Synthesis and biological evaluation of amphotericin B derivatives

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The preparation and biological properties of various AmB analogs and conjugates that have been studied since the introduction of AmB into the clinic over 50 years ago are reviewed. Amphotericin B (AmB) analogs prepared by semi-synthetic modifications as well as those produced *via* genetic modification of the AmB polyketide synthase cluster are discussed, thereby highlighting the complementary approaches. In addition, the review includes the synthesis of AmB conjugated to biomolecules such as sterols and reactive markers such as fluorescent dyes, as well as their application to further understand the mechanism of action of the parent compound.

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1 Introduction

In recent decades, the incidence of fungal infections acquired by patients during hospitalization, referred to as nosocomial infections, has been on the rise.[1,2] Currently, *Candida* is the fourth most common cause of bloodstream infections in the United States.[3] As a consequence, the importance of developing new antifungal drugs as well as efforts to map out their mechanisms of action is of importance and great relevance.

Antifungal agents can be divided into a number of categories according to their chemical structure, which often correlates to the presumed mode of action. The various drug classes manifest their activity on distinct organelles or biological processes (Fig. 1). For instance, the azole and triazole antifungal agents target ergosterol biosynthesis. Ergosterol is a major constituent of fungal cell membranes (between 5 and 30%) and is essential for controlling membrane stability and fluidity.[4] The triazole class of antifungal agents inhibits the fungal cytochrome P450-dependent enzyme lanosterol 14-α-demethylase, leading to diminution of ergosterol synthesis.[5,6] Examples of members of this class include flucanozole, ketoconazole, itroconazole and voriconazole (Fig. 2).

Another target for antifungal drugs is the cell wall and its network of crossed-linked sugars. One of the enzymes responsible for the synthesis of the cell wall is (1,3)-β-D-glucan synthase, whose production is inhibited by the echinocandins (Fig. 2).[7] This leads to destabilization of the cell wall, cell lysis, and ultimately cell death.

A third mode of action for antifungal agents is the inhibition of transcriptional and translational enzymes. These are responsible for the transcription of DNA to RNA and for the translation of RNA into protein. They may be targeted by the action of molecules spanning a wide range of structural motifs such as peptides (*e.g.* histatin-5[8]) and small molecules such as the antimetabolite 5-fluorocytosine[9] (Fig. 2). A serious and alarming consequence of the wide use of such drugs is the rapid emergence of resistant strains.[10]

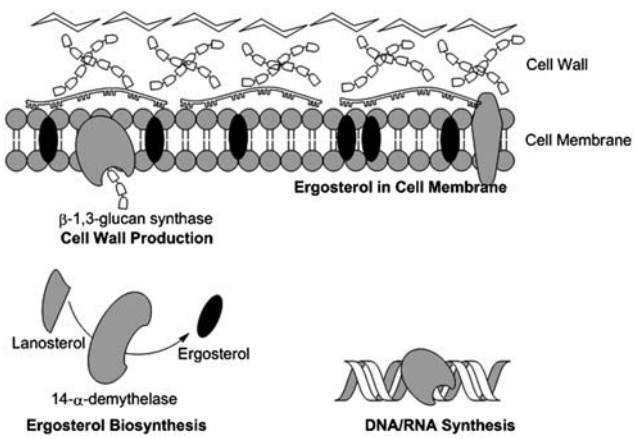

Fig. 1 Schematic representation of a fungus cell. Provided names in bold are the targets for systemic antifungal agents.

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A number of antifungal agents have the effect of interacting with and perturbing the cell membrane. The major drug group in this category is the polyene macrolides, of which the heptaene amphotericin B (AmB, **1**, Fig. 3) is a prominent member. Polyene macrolides have been suggested to form pores in fungal cells that allow ions and small molecules to leak out of the cell, thereby inducing cell death. Although some polyenes such as filipin show little cell specificity, others, notably AmB, exhibit remarkable differentiation between fungal cell membranes and mammalian cell membranes.[11] It is believed that this differentiation is related to the relatively high affinity of AmB towards ergosterol-containing membranes compared to those incorporating cholesterol.[12] However, the exact nature of the sterol–AmB interaction remains unresolved. Importantly, resistance against AmB has only rarely been observed.[13] Other polyene macrolides include the tetraene natamycin, the hexaenes nystatin, RK-397 and roxaticin and aromatic heptaene vacidin A and candicidin D.[14] The common motifs of AmB, natamycin, nystatin, vacidin A and candicidin D are both the extended chain of conjugated olefins along with the mycosamine sugar moiety.

2 Amphotericin B

Amphotericin B (AmB, **1**) was isolated in 1955 from a strain of *Streptomyces nodosus* found in the soil of the Orinoco River in Venezuela.[15–17] Due to its potent antifungal activity, AmB was rapidly introduced into the clinic to treat systemic mycosis. It received FDA approval in 1958, remarkably without its structure being known.

Extensive degradation studies established the overall connectivity of AmB.[15–20] However, only with the successful solution of the crystal structure of its *N*-iodoacetyl derivative, accomplished in 1970 by Schaffner and coworkers, was its structure unambiguously determined (Fig. 4).[21,22]

AmB owes its name to the amphiphilic nature of the structure. The hydrophobic polyene region (red) and the hydrophilic polyol (blue) region are stretched along the longitudinal axis of the molecule, whereas the polar sugar moiety, commonly referred to as the mycosamine unit (green), is attached at one of the turns (Fig. 3 and Fig. 4). Both the amine on the mycosamine and the carboxylic acid are ionized at physiological pH, with pK_a values of 10.0 and 5.5, respectively.[23]

Ten years after its isolation, the first mechanistic studies were undertaken to investigate the mode of action of AmB.[24] These studies revealed that treatment of cells with AmB leads to changes in the membrane permeability with concomitant leakage of mono- and divalent ions (most notably K^+, but also Ca^{2+}, Mg^{2+}, and PO_4^{3-}), and other small molecules. Subsequent to exposure, inhibition of glycolysis and protein synthesis has been observed. The fact that these events are followed by cell death led to the suggestion of channel formation and ion leakage as the basis of biological activity.[25]

Patients treated with AmB formulations frequently suffer from severe side-effects, including nephro- and hepatoxicity as well as anemia-related symptoms.[26] It has been suggested that this toxicity is linked to the aggregation state of AmB in the administered formulation. There are at least two self-associated forms of AmB in water, a water-soluble one and a water-insoluble one. In addition, there is a small quantity of monomer present.[27] The different forms of aggregation and the monomeric form of AmB molecules have been investigated by circular dichroism (CD) spectrometry and UV spectrometry.[28,29] However, the water-insoluble type of aggregates cannot be

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Erick Carreira obtained a B.S. degree in 1984 from the University of Illinois at Urbana–Champaign under the supervision of Scott E. Denmark and a Ph.D. degree in 1990 from Harvard University under the supervision of David A. Evans. After carrying out postdoctoral work with Peter Dervan at the California Institute of Technology through late 1992, he joined the faculty at the same institution as an assistant professor of chemistry and subsequently was promoted to the rank of associate professor of chemistry in the Spring of 1996, and full professor in Spring 1997. Since September 1998, he has been professor of chemistry at the ETH Zürich.

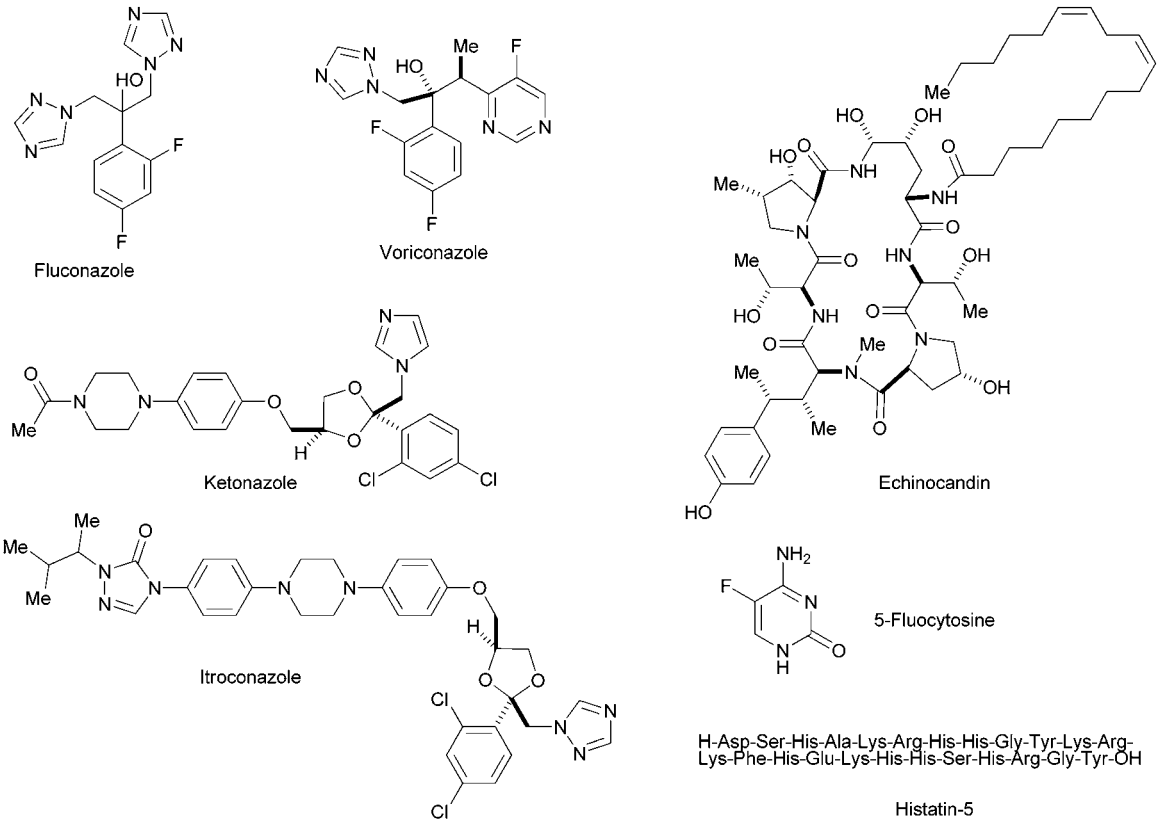


Fig. 2 Representative antifungal agents.

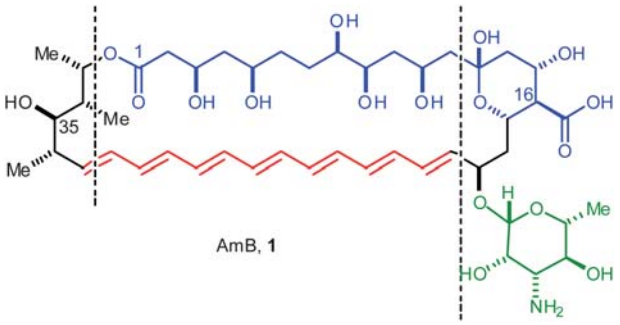


Fig. 3 Schematic representation of amphotericin B (AmB, **1**). Blue is the polyol, red is the heptaene and green is the mycosamine.

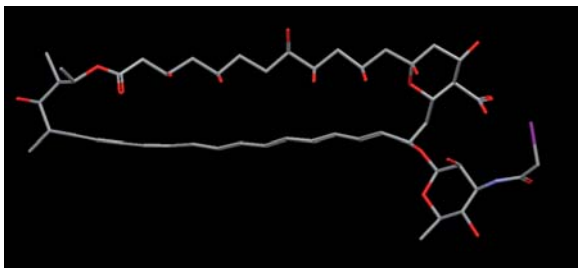


Fig. 4 Chem-3D representation of the crystal structure of *N*-iodoacetyl AmB.

visualized by UV- or CD-spectrometry. AmB monomer has a very distinct UV and CD spectrum as shown in Fig. 5 (i.a, ii.a and ii.b), with a strong absorption at 416 nm and three additional characteristic bands at 391, 371 and 348 nm in the UV spectrum. The positive bands in the CD spectrum above 370 nm of AmB in DMSO (a) and ethanol (b) are associated with the AmB monomer. In addition, the distinct vibrational fine structure is another indication that the compound exists as a monomer. The negative band at 272 nm corresponds to the C16-carboxylic acid and to the carbonyl at C1. Upon aggregation, the main UV absorption peak at 416 nm decreases in intensity, while the smaller monomeric peak at 348 nm increases in intensity and broadens. The CD spectra of AmB aggregates (Fig. 5 ii.c, d and e) show a strong dichroic signal centered around 342 nm in addition to the negative shoulders appearing above 370 nm. NMR studies on the aggregate performed by Balakrishnan and Easwaran showed that NOE enhancement between opposing ends of the aggregated molecules.[29] This suggests head-to-tail interactions, which is in agreement with the orientation of AmB molecules in the crystal structure.[21,22] The NMR was measured at 10^{-2} M in d_6-DMSO. Self-associated conglomerates are formed at this concentration as measured by CD spectrometry.

Bolard and coworkers found that toxic effects in cholesterol-containing membranes is restricted to water-soluble self-associated AmB oligomers, because at the concentration where aggregation occurs, they observed K^+ leakage from the liposome.[30] In addition, they claim that monomeric AmB and water-insoluble aggregates are inactive with respect to pore formation. Furthermore, activity towards ergosterol-containing

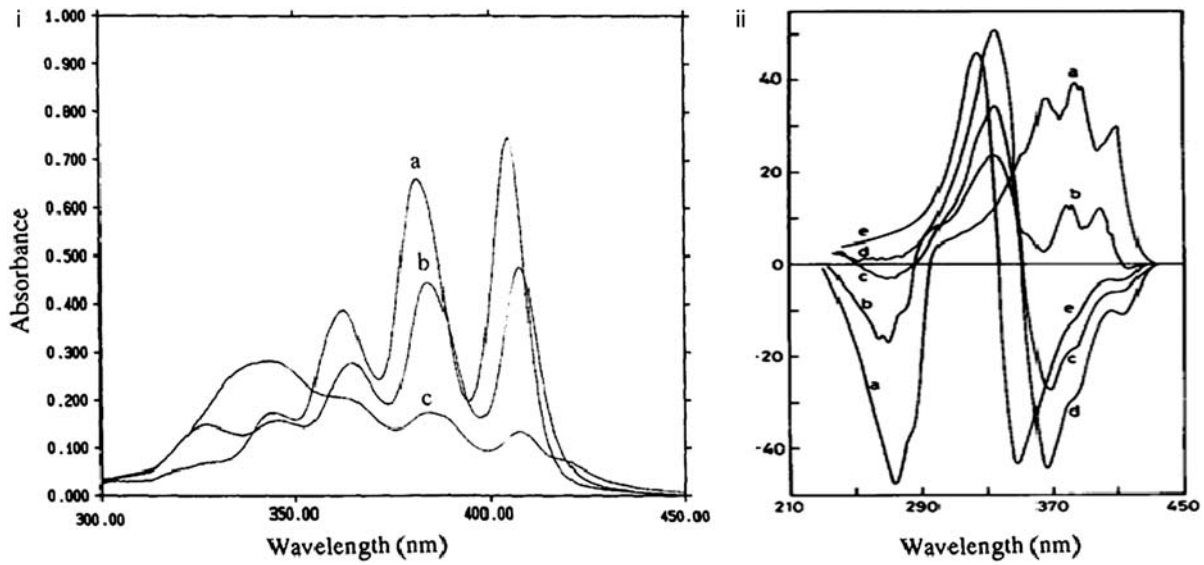


Fig. 5 i) UV absorption spectra of 10 μM AmB in MeOH with increasing water concentration: (a) 0% (monomer); (b) 70%; (c) 100% (aggregate). Taken with permission from ref. 28. ii) CD spectrum from AmB at a concentration of AmB of 100 μM in different solvents: (a) DMSO (monomer); (b) ethanol (monomer); (c) chloroform (aggregate); (d) acetonitrile (aggregate); (e) water (aggregate). Graphs taken with permission from ref. 29.

membranes is caused by both the monomeric form of AmB and by water-soluble aggregates, as K^+ leakage was observed at concentrations below the aggregation threshold. The water-insoluble aggregates are not active.[30] It likely that there is an equilibrium between the monomeric form, the water-soluble oligomeric form and the aggregated insoluble form, with the molecules dynamically equilibrating between the two states. Thus, the high tendency of AmB to self-associate is believed to play a major role in its mode of action.

3 Mechanism of action

3.1 Mechanism involving ion channel formation

In spite of more than five decades of investigations, the mechanism of action of AmB remains unclear. The various proposed modes of action as they relate to specific structural entities of AmB is illustrated in Fig. 6. Although it is clear that channel formation is an important feature, this effect may mask other modes of action that may be operative.

The most commonly accepted mechanism of action comprises several molecules of AmB undergoing self-assembly to form barrel-stave-like trans-membrane pores. However, the number of molecules involved, the overall structure of the assembly as well as the driving force for the self assembly process are far from clear. Studies have documented AmB-induced leakage of minor cell constituents, mono-valent electrolytes, and water.[24,31] The pore diameter has been estimated to be 3.6–4.6 Å when penetrating a cholesterol-containing membrane.[32–34] Mannitol (radius 3.6 Å) is released relatively easily, whereas glucose (radius 4.6 Å) was released very slowly. The number of molecules involved in forming the pores is not known; however, 4 to 12 AmB molecules have been proposed.[35,36]

An *ab initio* computational simulation was performed in order to estimate the number of molecules needed per channel.[37] In this study, the radius of the pore was fixed and the number of

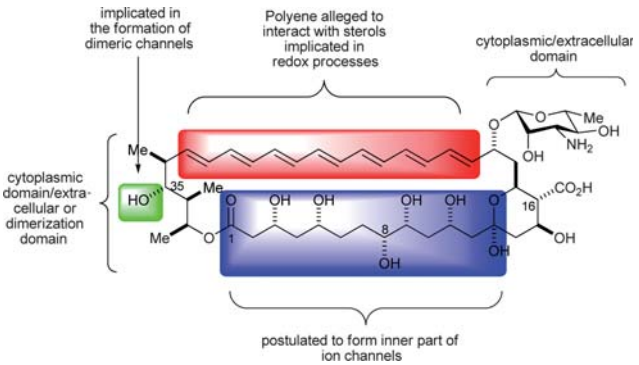


Fig. 6 Summary of proposed relations between structure and mechanism of action.

molecules varied. At the same time, the intermolecular interactions of the monomers forming the pores and the electrostatic potential of a cation moving through the pore were investigated. Both single- and double-length channels were considered. It was concluded that there is a upper limit of 12 monomers beyond which the formed pores are unstable. The optimal channel with respect to stability was proposed to be made up of 6–9 monomers, corresponding to a pore diameter of 4.5–7.2 Å.

A further complication with these models is the discrepancy between the width of the fungal membrane (50 Å)[38,39] and the length of AmB (21 Å).[20] In order to account for this, different models have been proposed.[18] In the model depicted in Fig. 7a, the membrane constricts around the site of the ion channel. According to a second model (Fig. 7b) two identical ion channel units latch onto each other in a tail-to-tail fashion. This dimeric pore is believed to be stabilized by hydrogen-bonding between opposing C35-hydroxyl groups (Fig. 7b).

The amine and carboxylic acid functions are suggested to be of importance for stabilizing the ion channel. It had been proposed that electrostatic interactions between neighboring AmB

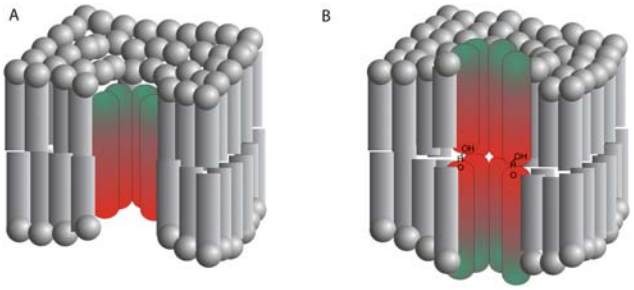


Fig. 7 a) A single pore of several AmB molecules. b) The double pore has the size of one glucose molecule (radius 4.6 Å).

molecules contribute to stabilize the ring, although this is difficult to reconcile with the fact that early work had shown that the methyl ester was of equal activity. Computational studies have suggested that the C8 hydroxy group is of importance for the lateral stabilization of the barrel structure of the ion channel.[40]

It has been observed that AmB ion channels form more rapidly in membranes containing ergosterol relative to those containing cholesterol.[41,42] This may be the basis for at least part of the selective toxicity of AmB towards fungal cells. The minimum inhibitory concentration (MIC) of AmB *in vitro* activity against *S. cerevisiae* is 0.3 μM. A rough estimate of the toxicity of AmB and its derivatives can be obtained by measuring the concentration of AmB needed to release 50% of the hemoglobin content from erythrocytes, known as the EH_{50}, which for the AmB 4.0 μM.[43]

The putative transmembrane channels formed by AmB molecules allow for the diffusion of both cations and anions. Interestingly, the rate of ion transport is dependent on the identity of the ion pair.[44] Monovalent anions enter the vacant AmB channel with the anionic conductance essentially independent of the presence of accompanying cations. In contrast, in the absence of a permeant anion, the vacant channel is impermeable to cations.[44] This observation has been explained by a model (Fig. 8) that emphasizes the partial positive potential at the center of the channel, which results from the alignment of OH-dipoles and the partial positive charge of the protons directed towards the center of the cavity. This favors anion binding in the cavity and prevents cations from binding in a vacant channel. However, when an anion is present, it counteracts the positive potential of the cavity and a cation can enter. When both ions occupy the cavity, the hydrophobic environment favors a tight ion pair.[45] Consequently, the direction of ion flow is determined by the concentration gradient.

Recent studies have shown that when AmB is administered to sterol-containing liposomes or biomembrane, pore formation rapidly ensues (within seconds).[46] In addition to K^+-ions, protons can also pass through the channel.[47] This can be exploited in NMR studies to estimate ion channel formation. Liposome suspensions with a pH gradient ($\Delta pH = 2$) can induce proton flow. AmB induced proton efflux converts intravesicular $H_2PO_4^-$ to HPO_4^{2-}, in turn leading to a shift of ^{31}P-NMR phosphate signal and allowing channel formation to be monitored (Fig. 9).

AmB ion channel formation has been shown to be dependent on the membrane thickness in liposomal vesicles using ^{31}P-NMR spectroscopy. For a 60 Å membrane prepared with didocosanoyl

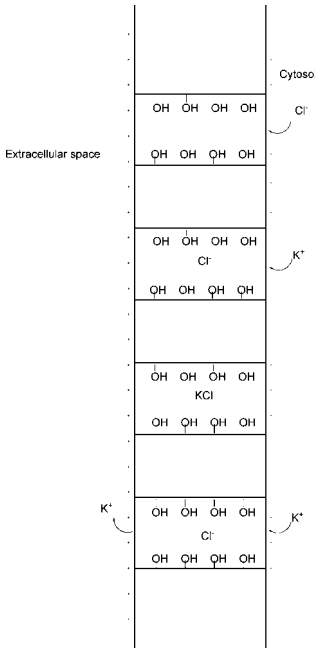


Fig. 8 A model presenting ion channeling by the AmB pore. A positive potential in the cavity allows anions to enter; the negative potential induced by the anion attracts a cation; a tight ion pair is formed and subsequently released along the concentration gradient.

phosphatidyl choline, AmB needs to be administered to both sides of the membrane in order to induce efflux of ions.[48a] This observation is consistent with the formation of a dimeric ion channel. For a thinner membrane prepared from oleyl phosphatidylcholine lipids, AmB only needs to be added to one side of

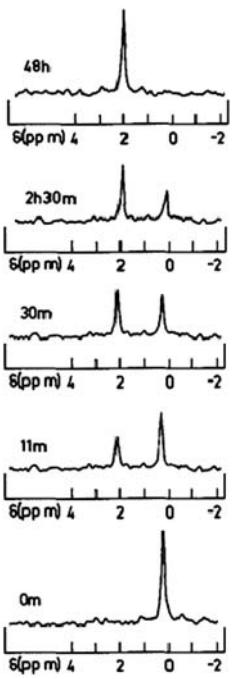


Fig. 9 ^{31}P-NMR studies of phosphate-filled liposomes, triggering a pH change, as a result of proton leakage induced by AmB. Concentration of AmB is 0.060 μM. The picture is taken from ref. 47.

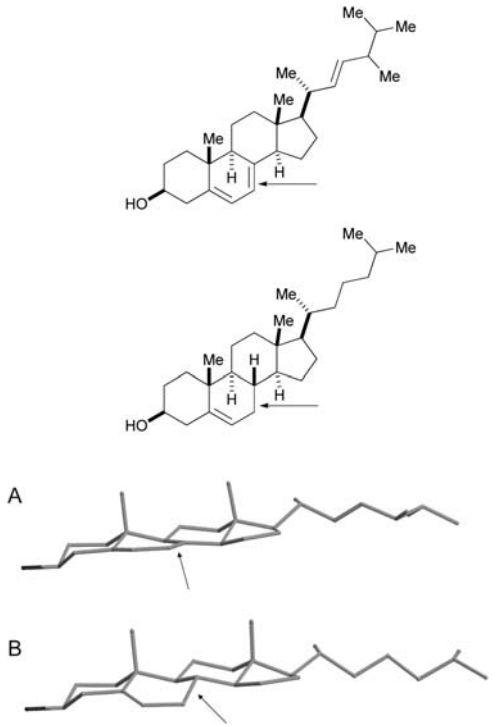


Fig. 10 The conformation of the ring structures in ergosterol (A) and cholesterol (B) viewed from the side at approximately the same angle, together with the flat structures. The arrow indicates the C7–C8 bond, resulting in different B-ring conformations; a 1,3-diplanar chair conformation in ergosterol and a half-chair in cholesterol. Picture taken with permission from ref. 1.

the membrane, suggesting that either a single ion channel unit is formed or rapid penetration of the membrane by AmB takes place.[48a]

The interaction between AmB and sterols in the membrane are complex and poorly understood. For example, the alkyl chain on the sterol is of great importance for inducing channel formation. Membranes containing sterols bearing an unsaturated alkyl chain are considerably better for ion channel formation. In addition, the conformation of the sterol is important for membrane packing (Fig. 10)[48b] Permeability studies have shown that ergosterol-containing membranes are less tightly packed than cholesterol-containing membranes.[49] Consequently, the differentiation of AmB for the two membranes might be a direct result of the membrane packing.

Bolard and coworkers showed that AmB induces K^+ efflux from erythrocytes (cholesterol-containing membrane) at concentrations at which self-association is observed by CD spectrometry.[50] In contrast, K^+ efflux from yeast cells (ergosterol-containing membranes) is observed at much lower concentrations where AmB is still in its monomeric form. It was found that on average, only one AmB molecule per vesicle is needed to observe K^+ efflux. This observation seems to further highlight the high propensity of AmB for self-association and implicates rapid exchange of AmB molecules among vesicles. The structurally related polyene macrolide nystatin can also easily exchange between the aqueous phase and the membrane.[51] In addition, they found that an average of 4 molecules of AmB are needed to interact per molecule of cholesterol in the vesicle membrane in order to induce K^+ efflux. Thus, these studies suggest that AmB may not need cholesterol for pore formation. Based on these results, a new model was proposed in which two types of mechanisms for channel formations where suggested (Fig. 11).[50] With ergosterol (a) the pore is constituted of AmB–ergosterol complexes, with alternating AmB molecules and sterols lined up parallel forming a ring. Furthermore, ergosterol is translocated out of the bilayer to assist in channel formation. With cholesterol (b), the pore is formed of AmB dimers or groups of dimers, positioned head-to-tail, cholesterol does interact with the pore and remains in the phospholipid phase. However no evidence has been forthcoming for the appearance of the polar head group of AmB on the inside of the cell, as hypothesized in Fig. 11b.[52]

Recently, a revised model has been proposed, based on interatomic distances derived from solid state NMR and computational analysis, that suggests that ergosterol is located along the outer rim rather than forming an integral part of the AmB pore.[53] According to this hypothesis it would be possible for two neighboring AmB molecules to interact with each other.

3.2 Other possible modes of action of amphotericin B

Although the barrel-stave ion channel model is the most commonly invoked mechanism of action for AmB, there are alternatives which are supported by experimental data. Lamy-Freund and coworkers found that concentrated samples of AmB (1.0–10 mM) can undergo partial auto-oxidation in aqueous media and protic solvents on a time-scale of hours.[55] Within the same time frame, loss of biological activity was observed when the samples were shaken under aerobic conditions at 37 °C. Slower inactivation was observed at 27 °C.[56] Importantly, this oxidative inactivation could be delayed by antioxidants and accelerated by oxidants. In contrast, it was found that the ability to induce potassium efflux is not dependent on the rate of oxidation.[57] A striking result was obtained in a potassium efflux experiment, where AmB showed strong potassium leakage from liposomes at 0.16 μg of AmB per ml, but no growth inhibition of yeast (*Candida albicans*). A similar phenomenon has been observed for inactive derivatives of AmB.[52] It has also been shown that the addition of AmB to cell cultures at concentrations below the MIC lead to cell growth stimulation, instead of cell growth inhibition.[58]

There have been some intriguing results involving a study of glycerol efflux induced in yeast.[59] When glucose is added to cultures of yeast *S. cerevisiae*, an increase in the production of glycerol in the cytosol can be observed. However, in an experiment wherein 1.0 μM AmB was co-added with glucose, the glycerol level decreased and with time approached zero. A known signaling pathway responsible for the induction of increased glycerol production involves the high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway.[60] This is triggered when yeast cells experience high osmolarity changes in their environment, for example by glucose exposure or salt stress. High osmolarity in the extracellular space leads to loss of water and results in shrinking of the cell, which may eventually lead to cell death. To compensate for the loss of water and to preserve cell size, production of glycerol is increased. However, if the fungal cells are exposed to high

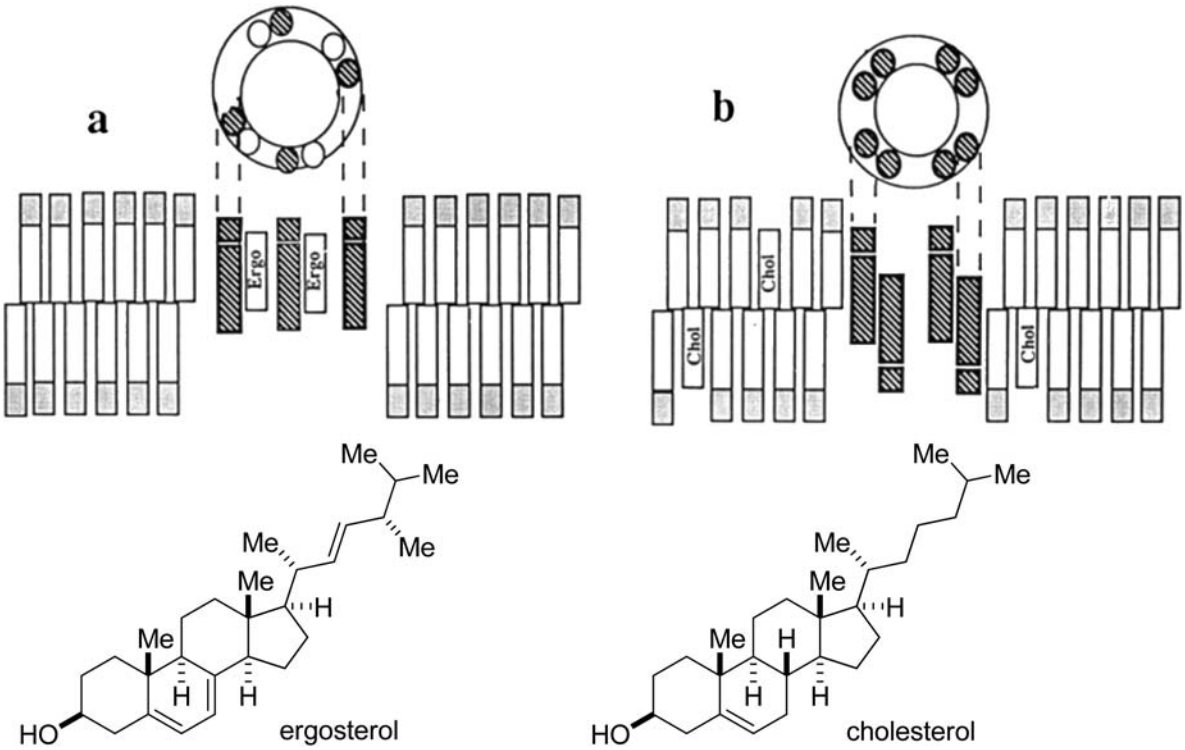


Fig. 11 Schematic representation of the AmB pores inducing K^+ permeability by one-sided action through ergosterol- and cholesterol-containing membranes. Amphotericin B is represented by black rectangles. a) Model for ergosterol-containing membranes. b) Model for ergosterol-containing membranes. The thickness of the bilayer is based on pure di-18:1 PC, and is 57 Å wide.[54] The longitudinal axis of AmB is 24 Å long. The picture is taken with permission from ref. 50.

osmolarity for a prolonged period of time, the production of glycerol is not able to rescue the cell. The breakdown of concentration gradients caused by the formation of AmB channels may trigger the signaling of the HOG MAP kinase pathway. However, a direct link to the HOG pathway has not yet been made.

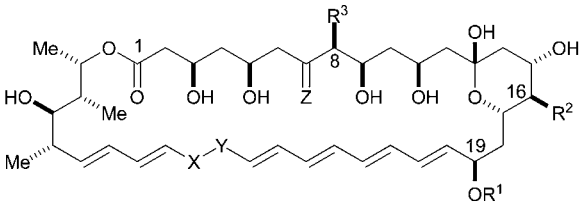
4 Biosynthetic derivatives of amphotericin B

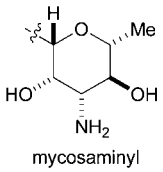
AmB is widely used as a therapeutic agent against systemic mycoses. Because of its pharmacological importance, great efforts have been made to allow for the efficient production of AmB by fermentation. In the course of developing the fermentation process, the biosynthetic pathway was elucidated. The polyketide synthase cluster (PKS) involved in the biosynthesis of AmB contains no fewer than 18 extension modules and is one of the larger PKS clusters known.[61–63] With the identification of the genes coding for AmB it became possible to transform a protoplast, containing the coding genes for enzymes required for the biosynthesis of AmB from *Streptomyces nodosus* into *Escherichia coli.* Industrial fermentation continues to rely on the fermentation of production strains of *S. nodosus*.[64,65]

Knowledge of the genes for the AmB PKS made it possible to obtain derivatives of AmB and AmA (**2**) by modification and deletion of specific genes (Fig. 12). Thus, biosynthetic derivatives were obtained with alterations at C8 and C16, saturation of the C28–C29 double bond, oxidation at C7, and missing the mycosamine.[66,67] Deletion of the *amphDIII* gene that codes for a GDP-mannose-4,6-dehydratase leads to disruption of mycosamine synthesis. The resulting *E. coli* strain instead produces compounds **3** and **4** that also lack the hydroxyl group at C8. This shows that the C8-hydroxyl group is installed only after glycosidation of the aglycone with the mycosamine and highlights the role of this moiety as a recognition element for the hydroxylation enzyme.[63] Analogues **3** and **4** displayed no antifungal activity (Table 1). In contrast, the *E. coli* mutant resulting from deletion of *amphL*, coding for a cytochrome P450 enzyme, yielded active 8-deoxyamphotericin B (**5**) and A (**6**). Importantly, this showed that in contrast to theoretical models,[40] the C8-hydroxy function is not important for biological activity.

Deletion of a second gene coding for a cytochrome P450 protein, *amphN*, responsible for oxidizing the C16-methylene to C16-carboxylic acid, led to compound **7**. This compound possessed the same antifungal activity as AmB but exhibited a 10-fold reduced hemolytic activity. Recently this same compound has been obtained *via* semi-synthetic methods, revalidating the previous results.[68] The reduced hemolytic activity supports the hypothesis that the carboxylic acid is involved in the toxicity associated with AmB (**1**).[69] With respect to the mechanism of action of AmB, this result cannot be squared with theoretical calculations,[40] which would have the carboxylic acid stabilizing the ion channel through electrostatic or hydrogen bonding interaction with the amine of a neighboring AmB molecule.

The deletion of a gene coding for a 3-oxoacyl reductase led to a strain producing 7-oxoamphotericin B (**8**). This compound





mycosaminyl

Entry	Compound	R^1	R^2	R^3	X—Y	Z
1	AmB, **1**	mycosaminyl	—CO_2H	—OH		H,H
2	AmA, **2**	mycosaminyl	—CO_2H	—OH		H,H
3	**3**	—H	—CO_2H	—H		H,H
4	**4**	—H	—CO_2H	—H		H,H
5	**5**	mycosaminyl	—CO_2H	—H		H,H
6	**6**	mycosaminyl	—CO_2H	—H		H,H
7	**7**	mycosaminyl	—Me	—OH		H,H
8	**8**	mycosaminyl	—CO_2H	—OH		O

Fig. 12 Amphotericin A, amphotericin B and bioengineered analogs.

exhibits reduced antifungal activity (3-fold less) but also a 10-fold reduced hemolytic activity compared to the natural product.[67]

In summary, genetic modification of polyketide synthase is a powerful method of preparing novel AmB analogs. Using this approach, AmB analogs may be accessed relatively rapidly and screened for improved pharmacological properties or used to investigate the mode of action of AmB.

5 Chemical semi-synthetic derivatization of amphotericin B

5.1 Small structural changes of amphotericin B

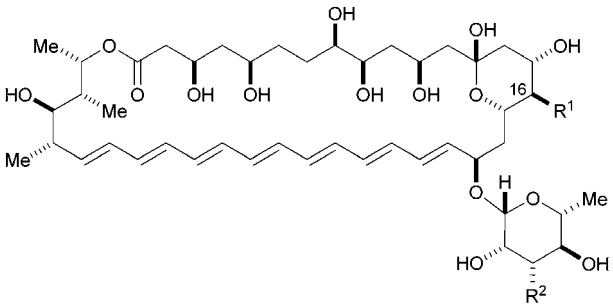
The large number of the structure–activity relationships studied to date have relied on chemical modification of natural AmB (**1**).[18] However, chemical modification of AmB is non-trivial because of its dense array of functional groups and low solubility in most common solvents. Additionally, the macrolactone is susceptible to saponification; the heptaene and the hydroxyl groups are prone to oxidation, and the hemiketal and the mycosamine are acid-sensitive. The C16-carboxylic acid is an easily accessible locus for chemical modification by *e.g.* esterification or amidation. In addition, the amine located on the mycosamine can be chemically modified by its condensation with a carboxylic acid or by alkylation strategies.

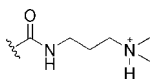
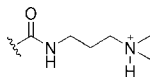
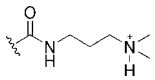
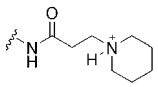
Borowski and coworkers found that the presence of a positively charged nitrogen (protonated or bearing a fixed charge) is essential for biological activity.[69–71] Acylation of the amine results in AmB derivatives with severely reduced activity (entries 4–6), whereas alkylation has a much more moderate effect (compounds **16**, **20** and **21**). Notably, acylation of the amine with a small amino acid leads to highly active AmB analogs (entries 7–11). The introduction of a positively charged group at C16 partially restores the antifungal activity, as can be seen for compound **14** compared to **13**. Thus, the presence, but not the location, of a free amine function on or near the mycsoamine moiety is essential for antifungal activity.

An important early observation was that the C16-carboxylic acid was not needed for biological activity (compare entries 1 and 2). Derivatization of the carboxylic acid as a methyl ester has no effect on activity (entry 2). Interestingly, toxicity is reduced three-fold for the ester in an *in vitro* red blood cell lysis assay. Furthermore, if a positively charged function is introduced at C16, (entry 3), giving the molecule an overall net charge of +2 at physiological conditions, there is no loss of activity and toxicity is reduced even further. A striking result was obtained with piperidine derivative **22** (entry 14), which exhibited good antifungal activity (IC_{50} = 0.15 μg/ml, 3-fold less than AmB) and notable reduced toxicity (175-fold).[72]

Several research groups have targeted semi-synthetic AmB analogs with the aim of producing derivatives with improved pharmacological properties and reduced toxicity. AmB analogs deriving from acylation of the amine of the mycosamine group with lysine, ornithine glycine, alanine and aminoethyl glycine ($RCOCH_2NHCH_2CH_2NH_2$), and methylation of the C16 carboxylic acid possess activity similar to that of the methyl ester of AmB. If the mycosamine is substituted with histidine or tryptophan or the carboxylic acid of AmB is benzylated, the activity drops.[73,74] However, reductive amination of AmB with

Table 1 Chemical and biological properties of charged AmB derivatives



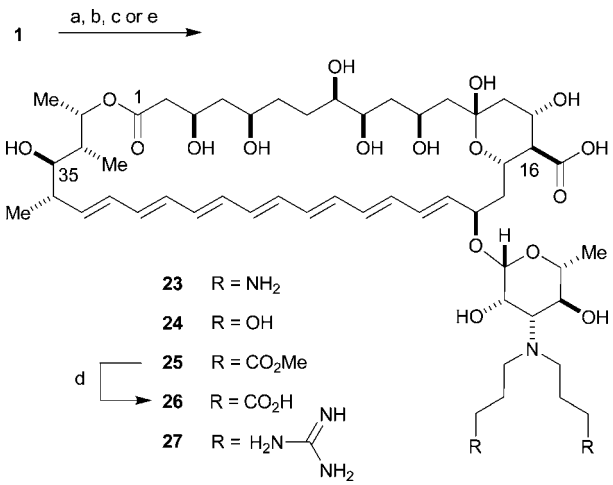
Entry	Compound	R^1	R^2	IC_{50} (μg/ml)[a]	EH_{50} (μg/ml)[b]
1	**9**	CO_2^-	NH_3^+	0.05	1.7
2	**10**	CO_2Me	NH_3^+	0.07	4.8
3	**11**		NH_3^+	0.08	6.5
4	**12**	CO_2^-	NHCOMe	0.35	25
5	**13**	CO_2Me	NHCOMe	0.55	115
6	**14**		NHCOMe	0.25	14
7	**15**	CO_2Me	$NHCOCH_2NH_3^+$	0.07	4.6
8	**16**	CO_2^-	$NHCOCH_2N^+HMe_2$	0.08	11
9	**17**	CO_2Me	$NHCOCH_2N^+HMe_2$	0.12	3.9
10	**18**		$NHCOCH_2N^+HMe_2$	0.13	15
11	**19**	CO_2Me	$NHCO(CH_2)_2N^+HMe_2$	0.1	2.9
12	**20**	CO_2^-	N^+Me_3	0.08	6.5
13	**21**	CO_2Me	N^+Me_3	0.07	8
14	**22**	CO_2Me		0.15	300

[a] IC_{50} was determined using *S. cerevisiae*, and indicates 50% of microbial growth inhibition. [b] EH_{50} indicates 50% hemoglobin release.

aromatic aldehydes bearing an electron-withdrawing group (such as halogen or nitro substituents) in the *para*-position of the aromatic ring leads to compounds with similar activity to AmB (**1**). If an electron-donating group, such as an alkoxy, dimethyl amino or alkyl group, is present in the *para*-position, the compounds are less active.[75]

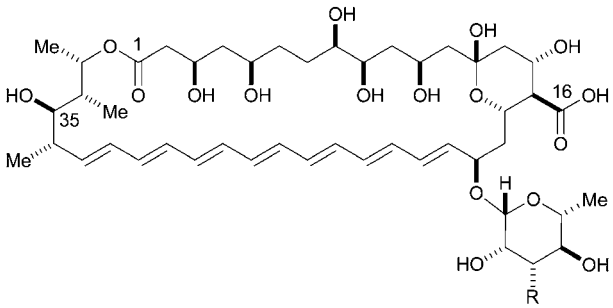
Carreira has documented the synthesis of analogs with significant improvement in antifungal activity through double reductive alkylation of the mycosamine with amino-propionaldehyde (Scheme 1).[76] The introduction of two amino propylene side-chains, to give the *N*,*N*-di-(3-amino-propyl)amphotericin B analog (**23**), caused a more than 10-fold improvement in activity (entry 2, Table 2). This analog displayed 2.5-fold reduction in EH_{50} (the concentration causing 50% hemoglobin release from human red blood cells).

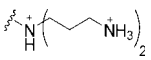
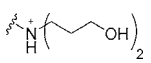
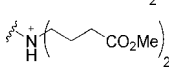
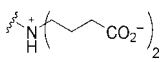
A collection of derivatives incorporating alcohol, ester and carboxylic acids in the mycosamine were prepared (Scheme 1).[77,78] The introduction of these groups had a deleterious effect on activity. The introduction of a guanidine provided conjugate **27** with similar antifungal activity to *N*,*N*-di-(3-aminopropyl)amphotericin B (**23**) (Table 2, entry 6). Even though



Scheme 1 *Reagents and conditions*: a) i. $FmocNH(CH_2)_2CHO$, $NaCNBH_3$, DMF; ii. piperidine, DMF, 76% for **23**. b) i. $TBSOCH_2CH_2CHO$, $NaCNBH_3$, DMF; ii. HF·pyridine, 28% for **24**. c) $MeO_2CCH_2CH_2CHO$, $NaCNBH_3$, DMF, 45% for **25**. d) LiOH, THF–H_2O, 43% for **26**. e) 1*H*-pyrazolecarboxamidine hydrochloride, DIPEA, DMF, 97% for **27**.

Table 2 Activity and toxicity of *N,N*-dialkylated AmB derivatives



Entry	Compound	R	MIC (μM)[a]	EH_{50} (μM)[b,c]
1	**1**	NH_3^+	0.30	4.0
2	**23**		0.020	10.0
3	**24**		0.50	n.d.
4	**25**		2.0	n.d.
5	**26**		>10	n.d.
6	**27**		0.020	n.d.

[a] MIC was determined using *S. cerevisiae*, and indicates the minimal concentration needed to inhibit growth, following the NCCLS protocol.[79] [b] EH_{50} represents 50% hemoglobin release. [c] n.d. – not determined.

the incorporation of a guanidine on the mycosamine enhanced potency, the observed effect was limited to AmB. Other polyene macrolides similarly modified with a guanidine did not benefit from this modification.

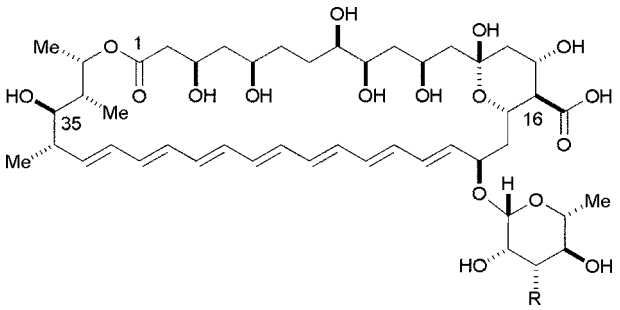
The reductive amination strategy described above was employed to generate alkylated mycosamine derivatives and several other polyamine analogs of AmB (Table 3). In order to examine the structural dependency on the topological distance between the core structure and the positive charge, compounds were prepared in which the length of the chain interconnecting the nitrogens on the side-chain with the mycosamine nitrogen was varied. The antifungal activity for the various polyamine derivatives was measured and documented. Two aminoethylene side-chains had no beneficial effect on the MIC value of compound **28** (entry 3). The decreased potency of **29** (entry 4) also suggests that the length of the spacer between the nitrogen atoms is a very important factor for activity. Furthermore, the reduced potency of derivative **30** compared to **23** (entries 4 *vs.* 2) gives some indication that a large number of primary amino groups are not necessary for optimal antifungal activity. In addition, the guanidylation of *N,N*-di-(3-aminoethyl)-amphotericin B (**28**) yielded compound **31**, which showed only a three-fold increase in activity compared with AmB (**1**).

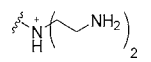
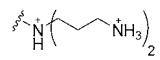
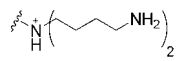
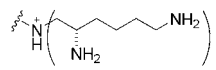
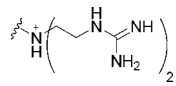
The effect of derivatization at the C16 carboxylic acid in AmB has been the subject of intensive studies. Masking the carboxylic acid with esters or amides has led often to derivatives with reduced hemotoxicity, as noted earlier. In addition, to modifications on the mycosamine, Carreira *et al.* have investigated cationic derivatization on the carboxylic acid. All amide coupling reactions were performed on the polyamine derivative **32**, where the mycosamine side-chains were protected with Fmoc groups (Scheme 2). Primary and secondary amines were used in the reaction in combination with peptide coupling reagents (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 1-hydroxybenzotriazole (HOBt).

Biological testing allowed the therapeutic index of the C16-conjugated analogs **33–41** to be compared to the free acid derivative **23** against *S. cerevisiae* and natural AmB (**1**) (Table 4). Most compounds exhibit antifungal activity similar to AmB where the MIC values were found to be between 0.20 and 0.60 μM. However, azole derivative **41** shows a clear improvement in the potency compared with AmB. Interestingly, derivatives **34** and **36**, both containing a primary amine in the amide side-chain, were also much more active, with MIC values of 0.040 μM and 0.060 μM.

The selected compounds were also screened against several *Candida* strains which include the wild-type strain as well as different clinical isolates and a mutant strain (Table 4). The majority of the derivatives were slightly less potent against *S. cerevisiae* as they were against all *C. albicans* strains, with the exception of azole **41** (MIC 0.040 μM). Overall, derivative **41** displays the best improvement in antifungal activity compared to AmB. For all the various yeast strains tested, this conjugate was typically 10 times more active; with the resistant strain the effect was even larger. The toxicity of the compounds were tested in a hemoglobin release test. All compounds showed greatly reduced toxicity towards human erythrocytes when compared to AmB.

Table 3 Minimum inhibitory concentration for compounds bearing a polyamine



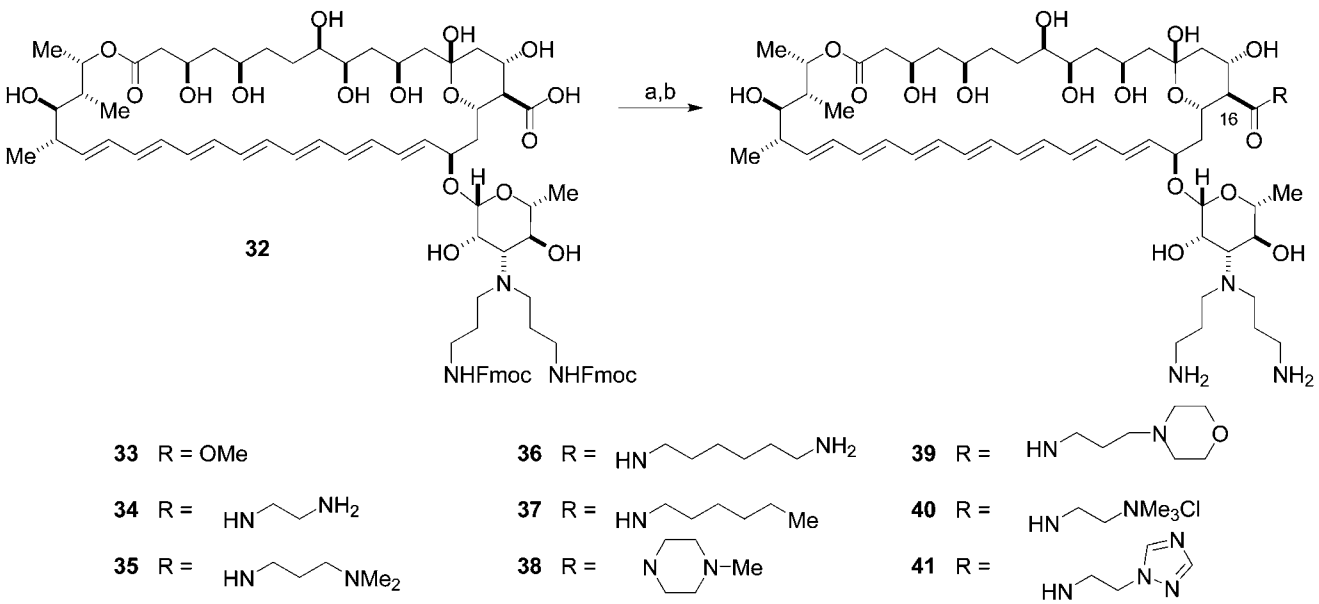
Entry	Compound	R	MIC (μM)[a]
1	**1**	NH_3^+	0.30
2	**23**		0.020
3	**28**		0.25
4	**29**		0.50
5	**30**		0.080
6	**31**		0.10

[a] MIC was determined using *S. cerevisiae*, and indicates the minimal concentration needed to inhibition growth, following the NCCLS protocol.[79]

Researchers at SmithKline Beecham (currently Glaxo-SmithKline) have reported a series of semi-synthetic analogs of AmB. This work relied on the ability to selectively modify the oxidation state of the C16 carbon and the hemiketal. Thus, reduction of the carboxylic acid function afforded 16-decarboxy-16-(hydroxymethyl)amphotericin B (Fig. 13).[80] Alcohol **42** had similar antifungal activity to AmB, and very low toxicity.

In an additional study, AmB ketone derivatives were synthesized (Scheme 3). AmB was persilylated with TES triflate. However, elimination took place at C13–C14, yielding 13,14-anhydroamphotericin B **43a**.[81] This TES-protected AmB derivative was reacted with 2-thiopyridyl chloroformate to give the mixed anhydride **43b**. Addition of commercially available Grignard reagents to the thioester afforded the corresponding ketones. Regeneration of the C13 hemiacetal was achieved by hydration with PPTS in THF–water. The derivatives formed in this method were active and showed reduced toxicity. Examples of derivatives include C16-phenyl ketone **44**, C16-pyrrol-2-yl ketone **45** and C16-vinyl ketone **46**.[82]

The introduction of fluorine can alter the electronic, lipophilic and steric parameters of drugs. This may in turn influence its pharmacological properties.[83] Furthermore, ^{19}F-labelled AmB is a valuable tool for NMR studies, including for the investigation of its interactions with micelles or even lipid bilayers. The first semi-synthetic fluorinated analog of AmB, where the C13-hydroxy group was replaced by fluorine, proved to be highly sensitive to hydrolysis in aqueous solutions.[84] A more stable fluorinated analog resulted from the selective reaction of Selectfluor with a protected Amb derivative.[85] The reaction was carried out in the presence of water in order to restore the hemiacetal, and provided compound **47** in good yield (Fig. 14). Compound **47** shows very similar antifungal and hemolytic activity to AmB. This makes it very suitable for NMR studies aimed at elucidating the mode of action of AmB. Compound **48**, an alternative fluorinated probe synthesized by the reaction of AmB with *p*-fluorbenzaldehyde under reductive conditions, proved to be almost devoid of antifungal activity. Its hemolytic activity was only two-fold higher than AmB. ^{31}P NMR studies were performed utilizing liposomes filled with phosphate ions. A shift of the ^{31}P-signal of the liposomal phosphate ions was



Scheme 2 *Reagents and conditions*: a) PyBOP, HOBt, DIPEA, RH (**34** and **36** are Fmoc-protected). b) piperidine, DMSO.

Table 4 Minimum inhibitory concentration against various yeast strains

		MIC (μM)[a]					
			C. albicans				
Entry	Compound	*S. cerevisiae* BY4741 (wt)	CAF2-1 (wt)	DSY294 (c.i.)	DSY296 (c.i.)	DSY1764 (AmB-resistant)	EH_{50} (μM)[b]
1	**1**	0.30	0.30	0.40	0.40	50	4.0
2	**23**	0.020	0.10	0.20	0.10	1.0	10
3	**33**	0.10	0.50	0.25	0.10	3.0	50
4	**34**	0.040	2.0	1.0	0.75	3.0	30
5	**35**	0.40	0.50	0.50	0.30	1.0	200
6	**36**	0.060	0.30	0.30	0.10	1.5	120
7	**37**	0.60	0.50	0.50	0.50	1.0	150
8	**38**	0.20	0.30	0.30	0.30	0.50	200
9	**39**	0.40	0.25	0.25	0.50	0.50	200
10	**40**	0.60	1.4	1.0	1.0	4.0	100
11	**41**	0.040	0.040	0.040	0.020	1.0	75

[a] MIC was determined using several yeast strains, and indicates the minimal concentration needed to inhibit growth, following the NCCLS protocol.[79]. wt – wild type; c.i. – clinical isolate. [b] EH_{50} indicates 50% hemoglobin release from red blood cells.

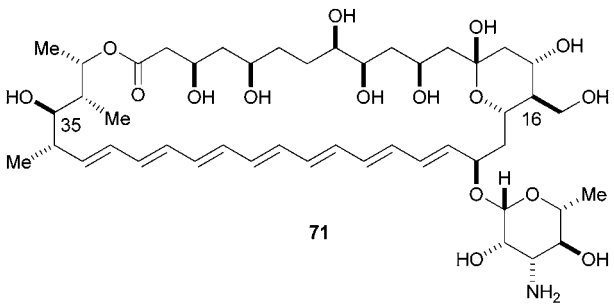


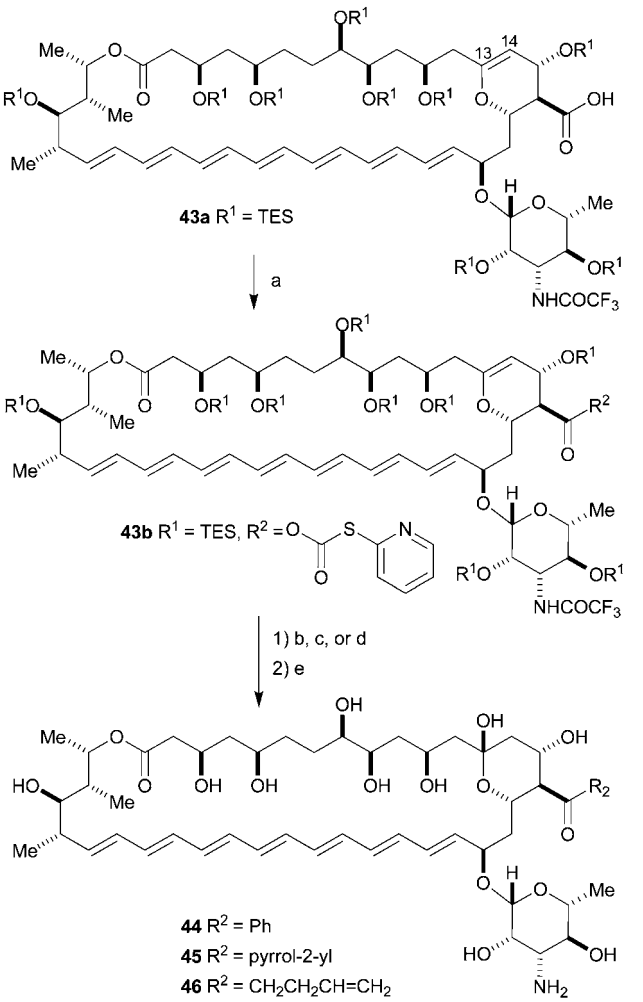
Fig. 13 16-Decarboxy-16-(hydroxymethyl)mphotericin B.

observed, due to pH changes, when the compound was administered to ergosterol-containing liposomes containing a transmembrane pH gradient. This indicates channel formation and proton leakage.[85]

5.2 Amphotericin B conjugation to biomolecules and polymers

Only in the last decade have conjugated derivatives of AmB been examined. The low solubility of AmB in water poses a serious problem for the development of useful formulations. In fact, renal toxicity of AmB has been linked to the form in which it is administered.[86,87] Even though lipid formulations such as AmBisome and Fungizone have been able to overcome some of these side-effects, a more soluble form of the drug could potentially alleviate these problems.

One possibility is to increase the water solubility of the drug by attachment of a carbohydrate.[71,88] A first example of this strategy was reported by Borowski and coworkers.[69,70] The mycosamine on AmB (**1**) was modified using glucose in an Amadori rearrangement and conjugating the intermediate aldehyde to AmB to yield the fructose derivative **49** (Scheme 4). The activity of **49** against *C. albicans in vitro* was similar to that of AmB (**1**). In addition, the toxicity was reduced two-fold. When the acid and the amine groups of AmB were alkylated with diazomethane, compound **50** showed only two-fold lower antifungal activity but a tremendous decrease in toxicity (125-fold) was found (Scheme 5).



Scheme 3 *Reagents and conditions*: a) NEt_3, 2-thiopyridyl chloroformate, Et_2O, 0 °C, 1 h, 76%. b) i. PhMgBr (15 equiv.), THF, 0 °C, 0.5 h; ii. $(CF_3CO)_2O$, NEt_3, CH_2Cl_2, 0 °C, 1–2 h, 73%. c) pyrrol-2-ylmagnesium bromide (20 equiv.), THF, 0 °C, 0.5 h, 82%. d) i. vinylmagnesium bromide (16 equiv.), THF, 0 °C, 1 h; ii. $(CF_3CO)_2O$, NEt_3, CH_2Cl_2, 0 °C, 1 h, 68%. e) i, HF·pyridine, THF, MeOH, pyridine, 25 °C, 16–24 h; ii, PPTS (6–8 equiv.), THF, H_2O, 25 °C, 1–2 h; iii. aq. NH_3, THF, MeOH, 25 °C, 6–12 h, 37–51%.

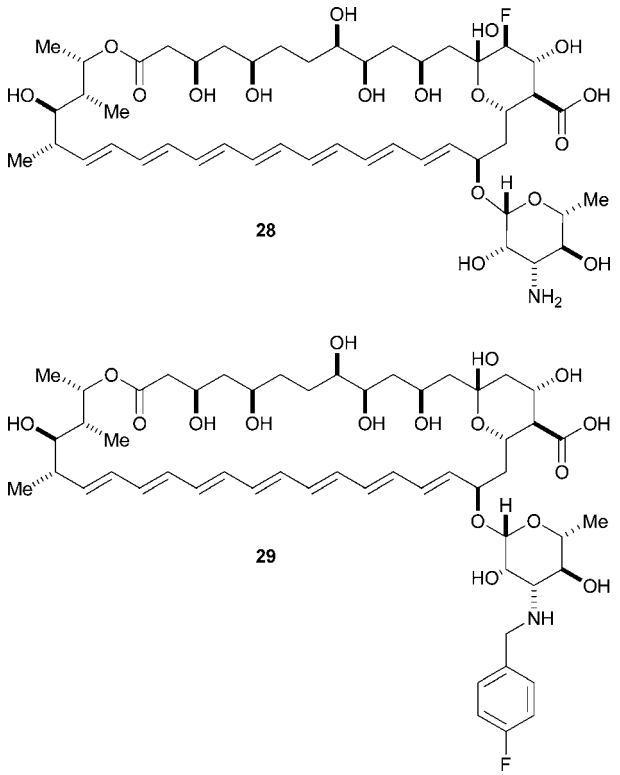
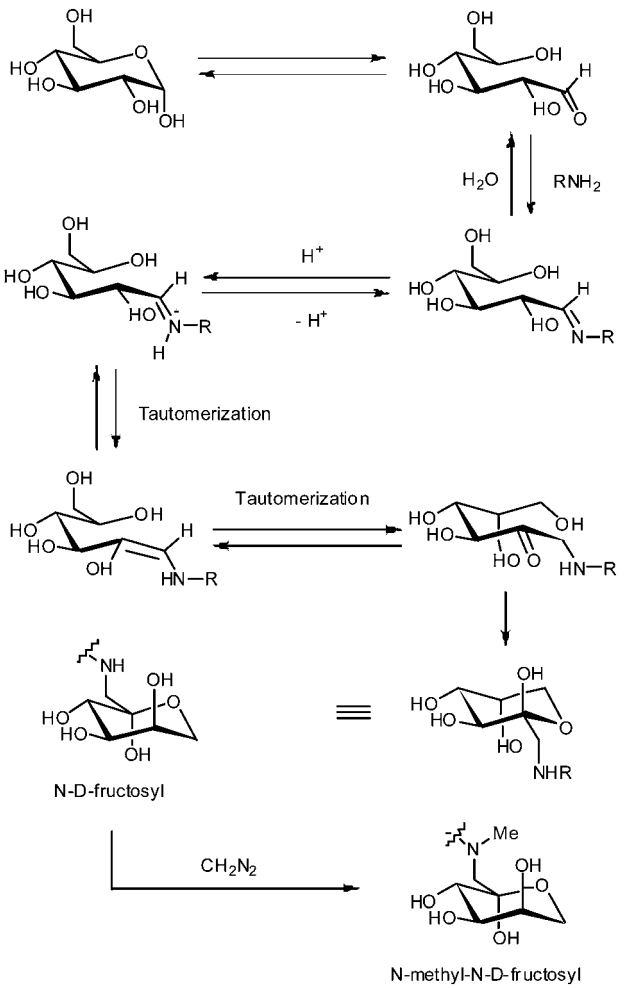


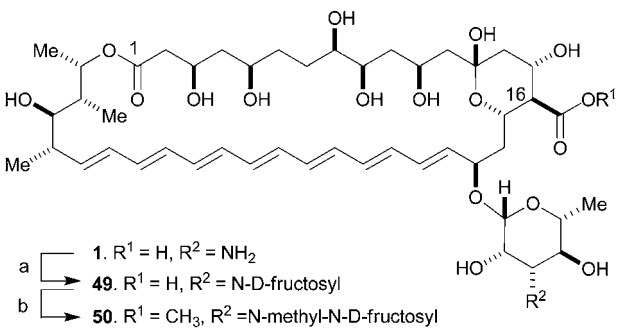
Fig. 14 Fluorinated AmB derivatives developed by Murata.

Carreira *et al.* have investigated the possibility of carbohydrate conjugation between AmB and glucosamine, based on the same principle of increasing solubility of AmB by conjugating it to a sugar (Fig. 15).[43] In addition, glycosamine conjugate **51** might benefit from the cationic amine at physiological pH to enhance solubility even more. However, the antifungal activity was similar to AmB.

Domb and coworkers have investigated the increased solubility resulting from the conjugation of arabinogalactan to AmB (**1**).[89,90] Arabinogalactan (AG) is a highly branched natural polysaccharide consisting of D-galactose and L-arabinose, with high water solubility (70% in water). AG consists of linked β-(1,3)-galactans with side-chains of galactose and arabinose units of various lengths.[91] The derivatization of AmB with AG can be achieved in three steps (Scheme 6): 1) Formation of an AG dialdehyde by oxidation with $NaIO_4$. About 50% of the saccharide was oxidized; 2) Conjugation by imine formation between the oxidized AG and AmB; 3) Reduction of the imine to the amine. This did not go to completion and as a result, the imine and the amine products were isolated separately. The least toxic conjugate was a 20 : 80 wt/wt reduced AmB–AG conjugate.[89] The antifungal activity of this conjugate was preserved, and *in vitro* studies showed that less than 1 μg AmB per mL was needed to fully inhibit growth of *C. albicans*. The toxicity was measured in mice and the maximum tolerated dose (MTD) was 20–40 mg of the reduced AmB–AG conjugate per kg per day for an 8 day period compared to 4 mg AmB per kg per day for Fungizone. In addition, total yeast eradication of the kidneys of the mice was already observed at 8 mg per kg per day with this reduced conjugate.[89,90]



Scheme 4 Amadori rearrangement to obtain a sugar conjugate of AmB.



Scheme 5 Synthesis of fructose derivatives of AmB. *Reagents and conditions*: a) glucose, DMF, 37 °C, 52%. b) CH_2N_2, DMF, 0 °C, 13%.

In later studies, an improved synthesis was developed, in which the primary alcohols of the arabinogalactan were reacted with tosyl or mesyl chloride. Subsequent substitution by the amine of the mycosamine yielded the AmB–AG product in a more reproducible manner and in a wt/wt ratio of about 20 : 80 through the mesylate and 13 : 87 through the tosylate (Scheme 6 ii). These conjugates proved to be slightly less active and more toxic compared to those produced by reductive amination.[92]

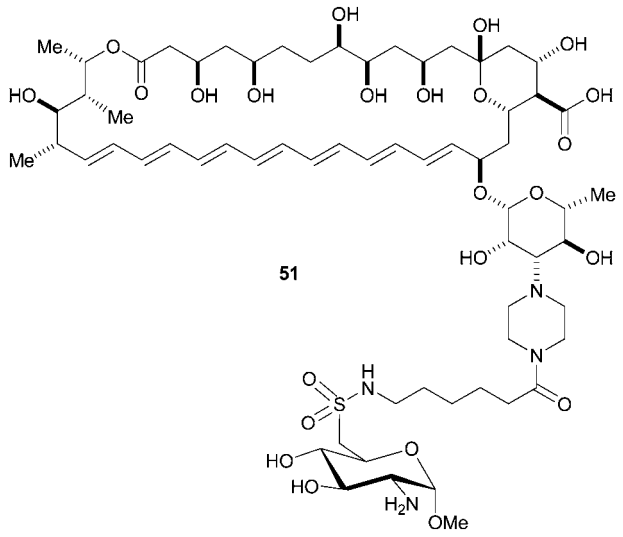
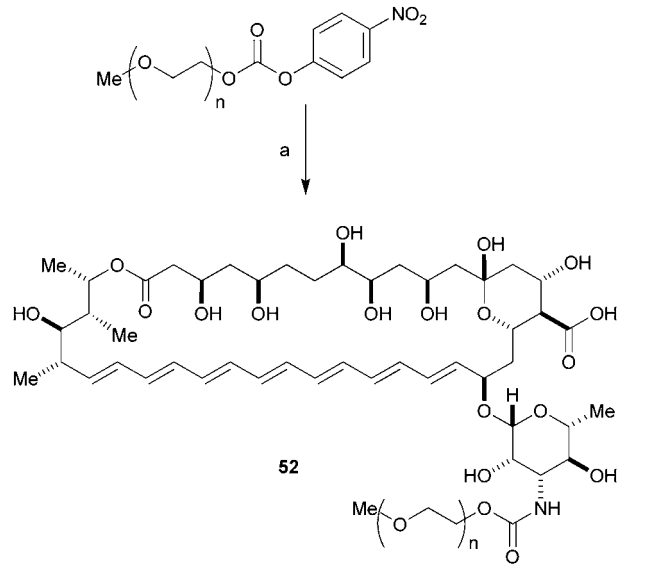
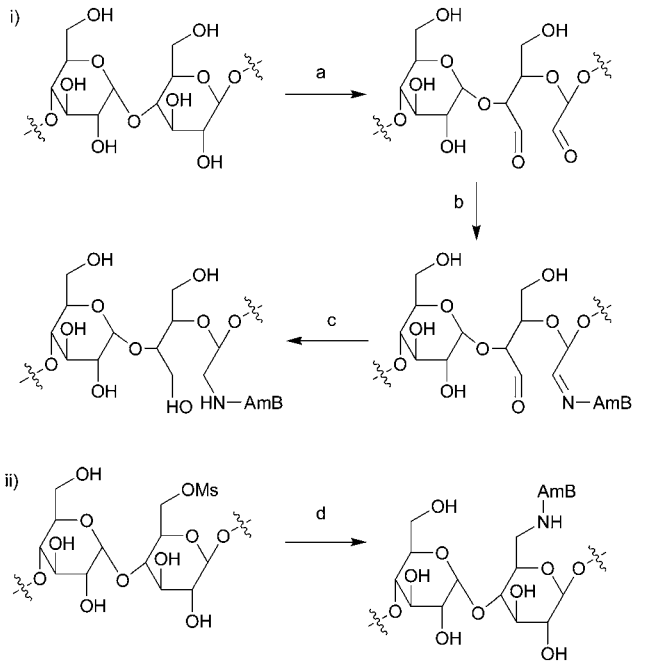


Fig. 15 Glucosamine–AmB conjugate **51**.



Scheme 7 Synthesis of methoxy-PEG–AmB. *Reagents and conditions*: a) AmB, DMAP, DMF.

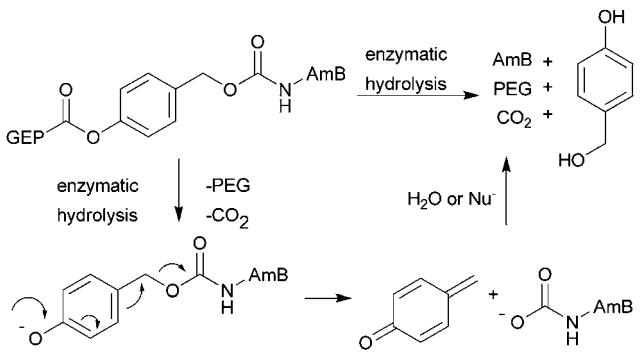


Scheme 6 i) AmB–arabinogalactan conjugation, *via* imine formation and reductive amination. ii) AmB–Arabinogalactan conjugation, *via* substitution. *Reagents and conditions*: a) $NaIO_4$. b) AmB. c) $NaBH_4$. d) AmB.

Solubility enhancement by conjugation of AmB polyethylene glycol (PEG) was initiated by Sedlak and coworkers. Carbamate formation was achieved by substitution of the 4-nitrobenzyl group of a methoxy-PEG–4-nitrobenzyl carbonate polymer with AmB (Scheme 7). The activity of conjugate **52** is comparable to that of Fungizone.[93] Presumably, the carbamate is hydrolyzed *in vivo*, leading to release of free AmB.

The results described above have led other researchers to develop a range of AmB–PEG prodrugs, capable of releasing AmB at a certain pH or by enzymatic cleavage.[94] The first examples relied on enzymatic cleavage of a PEG–benzyl ester bond. Ester hydrolysis took place with a half time of $t_{½} \sim 4$ h (Scheme 8). Ester hydrolysis was determined to be the rate-determining step. This was followed by fast 1,6-elimination and decarboxylation, to release the free drug. The rate of hydrolysis was dependent on the substitution pattern of the benzyl group.[95] The water solubility of these derivatives was 1.4–2.5 mg/mL compared to 0.01 mg/mL for the parent drug. This enhanced solubility makes it possible to achieve similar levels of effectiveness and reduced side-effects at lower doses.

In a second study, a PEG–AmB conjugate was developed that would release AmB in a pH-dependent manner.[96] This allows for selective cleavage in the vicinity of fungal pathogens, since these induce biochemical processes in which the pH is locally lowered from a normal pH of about 7.4–7.6 to ~5.8. A series of linkers was prepared that encompassed an acid-sensitive imine bond (Fig. 16). Analogs **53–55** proved to be stable at physiological pH for at least 24 h. While compound **53** undergoes relatively slow



Scheme 8 Enzymatic cleavage of AmB–PEG prodrug *via* 1,6-elimination.

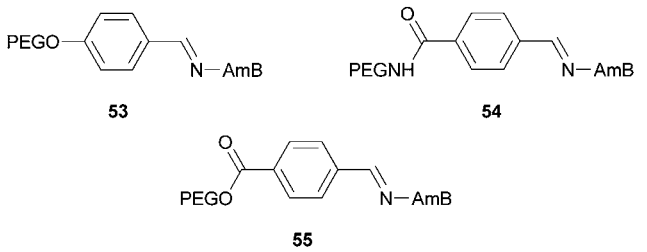


Fig. 16 pH-sensitive AmB–PEG conjugates.

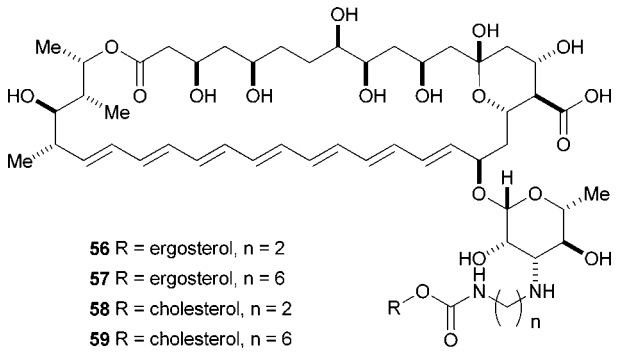
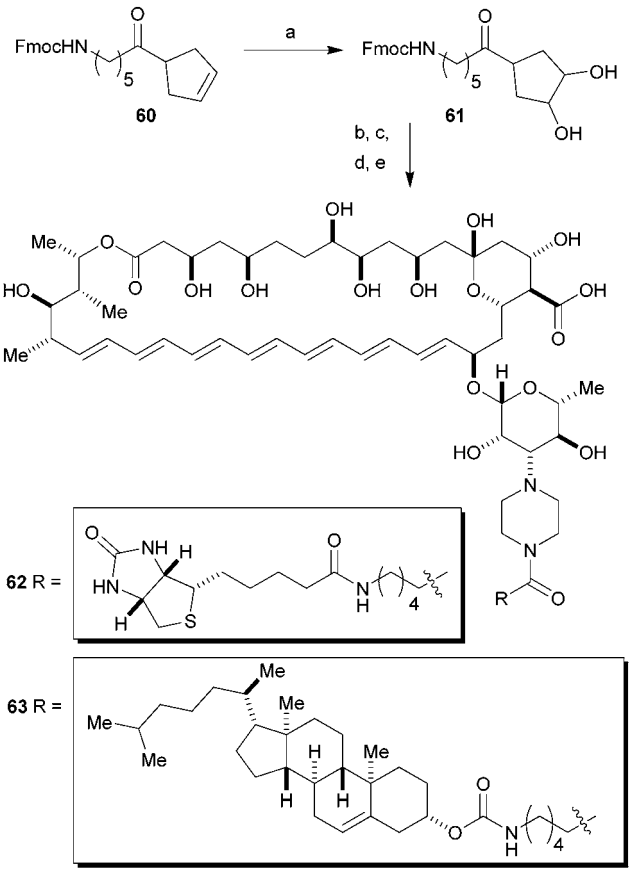


Fig. 17 AmB conjugated to sterol. $n = 2$ or 6; the sterol is either ergosterol or cholesterol.

acid-catalyzed hydrolysis at pH 5.5 ($t_{½} \sim 45$ min), derivatives **54** and **55** hydrolyzed with a half-life of approximately 2 min. *In vivo* mouse studies revealed that the toxicity of derivative **54** and **55** was 10-fold reduced compared to natural AmB.

Murata has examined the effect of conjugating AmB to the sterols it is believed to interact with in biomembranes, ergosterol and cholesterol (Fig. 17).[97] The sterols were attached with varying linker lengths to the mycosamine of AmB by reductive amination. This left the amine still capable of being protonated. The channel-forming properties of conjugates **56–59** was examined in liposomes with a transmembrane pH gradient by NMR. Changes in pH would indicate leakage. The cholesterol conjugates exhibited low channel formation properties, whereas the ergosterol conjugates were higly active in *in vitro* studies. It was found that the $n = 2$ conjugate with ergosterol gave the highest proton efflux compared to other sterol–AmB conjugates. However, none of the compounds showed any antifungal or hemolytic activity.

In a similar fashion, an AmB–phospholipid conjugate was prepared to study AmB–phospholipid interactions.[98] Different phospholipid acyl chain lengths (C_{12}–C_{18}) were used to investigate the influence of chain length on proton efflux. Interestingly, there is a dramatic difference between short and long lipids. Lipids with C_{12} and C_{14} acyl chains show highly concentration-dependent channel formation. There is a defined inflection for concentration, below which there is no leakage from channels and above which all liposomes suddenly rupture. However, the longer lipids (C_{16} and C_{18}) have a more uneven distribution of active channel-forming species, meaning that their aggregates are able to cause proton leakage, but at the same time leave some liposomes intact. The above examples show that it is not trivial to develop a conjugate that exhibits enhanced antifungal activity and reduced toxicity.



Scheme 9 Piperazinyl linker strategy. *Reagents and conditions*: a) K_2OsO_4, NMO, acetone, 66%. b) $NaIO_4$. c) AmB, $NaBH_3CN$, 63%. d) piperidine, DMF, 99%. e) Biotin *N*-hydroxysuccinimide ester or cholesterol chloroformate.

Carreira and coworkers have devised a modular approach for conveniently linking compounds to AmB. The strategy is based on double reductive amination with formation of a piperazine ring involving the amine of the mycosamine moiety.[99] The advantage of this strategy is the possibility of protonating the amine at physiological pH, which is important for antifungal activity. Several biomolecules where linked to AmB with this strategy, such as cholesterol and biotin (Scheme 9). Biotin piperazine conjugate **62** showed a 5-fold increase in activity compared to the corresponding amide linker, again confirming the necessity of a protonatable amine on the mycosamine.

Recently, a series of tryptophan-derivatized AmB derivatives has been investigated using the same linking strategy by the same group, based on the fact that tryptophan-rich peptides have been shown to possess antimicrobial ativity.[100] Conjugation to AmB might have an enhanced effect on the activity of AmB. Interestingly, monotryptophan-conjugated AmB had greater selectivity for ergosterol-containing membranes *vs.* cholesterol-containing membranes than the di- and tri-tryptophan-conjugated derivatives. However, no correlation was found between selectivity for different sterol-containing membranes and antifungal activity.

5.3 Controlled assembly of AmB

The putative ion channels believed to be responsible for the mode of action of AmB have been hypothesized to consist of anywhere between 4 and 12 AmB molecules (see above). A calix[4]arene covalently linked to four AmB molecules was developed in light of this hypothesis (Fig. 18).[46] AmB was attached to a linker by double reductive amination. This linker provided a handle for attachment to the phenol moieties of calix[4]arene.[99] The antifungal activity of the resulting derivative **64** was found to be similar to the parent structure, but with 10-fold reduced haemotoxicity. In addition, calix[4]arene **64** was able to induce ion channel formation in synthetic LUV (Large Unilamellar Vesicles) at rates comparable to those of free AmB. This dramatic result is presumably a result of the positive effect of preassembly, possibly with the added benefit of accumulative recognition of ergosterol-containing membranes.

Murata and coworkers have made a wide variety of dimers, in an effort to shed light on the mechanism of action of AmB (Fig. 19).[101] The first series of dimers linked the mycosamine of two AmB molecules together *via* amidation of an activated diacid with the amine on the mycosamine. Compounds **65–69** were all 10- to 20-fold less active than AmB and most of them exhibited strong toxicity towards human erythrocytes. In addition, NMR studies showed that the dimers assembled themselves into functional channels capable of proton release from sterol-containing liposomes. A second group of dimers (**70** and **71**), linked *via* tartrate–mycosamine conjugation, was synthesized. However, they exhibited no activity and similar hemotoxic effects were found as with the previous dimers.[102] Again, channel formation was found to take place. Based on the observation that a free amine is essential for activity, a third study shifted strategy to dimerization *via* coupling of the carboxylic acid residue of AmB with 1,6-hexadiamine, yielding dimers **72** and **73**. The compound obtained was highly toxic, and antifungal activity was not measured. Interestingly, there was no indication of a channel being formed. However, acylating both amines of the two mycosamines units with *N*-succinimidyl acetate led to a compound capable of acting as a weak antifungal agent and forming transmembrane pores.[103]

5.4 Fluorescent amphotericin B derivatives

Conjugation of AmB to fluorescent markers is an attractive means to visualize the interaction of AmB with living cells and natural biomembranes. Carreira and coworkers reported the conjugation of fluorescein to AmB and monitored its localization in cells with fluorescent microscopy. Using their piperazinyl linker strategy AmB–fluorescein conjugate **74** was prepared and shown to accumulate in yeast cell and mammalian cell membranes (Fig. 20).[52] Fluorescent derivative **74** showed no antifungal activity, but in a K^+ efflux LUV assay, it was found to induce leakage of potassium from ergosterol-containing membranes at 5 μM suggesting channel formation under these conditions. The most striking result is the uniform distribution of the compound over the yeast cell membrane as revealed with fluorescent microscopy. In contrast, in human Jurkat T lymphocyte cells the compound is mainly localized in the interior of the cell.

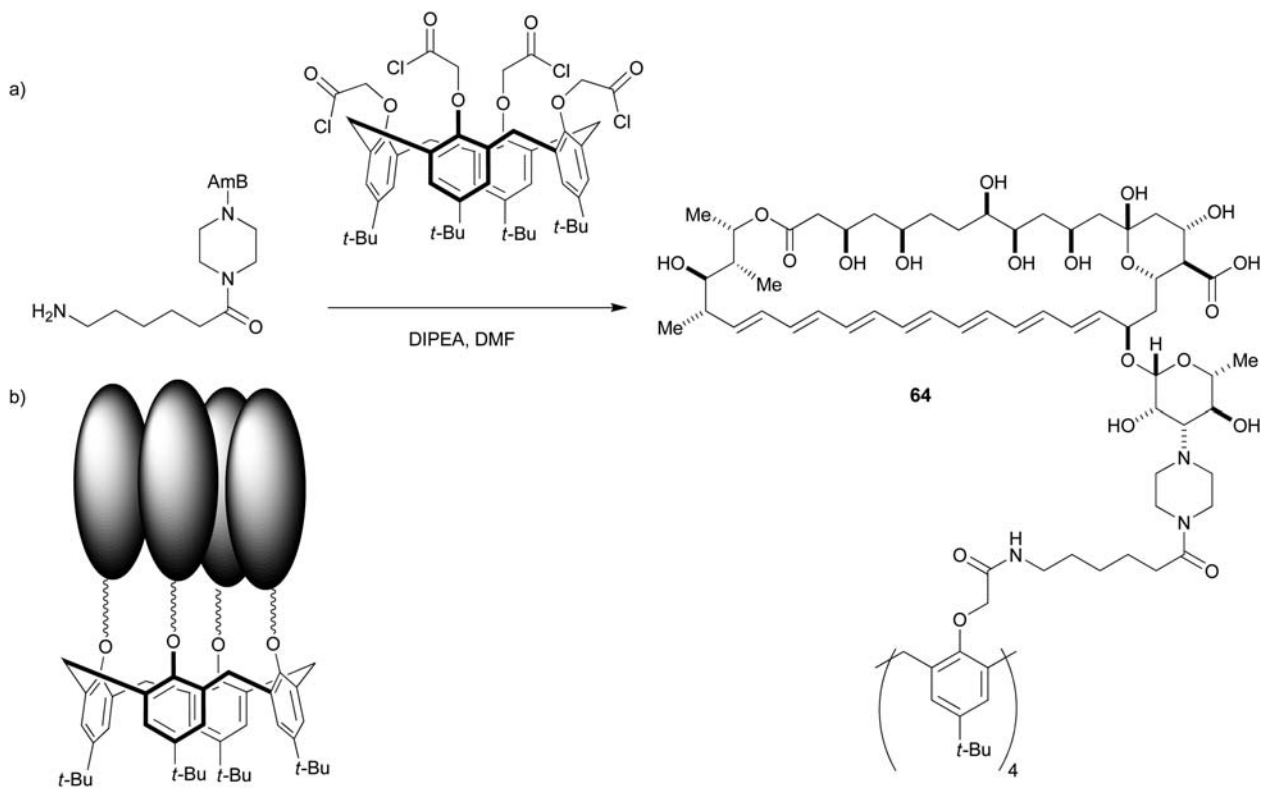


Fig. 18 a) Synthesis of AmB–*t*-butyl calix[4]arene **60** in 17% yield. b) Schematic representation of AmB conjugated to calix[4]arene. The ovals represent AmB, the spirals represent the linker.

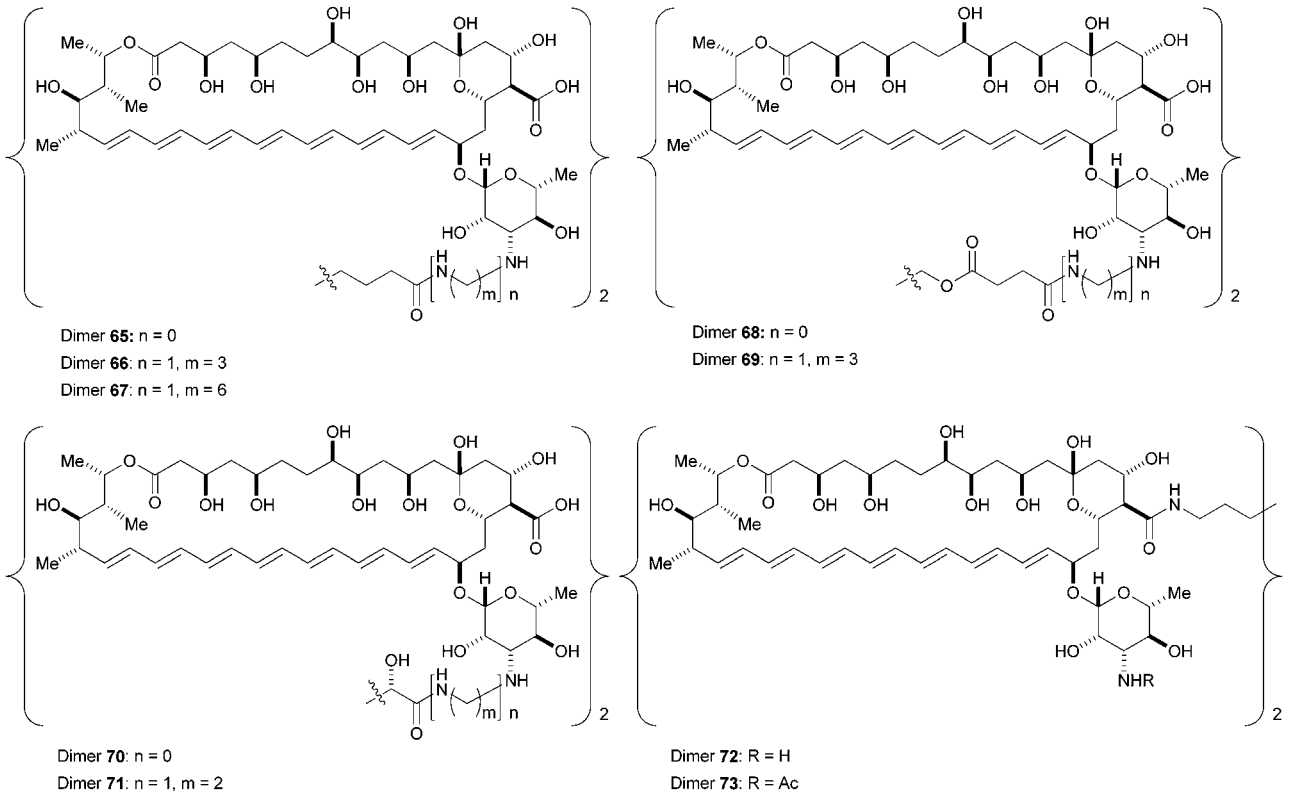


Fig. 19 Dimers synthesized and studied by Murata *et al.*[101]

In the last decade, functionalized carbon nanotubes have found multiple applications in drug delivery of therapeutic molecules.[38] Prato and coworkers reported the double functionalization of carbon nanotubes with AmB and fluorescein to afford conjugate **75**.[104] This allowed visualization of uptake of **75** in human Jurkat cells. An AmB–coumarin derivative (compound **76**) with 4-fold higher antifungal activity than natural AmB has also been shown to be internalized in mammalian HeLa cells as well.[105]

5.5 Conclusion

Semi-synthetic methods give access to a large number of different analogs. Knowledge of SAR combined with new strategies for selective chemistry on the amine, carboxylic acid and hemiketal functions have led to the development of a range of diverse AmB analogs and conjugates with an improved toxicity profile. Several other conjugates have provided clues for the mechanism of action of AmB or may serve as novel agents for probing the state of cell membranes. With the advent of more selective synthetic methods, it may become possible to functionalize other part of the structure in a selective manner. The use of a combination of semi-synthesis and *de novo* synthesis by the groups of Murata and Rychnovsky has led to synthetic/semisynthetic hybrids that have served as probes for the mechanism of action of AmB.

6 AmB derivatives made by diverted total synthesis

Another means of accessing AmB derivatives is through diverted total synthesis.[106] Carreira *et al.* developed a general strategy for the synthesis of AmB analogs (Fig. 21). This synthetic strategy was based on the development of highly efficient gram-scale syntheses of the C1–C20 fragment **80**,[107] C33–C37 fragment **77**[108] and mycosamine moiety **79** (Fig. 21).[109] 35-Deoxy C33–C37 fragment **78** was prepared and combined with fragments **79** and **80**, leading to 35-deoxy-amphotericin B methyl ester **81**.[110,111] This compound showed 18–26-fold lower antifungal activity than AmB methyl ester. Furthermore, it had no ability to form ion channels in a liposomal potassium efflux assay.

These experimental observations lend support to the hypothesis that a dimeric ion channel is necessary for antifungal activity and that the dimerization takes place through hydrogen-bonding between the C35 hydroxy groups of opposing tail-to-tail ion channel subunits. The observations cast some doubt on the oxidative damage mechanism, as this is a pathway that would not likely be affected by the removal of the C35 hydroxyl group.

The use of a diverted total synthesis strategy has several advantages, most importantly that it makes the modification of any functionality within the AmB structure possible virtually at will. In general, however, the labor intensive nature of the approach can be a detractor for a molecule of the sheer complexity of AmB. The strategy we describe was carefully identified to manage the challenge by employing easily accessible and storable synthetic intermediates as common building blocks in a convergent manner.

7 Conclusions

More than 50 years after its discovery, AmB remains one of the most important drugs for the treatment of severe fungal

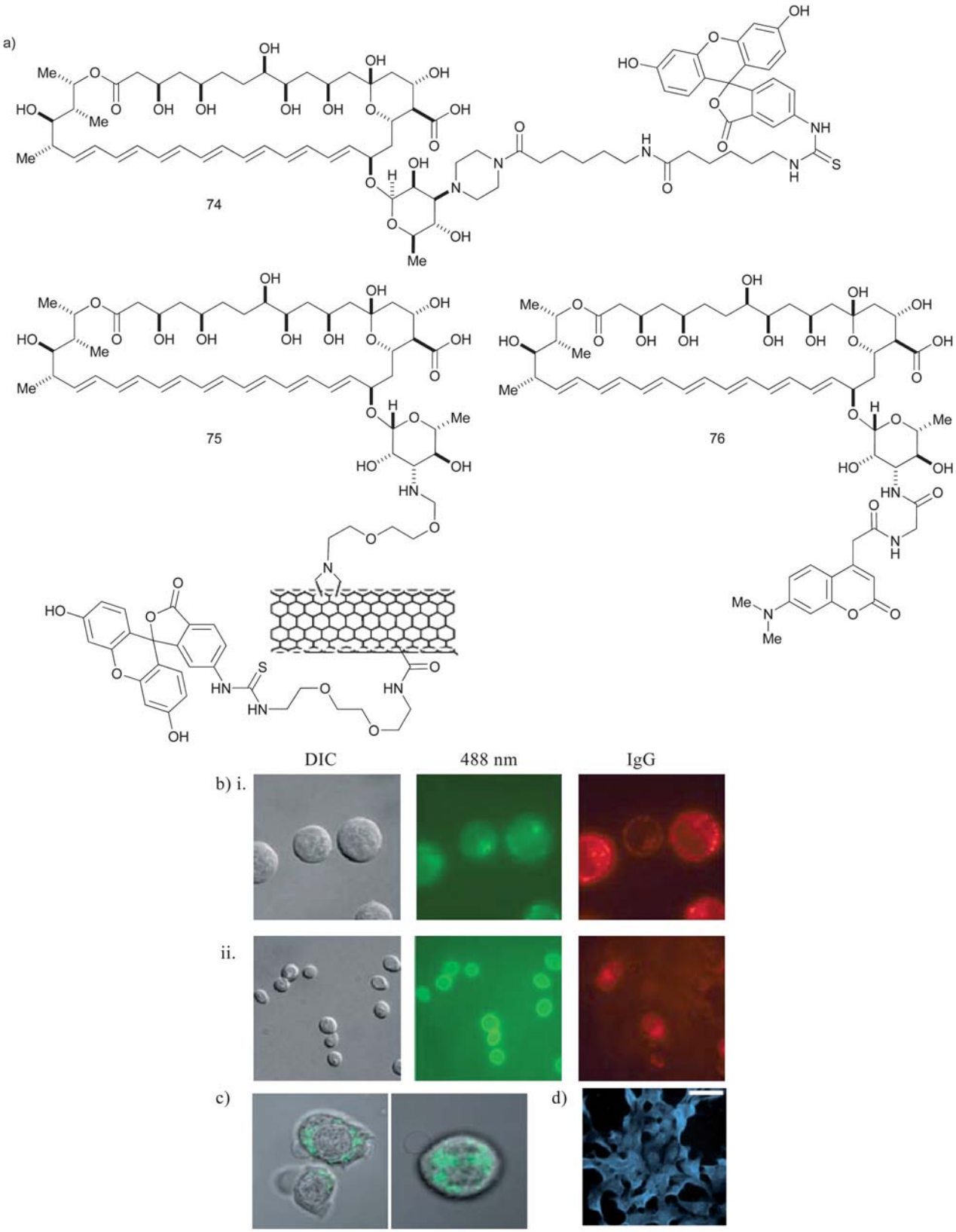


Fig. 20 Localization studies of fluorescent derivatives of AmB. a) Fluorescent conjugates **74**–**76**. b) (i) Jurkat T lymphocyte cells and (ii) yeast cells incubated with conjugate **74**. DIC: differential interference contrast microscopy, 488 nm (green) and antifluorescein IgG (red). c) Jurkat cells incubated with carbon nanotube conjugate **75**. d) HeLa cells incubated with fluorescent AmB **76**. Pictures taken with permission from refs. 52,104 and 105.

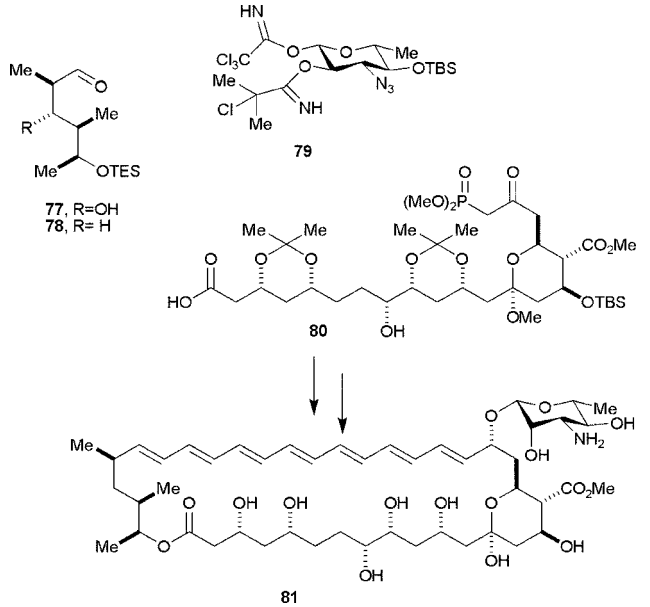


Fig. 21 Schematic of the synthesis of 35-deoxy-amphotericin B methyl ester.

infections. As discussed in this review, there is a treasure chest of observations that have been made with AmB and its derivatives; in some cases these observations are difficult to reconcile with the accepted models for its biological activity. It is therefore tempting to interpret these as portals into a collection of as-yet undiscovered cellular roles for this natural product. There is little doubt that channel formation is operative. However, it could very well be that to refer to a single "mode of action" may not do justice to the class of natural products AmB represents. The recent literature indicates a notable increase in the number of structural modifications designed to probe the activity at greater resolution. It is likely that AmB may be a 'molecular sphinx' which has many more secrets to reveal.

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