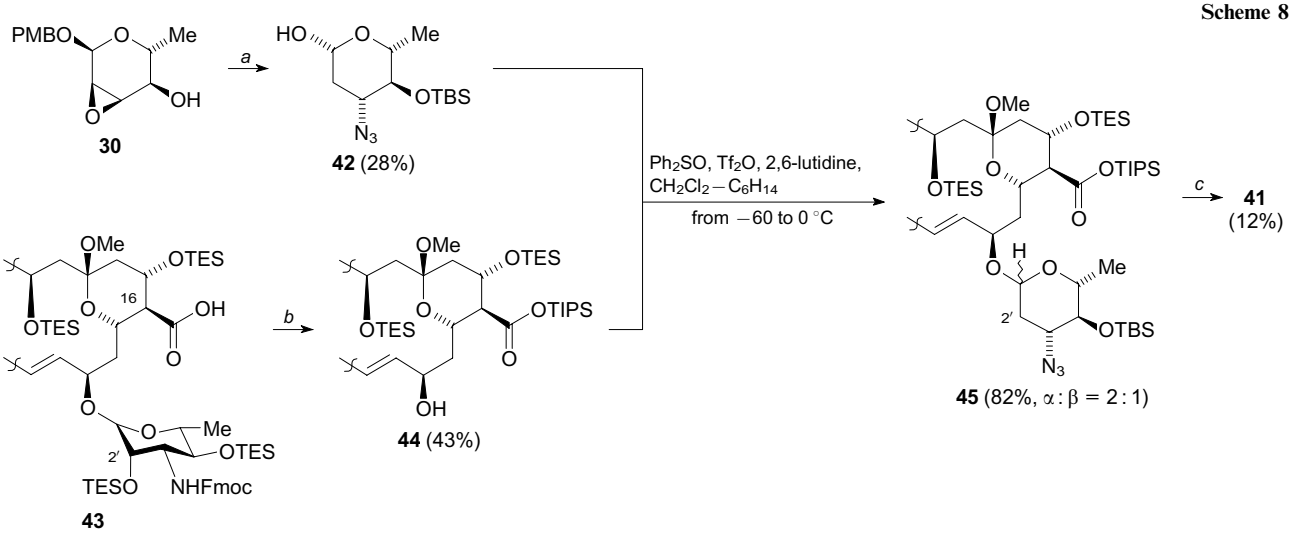
35 – **37**: R = H

Scheme 7



(*a*) 1) DEIPSOTf, 2,6-lutidine, $CH_2Cl_2 - C_6H_{14}$, 0 °C; 2) KCN, MeOH – THF (2 : 1), 40 °C; 3) triiodoimidazole, Im, PPh_3, PhMe, 70 °C; 4) $NaBH_4$, AgOAc, DMPU, 55 °C

Scheme 8



(*a*) 1) TBSCl, Im, CH_2Cl_2; 2) $LiHBEt_3$, THF; 3) MsCl, Py, THF, 0 – 23 °C; 4) NaN_3, DMF, 160 °C; 5) DDQ, $CH_2Cl_2 - H_2O$;
(*b*) 1) TIPSOTf, 2,6-lutidine, C_6H_{14}, 0 °C; 2) DDQ, $CaCO_3$, THF, 0 – 23 °C; 3) $NaBH_4$, THF – MeOH, 0 °C;
(*c*) 1) HF, Py, THF – MeOH, 0 °C; 2) PMe_3, H_2O, DMSO, 55 °C; 3) CSA, THF – H_2O

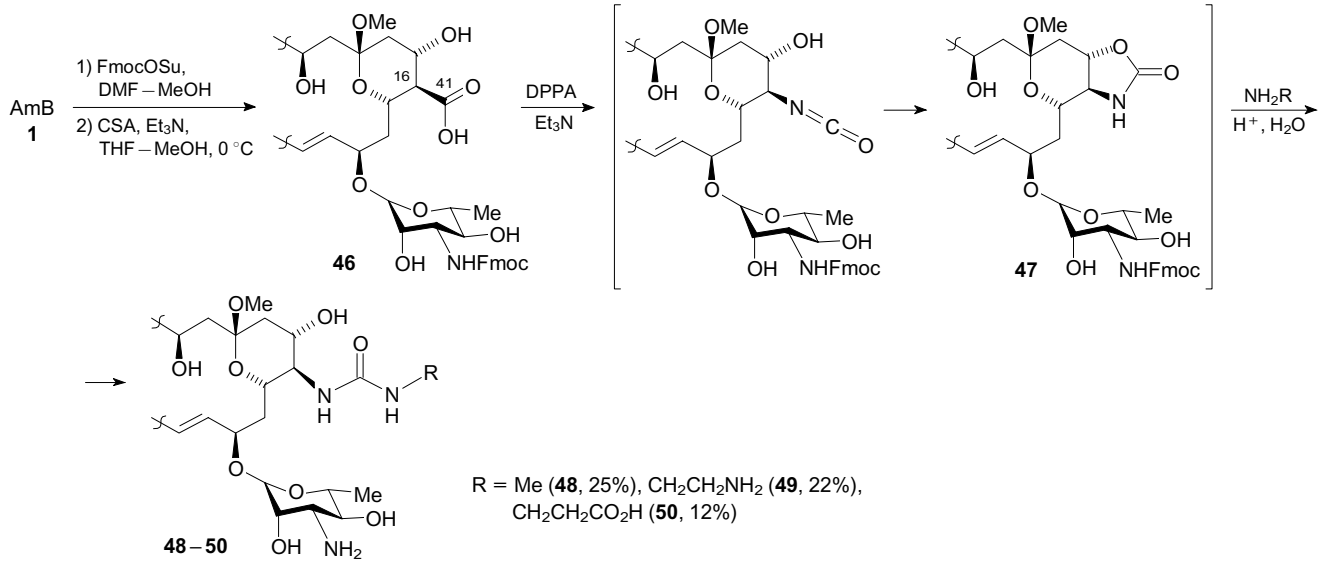
pared by the reaction of AmB derivative **46** with diphenylphosphoryl azide, easily undergoes a stereospecific Curtius rearrangement resulting in the C(16) – C(41) bond cleavage and the formation of intermediate isocyanate, which cyclizes to oxazolidinone **47** *via* an intramolecular attack of the adjacent C(15) hydroxy group. The treatment of the latter with primary amines leads to the cleavage of cyclic carbamate giving rise to a new class of carbamide derivatives of amphotericin B **48** – **50** (Scheme 9).[31]

Carbamide derivatives **48** – **50** proved to be less toxic than native AmB and be more effective in *in vivo* assays using a model of systemic mycosis. Moreover, attempts to obtain AmB-carbamide-resistant strains demonstrated that the new compounds, like AmB, do not cause resistance.[31] Since this new class of AmB-carbamides shows great promise, the method for their synthesis and the use for the treatment of mycoses are covered by a patent.[32]

Recently, Borowski's research group[33] patented a new type of AmB derivatives substituted at the amino group of the mycosamine moiety and their pharmaceutical formulations. New derivatives are characterized by the presence of bulky substituents that create steric hindrance between the amino and carboxyl groups, resulting in a decrease in antibiotic toxicity.

Later, this research team patented[34] N-substituted nystatin A_1 derivatives with improved pharmacological properties. Like in the case of amphotericin B, bulky substituents were introduced into the mycosamine moiety of nystatin A_1. The resulting nystatin A_1 derivatives are promising agents for the treatment of opportunistic oral infections caused by yeast fungi of the genus *Candida*. Boros-Majewska *et al.*[35] studied antifungal activity of these derivatives and their toxicity to human cells using an *in vitro* candidiasis model based on reconstituted human oral epithelium

Scheme 9



(RHOE). The measurement of lactate dehydrogenase activity released by RHOE showed a fourfold decrease in the toxic effect of nystatin A_1 derivatives on the epithelial tissue compared to native nystatin. Moreover, N-substituted nystatin A_1 derivatives proved to be more active against *Candida* spp. than the native antibiotic, as was demonstrated by estimation of *C. albicans* ATCC 10231 and epithelial cell viability after infection of RHOE by means of selective staining of dead or living cells. A confocal laser scanning microscopy study showed that in the absence of antibiotics, *C. albicans* extensively invaded oral epithelial cells. New nystatin A_1 derivatives can substantially reduce or completely prevent the fungal invasion.[35]

Burke's research group[36] synthesized 35-deoxyamphotericin B (**52**) *via* interative assembly of *N*-methyliminodiacetyl boronate (MIDA) building blocks (Scheme 10). It should be noted that the coupling of only twelve MIDA blocks allows the synthesis of conjugated double bond motifs found in 75% of natural polyene antibiotics.[37]

Derivative **52** retains the ability to bind ergosterol, but it is incapable of forming transmembrane channels in fungal cells. Antifungal activity of 35-deoxyamphotericin B (**52**) is comparable with that of the native antibiotic. These data support the hypothesis that ergosterol binding is the primary mechanism of antifungal activity of AmB, while the channel formation represents a second complementary mechanism.[36] Later, 35-deoxyamphotericin B (**52**) was used in the study of restoration of cell growth in fungi by replacing missing protein ion transporters with AmB. It was shown that at concentrations below the minimum inhibitory concentration (MIC), AmB restores cell growth in the impaired fungal culture, while derivative **52** does not cause the induction of cell division at any tested concentration.[26]

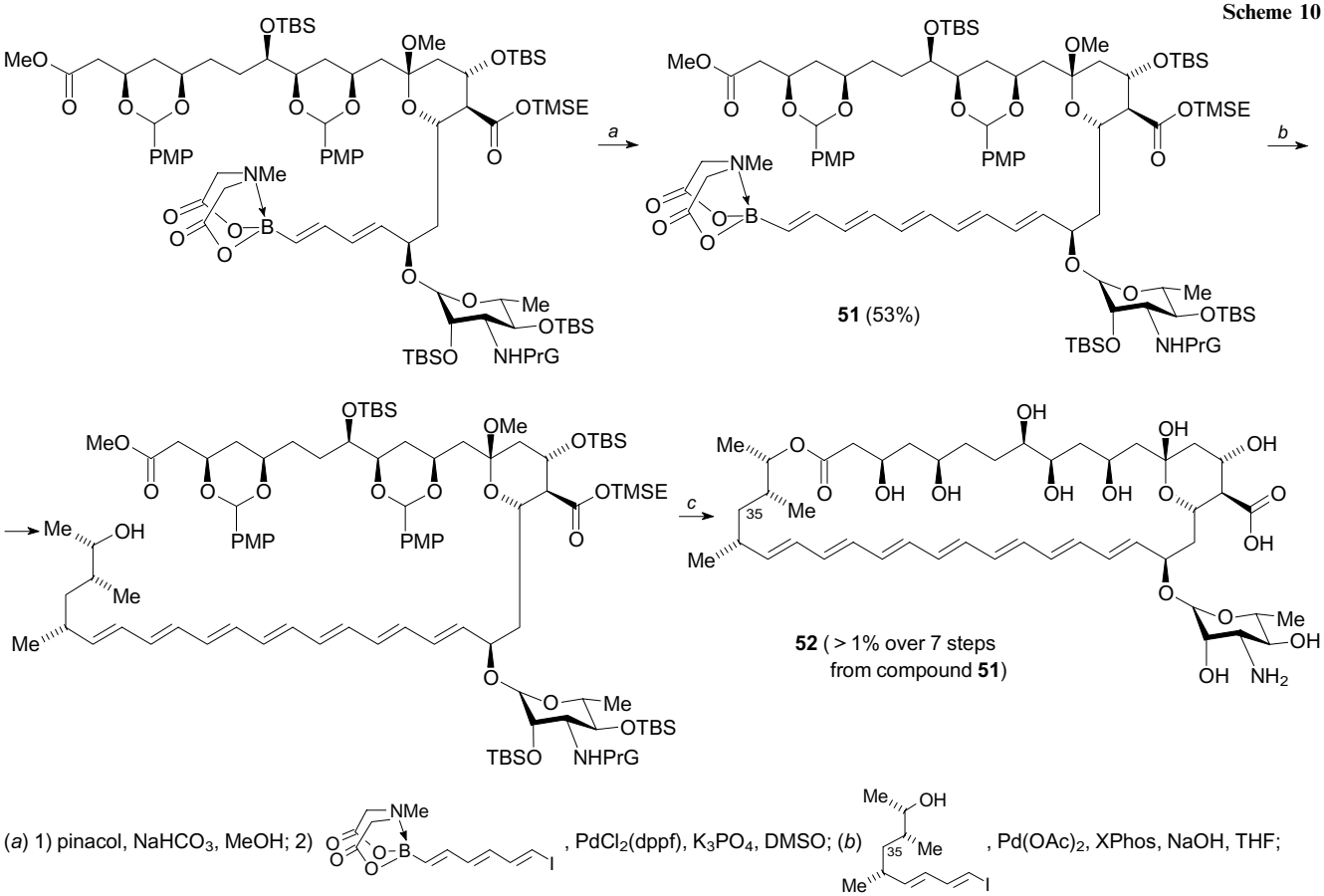
3.2. Synthesis of conjugates of polyene antibiotics

Janout *et al.*[38] reported an original strategy for enhancing selectivity of AmB towards fungal cells. It was expected that chemical modification of AmB with a bulky umbrella moiety would minimize undesirable effects associated with the formation of antibiotic aggregates. This assumption was based on the fact that antibiotic aggregates are more toxic because they tend to form transmembrane channels, whereas monomeric forms selectively extract ergosterol from the membrane and are toxic exclusively to fungal cells. This authors used spermidine *N*(1),*N*(3)-dicholoyl-*N*(2)-(8-amino)capryloyl triamide (**54**) as a molecular umbrella, which was derived from one spermidine molecule (**53**), one 8-aminooctanoic acid molecule and two cholic acid molecules. Target compound **56** was synthesized by amidation of the carboxy group at the C(16) atom of amphotericin B, which was activated by 1,2,3-benzotriazin-4(3*H*)-one (compound **55**), with spermidine triamide **54** (Scheme 11).

The spermidine dicholoyl triamide – AmB conjugate (**56**) has antifungal activity comparable to that of the native antibiotic, while haemolytic activity and cytotoxicity of this conjugate towards mammalian cells are significantly reduced.[38]

Bifunctional compounds and their formulations composed of the polyene antibiotic attached *via* a linker to a

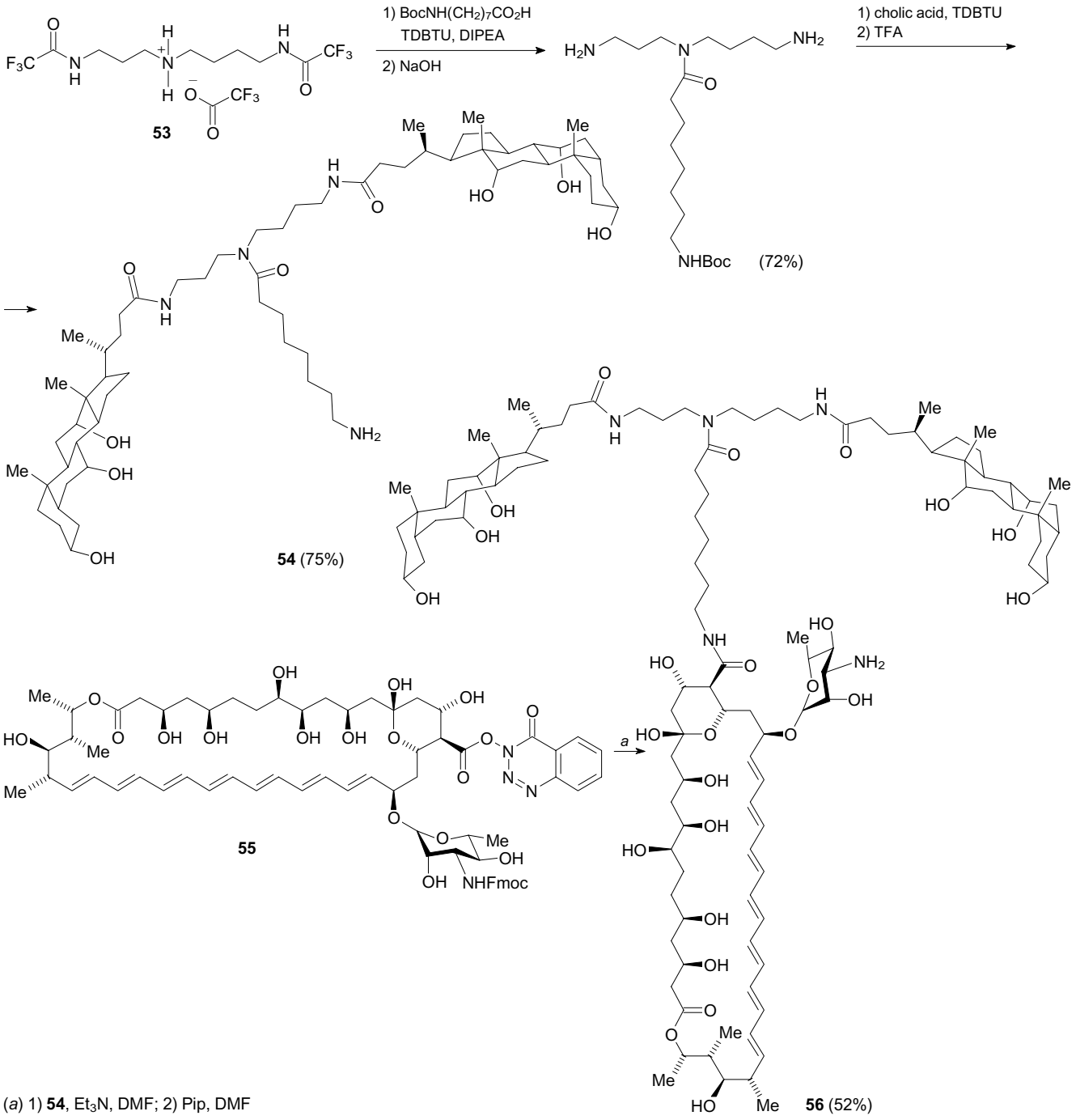
Scheme 10



(*a*) 1) pinacol, $NaHCO_3$, MeOH; 2) , $PdCl_2(dppf)$, K_3PO_4, DMSO; (*b*) , $Pd(OAc)_2$, XPhos, NaOH, THF;

(*c*) 1) LiOH; 2) MNBA; 3) TBAF; 4) HF, Py; 5) HCl; 6) penicillin G amidase

Scheme 11



(*a*) 1) **54**, Et_3N, DMF; 2) Pip, DMF

ligand that specifically interacts with chemokine receptors were patented as new promising antifungal agents. Examples of structures of such compounds are shown in Fig. 4.

The conjugation to chemotactic ligands increases the efficiency of polyene antibiotics due to ligand-associated chemotaxis of neutrophils to a site of infection. Bifunctional compounds can be used to treat invasive aspergillosis, pulmonary aspergillosis, peritonitis, candidemia, invasive candidiasis, intra-abdominal abscess, pleural cavity infections and esophagitis.[39]

One of strategies currently employed for optimization of pharmacological properties of antimicrobial agents relies on the design of dual-action antibiotics or 'chimeric antibiotics' — heterodimers based on derivatives of different classes of antimicrobial agents conjugated together *via* a covalent bond.[40] Recently, a series of hybrid polyene antibiotics, AmB – benzoxaborole conjugates, were reported. Benzoxaboroles exhibit pronounced antifungal activity, and 5-fluoro-1-hydroxy-1,3-dihydro-2,1-benzoxaborole was approved by FDA for the treatment of onychomycoses.[41] It was expected that the conjugation of the polyene skeleton to the benzoxaborole moiety would lead to synergistic enhancement of antifungal activity and a decrease in toxicity. Five types of hybrid compounds, such as C(16)-

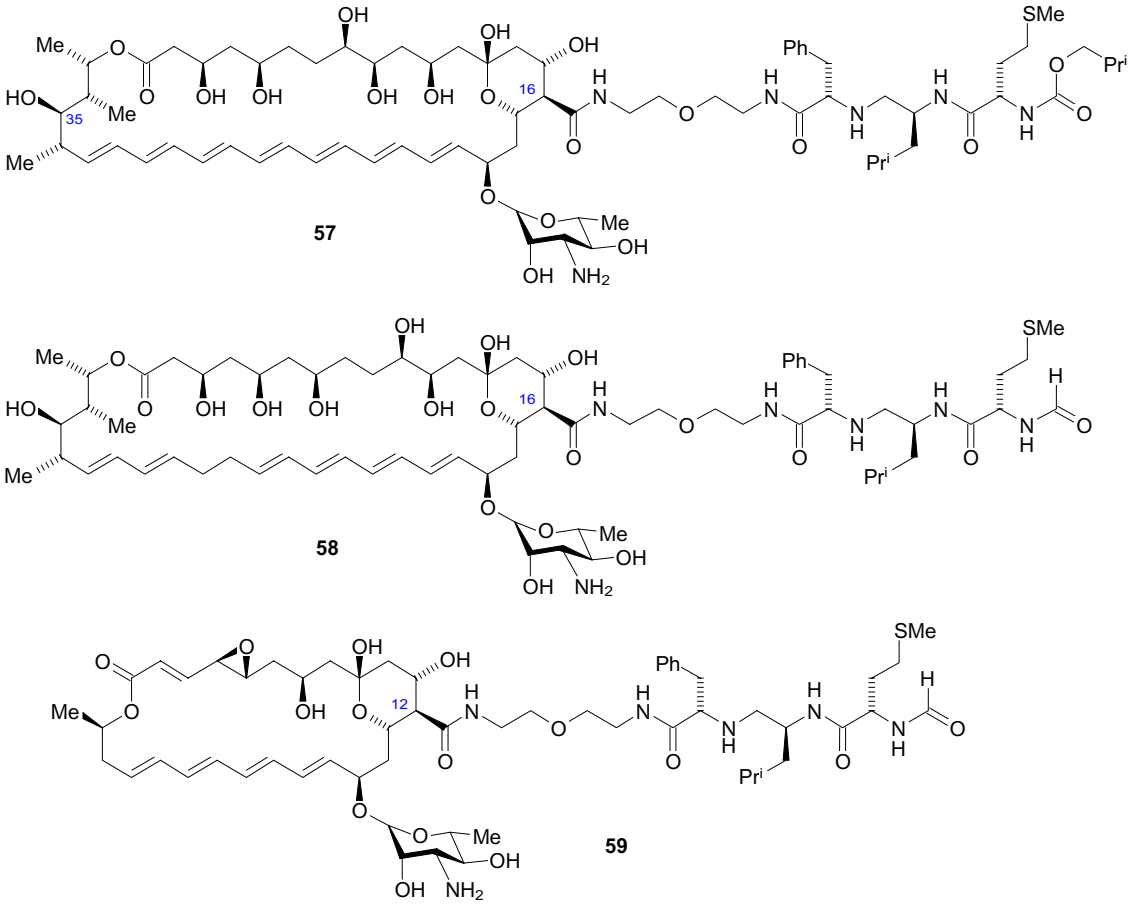


Figure 4. Conjugates of amphotericin B (**57**), nystatin A_1 (**58**) and natamycin (**59**) with a chemokine receptor ligand.

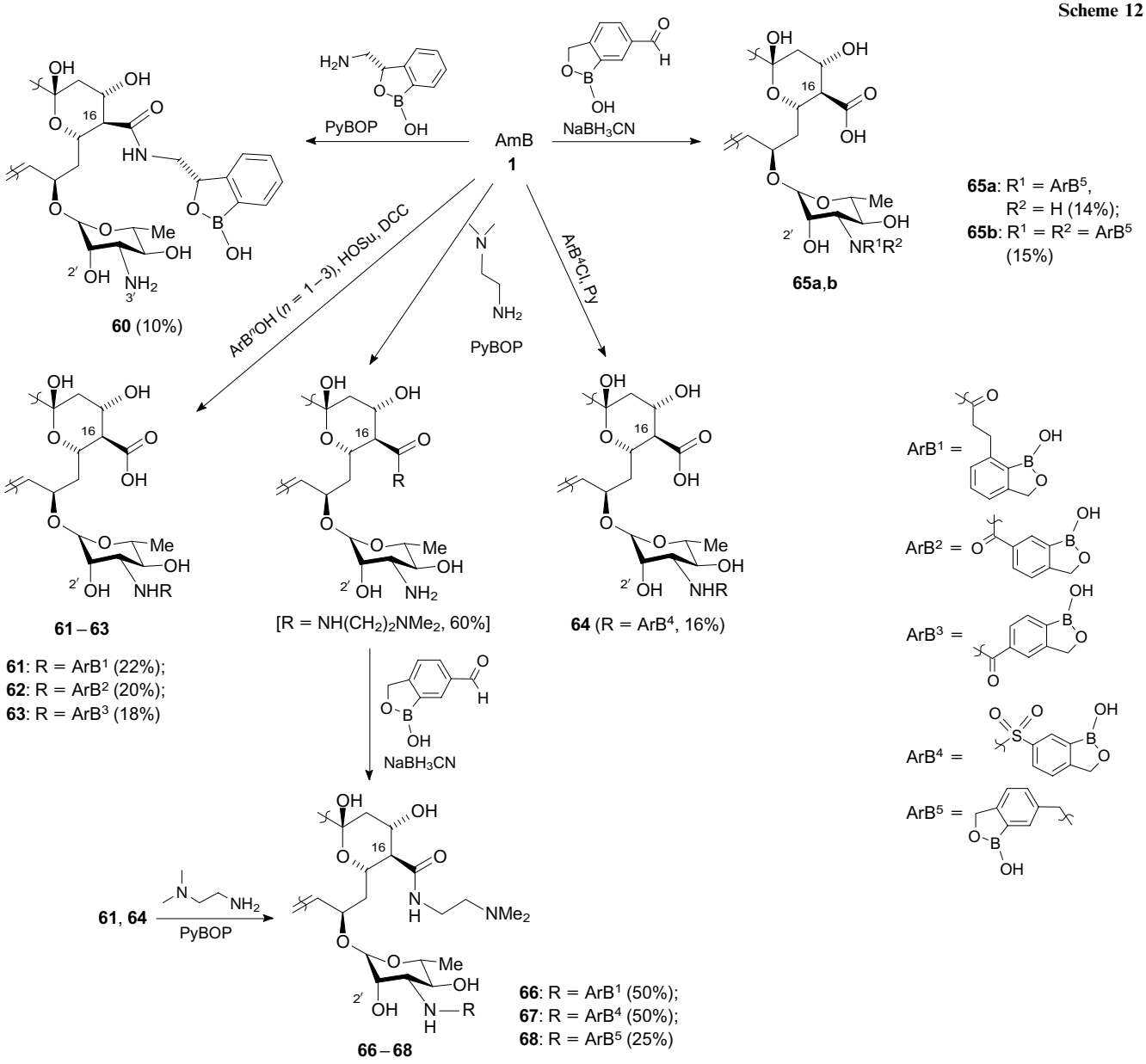
amide, N(3′)-acyl, N(3′)-alkyl, N(3′),N(3′)-bis(alkyl) and N(3′)-sulfo AmB derivatives, were synthesized[42] (Scheme 12). Conjugates **60**, **61**–**64** and **65b** were less active than native amphotericin B. The amidation of the C(16)-carboxyl group with *N*,*N*-dimethylethylenediamine enhanced the antifungal activity (compounds **66** and **67**). Lead compound **66** was identified in the series of these derivatives. It has high therapeutic efficacy and exhibits lower haemolytic and cytotoxic activity compared to AmB.[42]

The authors studied the effect of the most active AmB–benzoxaborole conjugates on diphytanoylphosphatidylcholine lipid bilayers containing 33 mol.% ergosterol or cholesterol and on giant monolayer vesicles composed of phosphatidylcholine or a mixture of phosphatidylcholine and ergosterol (67 : 33 mol.). It was shown that AmB–benzoxaborole conjugates can form channels with a larger radius and shorter lifetime compared to the native antibiotic. The modification of one of the polar groups of amphotericin B [at the position C(16) or N(3′)] leads to a more significant decrease in the channel lifetime and has a more pronounced effect on the ability of the compound to induce phase separation in the vesicle membrane. Apparently, a single charge in amphotericin derivatives [free C(16)-carboxyl or 3′-amino group] prevents aggregation of molecules, whereas zwitterionic molecules of the native antibiotic and neutral derivatives more readily interact with each other and with lipids in the membrane. The reliable correlation between the channel-forming ability and antifungal activity of AmB conjugates was revealed. It was shown that lead compound **66** has an enhanced selectivity towards ergosterol-containing membranes compared to AmB.[43]

3.3. Other methods for modification of polyene antibiotics

One new approach to chemical modification of AmB is based on complexation with transition metal ions. Chudzik *et al.*[44] hypothesized that the insertion of copper ions as a complex with amphotericin B into fungal cells may lead to enhancement of antifungal activity of the antibiotic due to excess copper uptake by the cells and inhibition of their defense mechanisms. The complex was synthesized as follows. An alkaline AmB solution (1.1×10^{-5} mol litre^{-1}) was adjusted to pH 10.7 with hydrochloric acid. Then copper(II) chloride was added in an amount sufficient for the preparation of a solution of the complex at a concentration of 0.6×10^{-5} mol litre^{-1}. The complexation was performed in the dark under inert nitrogen atmosphere for 1 h. The formation of the complex was confirmed by UV-Vis spectroscopy. The amphotericin B complex with divalent copper (Cu-AmB) proved to be more active against *C. albicans* ATCC 10261 than AmB and its formulation Fungizone. Moreover, increased activity of the Cu-AmB

Scheme 12

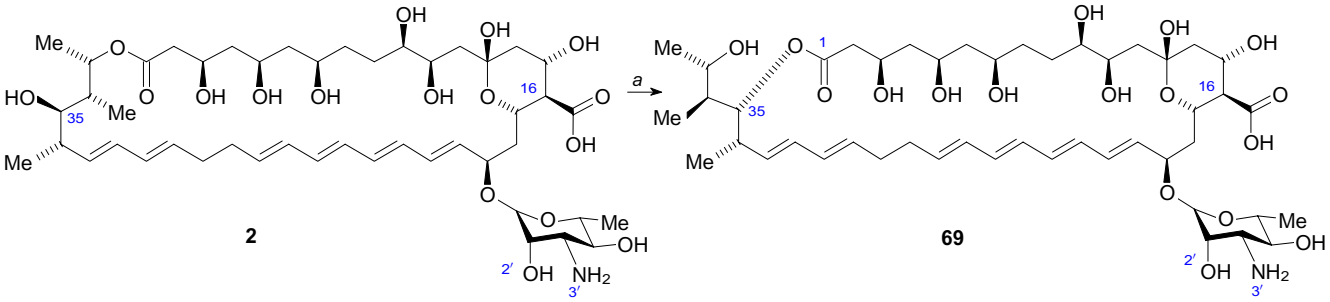


complex is not a sum of toxic effects of copper and AmB, but is a result of the unique action of the complex.[44]

Due to the presence of the polyene chromophore in AmB, the antibiotic molecule can undergo oxidation. Czernel *et al.*[45] studied the oxidation of the AmB chromophore catalyzed by free iron ions ($FeCl_3$, pH 2.5) and iron ions incorporated in haems (methaemoglobin $HbFe^{III}$ and haemin). The results of the reaction of oxidizers with AmB were interpreted by means of molecular spectroscopy (UV-Vis absorption and fluorescence spectra) and mass spectrometry. The use of 1,10-phenanthroline as a chelating agent allowed the authors to ultimately establish the oxidative effect of free Fe^{III} ions in an acidic solution of amphotericin. Changes in the fluorescence spectrum at a wavelength of 470 nm are indicative of oxidation of the AmB chromophore catalyzed by free Fe^{III} ions. Similar changes in the spectral properties of AmB were observed upon treatment with methaemoglobin and haemin at physiological pH. At a physiological concentration of 8×10^{-7} mol litre^{-1}, methaemoglobin and haemin cause a substantial increase in the rate of AmB oxidation, approaching the auto-oxidation rate.[45] A comparative analysis of the results of biological assays of the oxidized and native forms of AmB demonstrated that the oxidized form of AmB has 16-fold lower antifungal activity and fivefold lower toxicity to mammalian cells. Therapeutic safety assessed as the ratio of the cytotoxic concentration (CC_{50}) to antifungal activity (MIC) shows that the oxidation of AmB is a very unfavourable process because the oxidized derivative possesses severely reduced antifungal properties, while its toxicity decreases less strongly.[46]

Borowski and co-workers[47] found that, in slightly acidic or basic solutions, the nystatin A_1 molecule undergoes intramolecular translactonization, resulting in contraction of the macrocycle by two carbon atoms (Scheme 13). The structural isomer iso-nystatin A_1 (**69**) thus formed exhibits lower activity compared to the native antibiotic.[48]

Scheme 13



(*a*) MeOH, DMSO, Na_3PO_4 (buffer solution), pH 7.5 or 6.5, 37 °C

4. Polyene antibiotic derivatives produced by engineered biosynthesis

Engineered biosynthetic approaches provide a promising alternative to chemical modification for the synthesis of new polyene antibiotics with improved pharmacological properties. Microbiological methods for production of semisynthetic antibiotics are based on identification and annotation of gene clusters responsible for their synthesis and directed modulation of their functions. Studies aimed at producing polyene antibiotics using modified genetically engineered producer strains, which were published before 2011, are summarized in a review.[10]

Kim's research group[49] described the biosynthetic preparation of new nystatin A_1 derivatives, NPP A_1 (**70**), NPP A_2 (**71**) and NPP A_3 (**72**) (Fig. 5).

The derivative NPP A_1 (**70**) is a structural analogue of nystatin A_1 (**2**). These compounds differ only in that NPP A_1 contains the second carbohydrate residue (acetylglucosamine). The mycosaminyl-*N*-acetylglucosamine disaccharide moiety of NPP A_1 (**70**) provides higher solubility and lower haemolytic activity of this antibiotic compared to nystatin. However, NPP A_1 (**70**) is less active against fungi than nystatin A_1. The derivative NPP A_3 (**72**) in which the *N*-acetylglucosamine residue is replaced by mannose, was prepared through genetic manipulations of the NPP *Pseudonocardia* KCTC9441 producer. The evaluation of biological properties of derivative **72** demonstrated that this derivative has reduced *in vitro* antifungal activity, while exhibiting higher toxicity against human hepatocytes. Therefore, the biological activity of polyene antibiotics

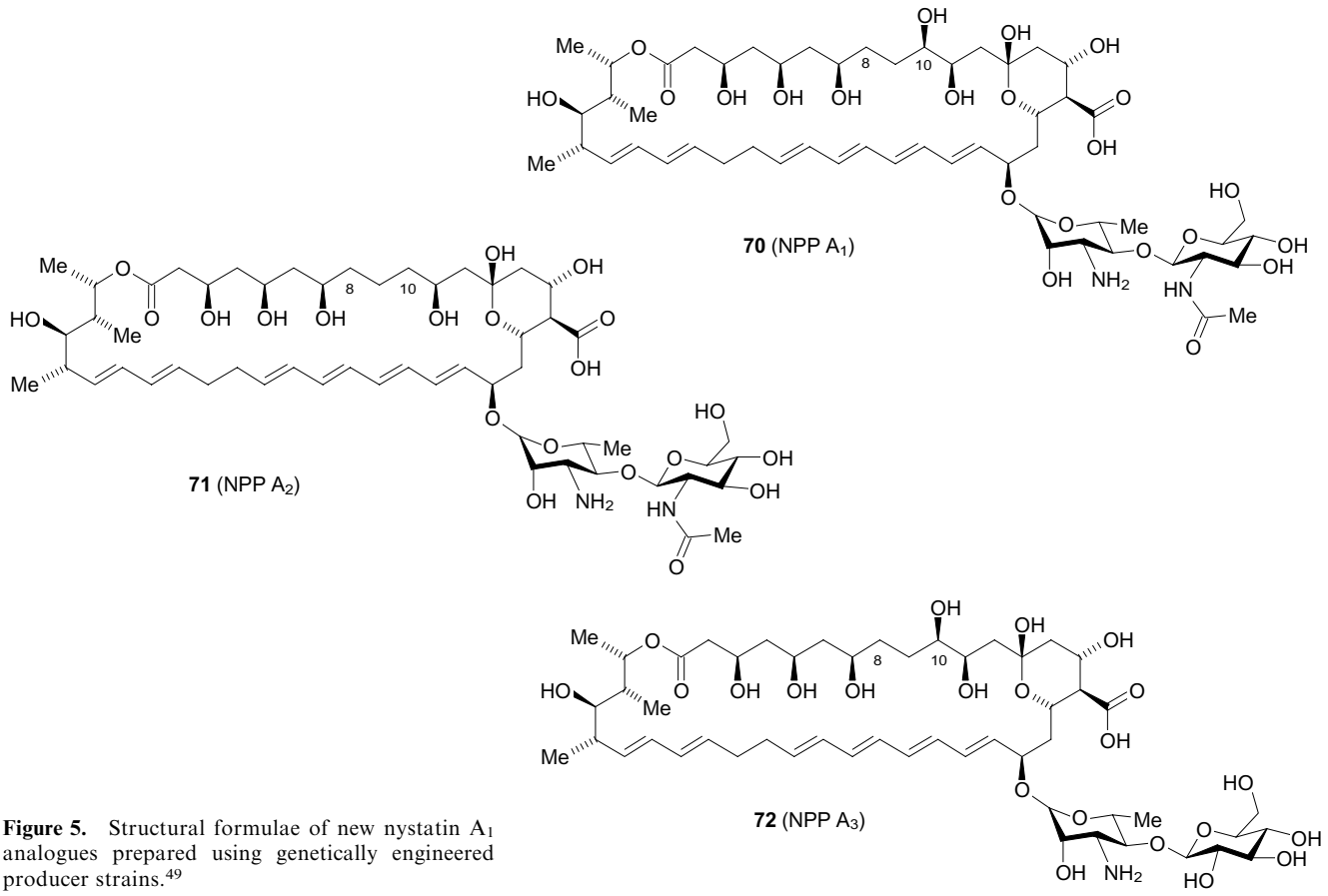


Figure 5. Structural formulae of new nystatin A_1 analogues prepared using genetically engineered producer strains.[49]

depends not only on the number of carbohydrate residues but also on the nature of the second sugar moiety.[49]

Kim and co-workers[50] performed molecular genetics study of the biosynthesis of the NPP antibiotic in the *P. autotrophica* KCTC9441 producer and found that the deletion of the *nppL* gene encoding P450 monooxygenase gives rise to the 10-deoxy derivatives of NPP A_1 — NPP A_2 (**71**). Later, this research group developed an efficient crystallization method for NPP A_2, which allowed them to prpepare this antibiotic of a purity suitable for biological assays.[51] It was found that the removal of the OH group in position 10 leads to a twofold increase in both antifungal and haemolytic activity.

The role of P450 monooxygenases in natamycin biosynthesis in the industrially used *Streptomyces chattanoogensis* L10 producer was studied in detail. The following derivatives of this antibiotic were produced by inactivation of different genes encoding monooxygenases: the previously known 4,5-des-epoxynatamycin (**73**), its aglycone **74** and three new derivatives, namely, 12-decarboxy-12-methylnatamycin (**75**), 4,5-des-epoxy-12-decarboxy-12-methylnatamycin (**76**) and 3-hydroxy-4,5-des-epoxy-12-decarboxy-12-methyl-2,3-dihydronatamycin (**77**) (Fig. 6).[52, 53]

The evaluation of biological activity of biosynthetic precursors of natamycin against *S. cerevisiae* showed that the mycosamine moiety is essential for biological activity of natamycin. The replacement of the carboxyl group by the methyl substituent in position 12 (compound **75**) led to a twofold increase in activity against *C. albicans* ATCC 14053 and a fivefold decrease in cytotoxicity against erythrocytes. This modification accompanied by reduction of the epoxide ring (compound **76**) has no significant effect on biological properties (high antifungal activity is retained and haemolytic activity is reduced). The hydration of positions 2 and 3 in 4,5-des-epoxy-12-decarboxy-12-methylnatamycin (compound **77**) led to loss of both antifungal activity and cytotoxicity. Genetic engineering studies gave insight into the natamycin biosynthetic pathway and resulted in the identification of genes responsible for each stage. These data provide opportunities for producing different semisynthetic analogues of this polyene antibiotic.[52, 53]

5. Drug formulations of polyene antibiotics

Amphotericin B has low solubility in pharmaceutically acceptable media. Hence, it was first introduced in the market and clinical practice in 1959 as a soluble complex[54] with sodium deoxycholate (Fungizone®). This drug formulation is used for parenteral administration because of low oral bioavailability of this antibiotic. Fungizone has serious adverse effects associated with its administration by intravenous infusion and nephrotoxicity. For more than 30 years, Fungizone remained the only available drug formulation of AmB. A liposomal formulation of AmB (AmBisome®) with an improved toxicological profile and activity comparable with that of Fungizone was developed in the 1990s. The improvement of the treatment safety using liposomal formulations of amphotericin B was proven clinically.[55] Later, several lipid formulations of AmB (Fungisome iv, Fungisome gel) were patented.[56] This discovery has stimulated extensive studies aimed at developing lipid, nanoemulsion and other drug formulations of AmB with reduced toxicity and designing targeted drug delivery systems. Emphasis is given to the production of drug formulations for oral, topical and inhalation administration of AmB, because these routes of administration will ensure safety of the treatment of mycoses. Advances in the development of formulations achieved before 2013 are considered in reviews.[56, 57] The most promising formulations of AmB used in clinical practice and those that are currently under clinical trials are described in another review.[58]

5.1. Amphotericin B formulations for the treatment of fungal infections

One of extensively studied approaches to improving pharmacological properties of amphotericin B is that based on

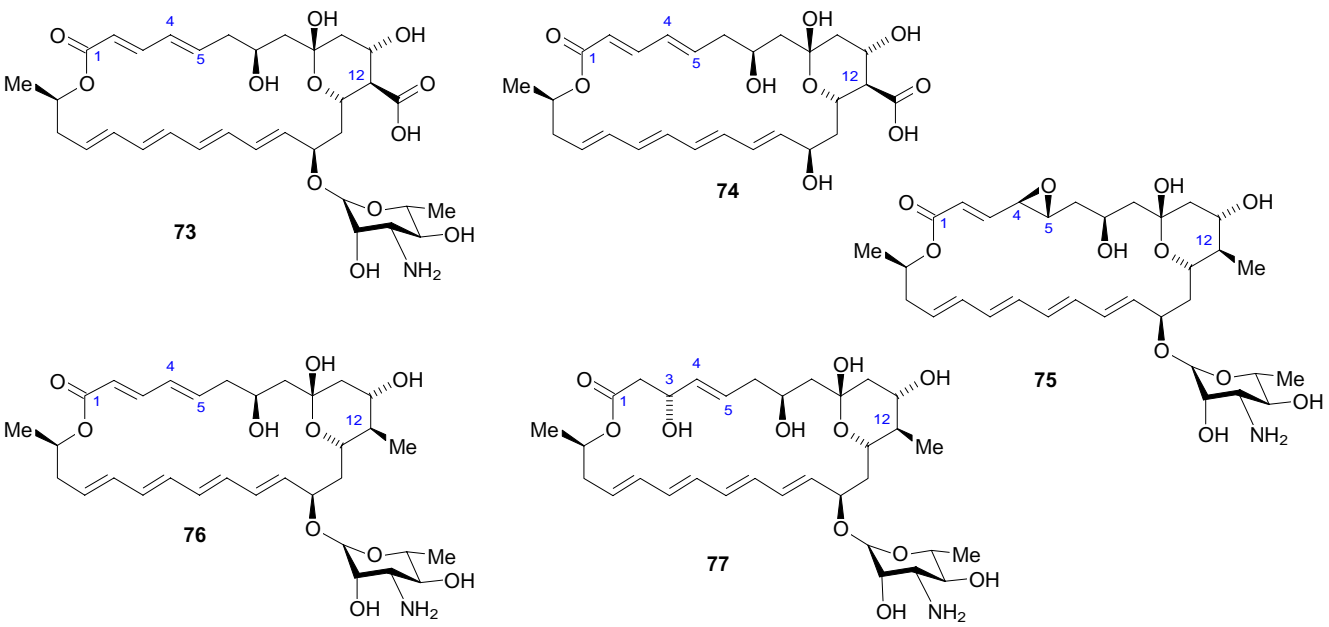


Figure 6. Structural formulae of natamycin derivatives prepared using modified genetically engineered producer strains.[52, 53]

conjugation of AmB to different water-soluble biomolecules and polymers. Early studies in this area are described in reviews.[9,10] Bioconjugates of AmB still continue to attract interest. In 2017, Dinh *et al.*[59] synthesized water-soluble nanosized AmB complexes based on poly(γ-glutamic acid) (PGGA). In *in vitro* haemolysis assays, these complexes showed toxicity comparable to that of liposomal AmB and had a better safety profile than Fungizone. *In vivo*, they were more efficacious than both AmB and Fungizone in a candidiasis model.

Investigations aimed at improving pharmacological properties of AmB by condensation with polysaccharides are currently ongoing. Ravichandran *et al.*[60] described the preparation of AmB conjugates with polyglucose (PG) *via* periodate activation of the polymer followed by condensation with the amino group of the mycosamine moiety of AmB (imino conjugates) or subsequent reduction (amino conjugates). Both the imine and reduced (amine) forms of AmB–PG conjugates exhibit high antifungal and antileishmanial activity and much lower *in vitro* haemolytic activity. An advantage of polyglucose–AmB conjugates over the AmB conjugates with arabinose or arabinogalactose polymers described earlier is their higher (4–5 times) solubility in water.[60]

As mentioned above, AmB has low oral bioavailability (~0.3%)[56] and, hence, it is administered only parenterally, causing undesirable side effects. The development of other routes of administration is a possible way of reducing toxicity of this antibiotic. Egito's research group[61] developed oil-in-water microemulsions (ME) of AmB for oral administration, leading to an increase in the solubility of the antibiotic by three orders of magnitude. The evaluation of biological properties of microemulsion formulations of amphotericin B (ME-AmB) revealed their dose-dependent toxicity against J774 macrophage cells, which is apparently associated with the presence of AmB aggregates in ME. However, toxicity of ME-AmB was lower than that of a plain solution of AmB in DMSO, and the tested formulations were well tolerated by J774 cells at concentrations < 25 μg ml^{-1}.[61]

So-called 'stealth' nanoparticles[62] are another example of AmB formulations suitable for oral administration. In this case, solubility of AmB was enhanced by its encapsulation in poly(ethylene glycol)-block-poly(lactide-co-glycolide) nanoparticles. The drug entrapment efficiency of AmB in this copolymer reaches 57% with 93% drug yield. The developed pharmaceutical formulations provide a feasible, effective and improved alternative to other carriers for oral delivery of AmB.

Butani *et al.*[63] developed a ME-AmB formulation for topical administration. Isopropyl myristate was chosen as a lipid fraction due to its ability to enhance AmB resorption and solubility. This ME-AmB formulation exhibited higher drug permeation through intact mouse skin as compared to plain antibiotic solution and showed minute skin irritation. The ME-AmB formulation is highly active against fungal strains of *Trichophyton rubrum* and is stable both at 2–8 °C and room temperature over a period of 3 months. Therefore, the developed prototype of the ME formulation of AmB is promising for the topical treatment of mycoses.

Gangadhar *et al.*[64] examined the possibility of using amphotericin B lipid formulations for the treatment of invasive pulmonary mycoses *via* inhalation. Sodium 3α,12α-dihydroxy-5β-cholan-24-ol sulfate (SDS), which was prepared from cholic acid in 82% yield, was used as a lipid component. The physicochemical parameters of the lyophilisate of the SDS-AmB formulation dissolved in distilled water attest to high stability of the resulting emulsion, higher than that of the corresponding sodium AmB-deoxycholate (Fungizone) solution.The mass median aerodynamic diameters of the SDS-AmB and AmB-deoxycholate particles are 1.70 and 1.74 μm, respectively, indicating that these formulations can ensure effective antibiotic delivery to the lungs *via* inhalation. It was found that the drug does not undergo chemical degradation and retains antifungal activity during lung aeration. These results suggest that lipid formulations of AmB can be administered *via* inhalation and that sodium 3α,12α-dihydroxy-5β-cholan-24-ol sulfate is a promising excipient of inhalation formulations.[64]

Bone cement is widely used for delivery of antibiotics to a site of bone infection.[65] Roberts, Bingham and co-workers[66] showed that liposomal amphotericin B loaded in bone cement is promising for the treatment of deep mycoses of the musculoskeletal system. This formulation, although being toxic *in vitro* to fibroblasts and osteoblasts, has no serious toxic effect in *in vivo* assays in mice.[66]

The results of an extensive study of the solubilization behaviour of polyene antibiotics in a nanomicellar system were published in 2016. To reveal specific features of the formation of nanomicellar solutions of polyene antibiotics and determine their quantitative characteristics that are necessary for the development of formulations, Mobasheri *et al.*[67] modelled the interactions of AmB and nystatin with polysorbate 80 by molecular dynamics simulation. The results of these simulations provide an explanation for the experimentally observed ability of polysorbate 80 to enhance solubility of polyene antibiotics. Thus, the rate of antibiotic encapsulation in micelles is higher than the rate of self-association of the antibiotic molecules, thereby facilitating high loading efficiency of drug encapsulation in micelles. The antiobiotic molecules are mainly solubilized in monomeric forms, with antibiotic–surfactant interactions increasing the antibiotic solubility. The equilibrium is substantially shifted towards encapsulation, and the level of back diffusion of antibiotics to solution is insignificant. It was found that polyenes are preferably localized in the peripheral part of the micelle, the degree of penetration being rather low (approximately 1/4 of the micelle radius). Deeper penetration of polyenes is impeded by the synergistic effect of limited diffusion of water and the perpendicular orientation of the antibiotic molecules with respect to the micelle radius. Polyenes are almost completely dissolved in the hydration shell of polyoxyethylene because the antibiotic molecules and the micelle core are spaced rather far apart, resulting in the almost complete absence of interactions between them. This fact accounts for the earlier observation that the solubilization of nystatin does not depend on changes in the micelle core. The controlled promotion of micelle permeability and optimization of chain crowding in the palisade layer apparently facilitate more efficient solubilization of polyenes.[67]

5.2. Amphotericin B formulations for the treatment of leishmaniasis

The medical potential of AmB is not restricted to antifungal therapy. Amphotericin B is active against leishmaniasis and is an alternative to highly toxic pentavalent antimonial

drugs for the treatment of this disease. Leishmaniasis is a common poverty-related infection in tropical countries. According to the WHO bulletin, on March 14, 2018, >90% of new cases of this disease reported in 2015 occurred in 7 countries: Brazil, India, Kenya, Somalia, Sudan, Ethiopia and South Sudan.

Recently, Cunha *et al.*[68] performed a retrospective analysis of the efficacy and toxicity of liposomal AmB used in the therapy of mucosal leishmaniasis and showed high efficacy (the cure rate was 93.1%; 27 of 29 patients were cured) and safety of liposomal AmB for the treatment of patients with this disease who had contraindications to the use of other agents. However, high cost of parenteral liposomal formulations of amphotericin B limits the use of AmB in less developed countries. Hence, the elaboration of commercially available formulations of AmB for oral administration is a challenging problem. Wasan *et al.*[69] gained some success in this field. The authors designed a prototype of an AmB lipid formulation for oral administration (iCo-010) that is stable during storage in tropical conditions and exhibits high activity in *in vivo* assays in mice infected with visceral leishmaniasis. This pharmaceutical formulation is composed of a mixture of AmB with mono- and diglycerides (60 : 40 vol.%) (Peceol and Gelucire 44 : 14) and a water-soluble derivative of vitamin E (TPGS).[69] The new formulation iCo-010 was shown to be quite stable and retained its pharmacological activity after 60-day storage at 43 °C.[70]

Nanoemulsions loaded with amphotericin B (NE-AmB) are considered as another commercially available alternative to liposomal formulations. Ferreira's research group[71] prepared an AmB nanoemulsion containing cholesterol and stearylamine. This formulation is promising for the treatment of leishmaniasis. Cholesterol plays an important role in stability of nanoemulsion, while stearylamine enhances biological activity of the formulation. Later, the procedure for the preparation of this nanoemulsion was optimized by a gradual increase in the pressure using a a high-pressure homogenizer, resulting in a decrease in the polydispersity index and particle size.[72] Haemolytic activity of the new formulation is lower than that of Fungizone. In *in vivo* assay in a mouse model infected with *Leishmania infantum chagasi* (pathogen that causes visceral leishmaniasis), NE-AmB at a dose of 2 mg kg^{-1}, unlike Fungizone, did not show any sign of acute toxicity. Therefore, NE-AmB holds considerable promise for the development of alternative agents for the treatment of visceral leishmaniasis due to enhanced efficacy and lower toxicity.[72]

Kumar *et al.*[73] prepared bioconjugates of amphotericin B-encapsulated poly(lactide-co-glycolide)-block-polyethylene glycol (PLGA-PEG) nanoparticles with antibody to the macrophage CD14. These conjugates are suitable for the treatment of leishmaniasis. This formulation is characterized by higher solubility of the antibiotic and its affinity for macrophages of tissues infected with visceral leishmaniasis. In *in vitro* assays, nanoparticles were much more effective against extracellular leishmaniasis (promastigotes) compared to native AmB. Similar results were obtained against leishmania amastigote in assays in hamster spleen tissues.[73]

5.3. Formulations of other polyene antibiotics

Studies aimed at developing formulations of other antibiotics were also reported in the literature. Liposomal formulations of natamycin based on soybean lecithin were prepared by solvent injection, enabling the production of unilamellar vesicles of small size (< 130 nm). These vesicles are able to encapsulate natamycin without significant modification of their size characteristics. In this formulation, natamycin is quite labile, resulting in uncontrolled leakage of the antibiotic out of the liposomes. The stability of the formulation was increased by incorporation of sterols (cholesterol, ergosterol) in the lipid mixture, which led to both higher entrapment levels of the drug in liposomes and reduced permeability of the phospholipid membrane due to hydrophobic interactions of the lipid layer with sterols. A comparative analysis of the use of different sterols showed that ergosterol is a sterol of choice because the efficient stabilization of natamycin is observed at ergosterol concentrations 2.5-fold lower compared to cholesterol, thereby providing higher cell membrane permeability. The fine tuning of the sterol concentration allows the preparation of liposomal suspensions which show good *in vitro* drug release kinetic curves and exhibit enhanced antifungal activity against a model strain of the yeast fungi *S. cerevisiae* ATCC 9763.[74]

The use of molecular containers for the targeted drug delivery and improvement of pharmacological properties of the drug is another promising approach to the development of drug formulations. Li *et al.*[75] found that the β-cyclodextrin – acrylamide heteropolymer, which was prepared by polymerization of β-cyclodextrin with acrylamide, substantially enhances solubility and antifungal activity of natamycin, resulting in increased bioavailability of the antibiotic.
A decrease in the particle size of an AmB suspension is known to improve its pharmacological properties.[58] This is also valid for nystatin. Melkoumov *et al.*[76] described the preparation of a nanosized nystatin formulation from a commercially available antibiotic suspension by wet-media milling. The nanoemulsion (particle sizes from 6577 to 137 nm) exhibited higher *in vitro* activity against the *C. albicans* SC5314 strain compared to the conventional nystatin suspension. In *in vivo* assays, the new formulation caused less adverse effects in immunocompromised mice infected with oral candidiasis. Besides, mice treated with the nystatin loaded in nanoparticles had improved survival compared with other groups of animals.[76]

The preparation of lipid formulations of nystatin were shown to be a promising approach. Semis *et al.*[77] developed a stable emulsion formulation of nystatin based on commercially available intralipid and showed that this formulation is less toxic and more active against pathogenic strains of *Candida*, *Aspergillus* and *Fusarium* species compared to the native antibiotic both in *in vitro* and *in vivo* assays. The mechanism of activity of this formulation against fungi is similar to that of the native nystatin, while nystatin-intralipid loses channel-forming activity in mammalian cell membranes, which leads to a considerable decrease in toxicity of the antibiotic.[77]

6. Conclusion

Knowledge of the mechanisms of biological action of polyene antibiotics has been greatly improved in the last five years. The results of recent studies confirm the assumption that the antifungal affect of polyene antibiotics is associated with extraction of ergosterol from the cell and induction of oxidative stress, while the channel formation

represents a second complementary mechanism, facilitating the penetration of antibiotics into the cells and increasing its toxicity to mammalian cells. This model can provide an explanation for a low level of resistance to amphotericin B. Thus, simultaneous gene mutations that affect all ergosterol-dependent intracellular metabolic processes are unlikely. Besides, in some cases the emergence of resistance leads to invirulence of the mutant strain.

According to current concepts on the mechanism of action of polyene macrolides, the separation of antifungal and channel-forming activity by a targeted alteration of the structures of these antibiotics is most promising for the preparation of antifungal agents with an improved toxicological profile. Some advances were achieved in this field. Thus, amphotericin B and nystatin A_1 derivatives at positions 16 and 3′, which were prepared mainly by chemical modification, showed significantly lower toxicity in *in vitro* and *in vivo* assays compared to the native antibiotics, with retention of high antifungal activity. In many cases, experimental assays showed that a decrease in toxicity of semisynthetic derivatives of polyene antibiotics is achieved due to an increase in the affinity for ergosterol and a decrease in ability to form transmembrane channels.

A number of nanosized formulations of polyene antibiotics ensure an increase in therapeutic efficacy and safety of these drugs in *in vivo* assays. Moreover, new oral and topical formulations of AmB hold promise since they can significantly reduce side effects of this antibiotic.

A large body of recent studies demonstrated that polyene macrolides are still of great scientific and applied interest. This class of antibiotics has a considerable potential for the development of new-generation antifungal agents with improved pharmacological properties due to their commercial availability, the possibility of modulating desired pharmacological properties and rare occurrence of resistance to polyenes.

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