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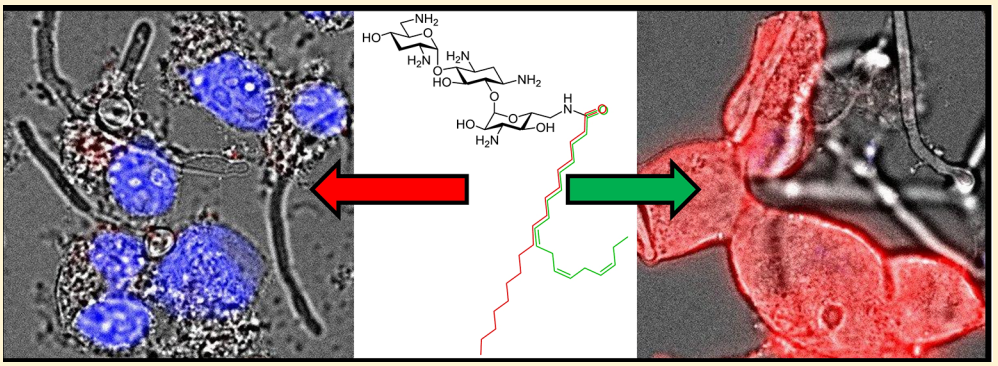
Increased Degree of Unsaturation in the Lipid of Antifungal Cationic Amphiphiles Facilitates Selective Fungal Cell Disruption

Kfir B. Steinbuch,[†] Raphael I. Benhamou,[†] Lotan Levin,[‡] Reuven Stein,[‡] and Micha Fridman*[,†]

[†]School of Chemistry, Raymond and Beverley Sackler Faculty of Exact Sciences, Tel Aviv University, Tel Aviv, Israel 6997801

[‡]Department of Neurobiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel 6997801

Supporting Information



ABSTRACT: Antimicrobial cationic amphiphiles derived from aminoglycosides act through cell membrane permeabilization but have limited selectivity for microbial cell membranes. Herein, we report that an increased degree of unsaturation in the fatty acid segment of antifungal cationic amphiphiles derived from the aminoglycoside tobramycin significantly reduced toxicity to mammalian cells. A collection of tobramycin-derived cationic amphiphiles substituted with C_{18} lipid chains varying in degree of unsaturation and double bond configuration were synthesized. All had potent activity against a panel of important fungal pathogens including strains with resistance to a variety of antifungal drugs. The tobramycin-derived cationic amphiphile substituted with linolenic acid with three *cis* double bonds (compound **6**) was up to an order of magnitude less toxic to mammalian cells than cationic amphiphiles composed of lipids with a lower degree of unsaturation and than the fungal membrane disrupting drug amphotericin B. Compound **6** was 12-fold more selective (red blood cell hemolysis relative to antifungal activity) than compound **1**, the derivative with a fully saturated lipid chain. Notably, compound **6** disrupted the membranes of fungal cells without affecting the viability of cocultured mammalian cells. This study demonstrates that the degree of unsaturation and the configuration of the double bond in lipids of cationic amphiphiles are important parameters that, if optimized, result in compounds with broad spectrum and potent antifungal activity as well as reduced toxicity toward mammalian cells.

KEYWORDS: *amphiphilic aminoglycosides, membrane disruption, antifungal drugs, lipid unsaturation*

Fungal infections such as candidiasis are a major cause of nosocomial infections and are especially prevalent among patients with compromised immune systems.[1,2] The high evolutionary similarity between fungal and mammalian cells greatly limits the number of specific targets available for antifungal drug development, and there are currently very few classes of antifungal drugs in clinical use (Figure 1).[3] Among these antifungal drugs are polyenes such as amphotericin B (AmB, Figure 1). These drugs are potent and last resort antifungal drugs that directly bind to ergosterol, an essential fungal membrane lipid that has a similar function to that of cholesterol in mammalian cells.[4−7] Echinocandins such as caspofungin (CASP, Figure 1) are a lipopeptide class of antifungal agents that act through inhibition of the (1−3)-β-D-glucan synthase complex involved in the biosynthesis of the (1−3)-β-D-glucan layer that protects fungal cells from osmotic pressure.[8] Azoles such as fluconazole (FLC, Figure 1) inhibit the fungal cytochrome P450 enzyme 14α-demethylase involved in the biosynthesis of the fungal membrane sterol ergosterol.[6] Toxic side effects caused by antifungal drugs, especially polyenes, and evolutionary pressure that has enhanced the emergence of resistance to the current repertoire of clinically approved antifungal drugs highlight the urgent need for new additional antifungal agents.[9−11]

In recent years, we as well as other research groups have developed antimicrobial cationic amphiphiles derived from amino-sugars or aminoglycoside (AG) antibiotics or poly-β-peptides. Several of the reported cationic amphiphiles display broad spectrum and potent antibacterial[12−21] and/or anti-

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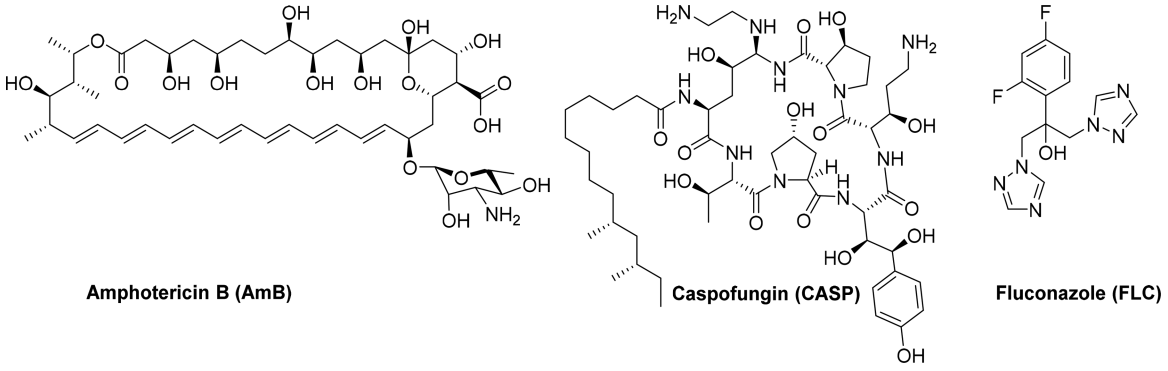
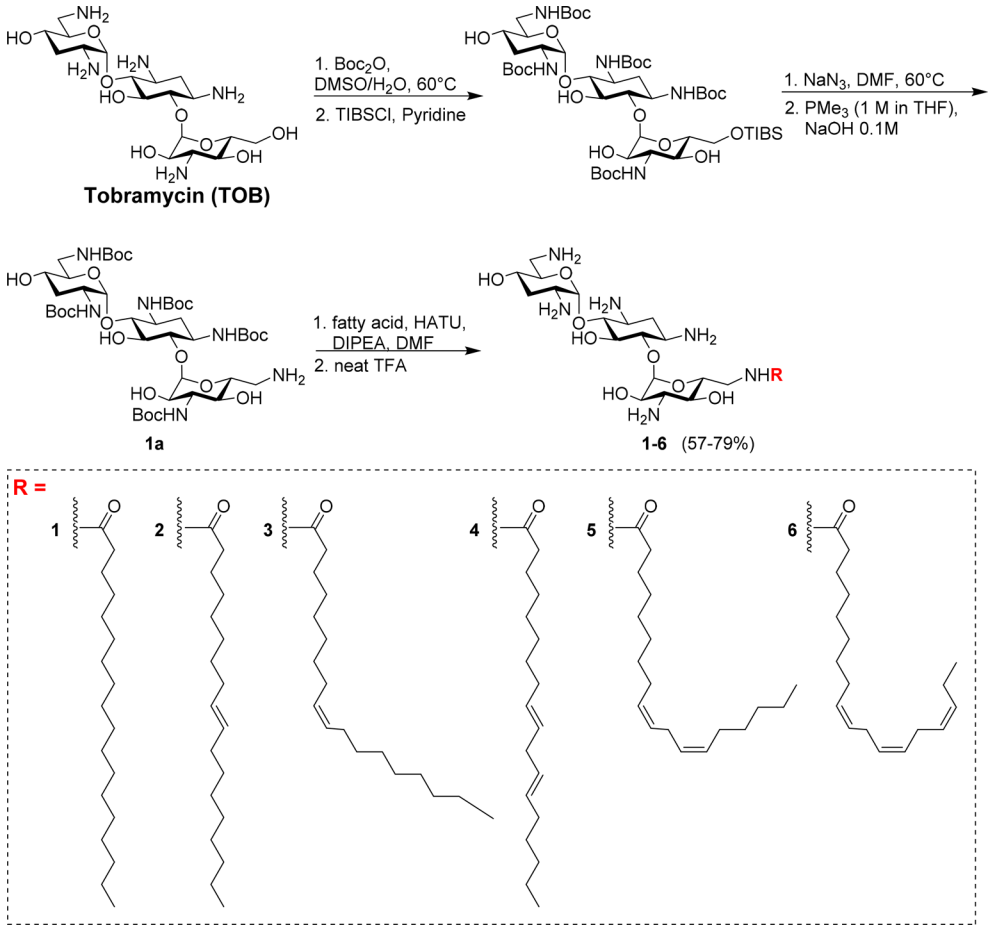


Figure 1. Structures of major clinically used antifungal drugs.

Scheme 1. Synthesis of TOB-Derived Cationic Amphiphiles



fungal[22−29] activities against strains with high levels of resistance to clinically used antimicrobial drugs; however, limited membrane selectivity prevents most of these compounds from being considered for further clinical development for systemic use. We recently reported that conversion of the primary amines of cationic amphiphiles derived from the AG nebramine to the corresponding imidazoles resulted in compounds with potent antifungal activity against *Candida glabrata*, an important fungal pathogen.[30] Notably, compared to other types of AG-derived antimicrobial cationic amphiphiles, the reported imidazole-decorated cationic amphiphiles caused only minor damage to mammalian red blood cells (RBCs). In search of strategies to further improve the safety profile of AG-derived cationic amphiphiles through chemical modifications, our attention was drawn to the degree of unsaturation and the configuration of the double bond in the lipid segment of these antimicrobial agents. It is well established that the degree of *cis* unsaturation of membrane lipids regulates fundamental biophysical and biological properties such as fluidity, permeability, endo- and exocytosis, cell division, signal transduction, and membrane protein activities.[31−35] Saturated fatty acids increase membrane rigidity, and *cis* unsaturated fatty acids increase membrane fluidity.[33,34] We therefore hypothesized that displacement of the saturated lipid chains in AG-derived cationic amphiphiles with *cis* unsaturated lipids would lead to a higher selectivity in the membrane permeabilizing activity of these antimicrobial agents, potentially reducing their toxicity to the host tissues. To test our hypothesis, we

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Table 1. Antifungal Activity: Minimal Inhibitory Concentration (MIC) Values [μg/mL][a]

Strains	Compounds									
	1	2	3	4	5	6	FLC	AmB	CASP	TOB
A. *C. albicans* ATCC 90028	8	8	8	8	8	8	0.5	0.25	0.25	>64
B. *C. albicans* ATCC 24433	4	8	4	8	8	8	1	0.125	0.25	>64
C. *C. albicans* SC5314	4	4	4	8	4	8	1	0.125	0.5	>64
D. *C. parapsilosis* ATCC 90018	8	8	8	8	8	8	1	0.25	2	>64
E. *C. tropicalis* Clinical isolate 660[b]	2	4	4	4	4	4	0.5	0.25	1	>64
F. *C. dubliniensis* Wu284[b]	4	4	4	4	4	8	1	0.5	0.125	>64
G. *C. guilliermondii* B3163[b]	4	4	4	4	4	4	0.25	0.25	0.25	>64
H. *C. albicans* Clinical isolate 280[b]	4	4	4	4	4	4	32	0.25	0.5	>64
I. *C. glabrata* ATCC 66032	4	4	4	4	4	8	>64	0.5	0.25	>64
J. *C. glabrata* Clinical isolate 50[b]	4	4	4	4	4	8	32	0.25	0.25	>64
K. *C. glabrata* ATCC 2001	4	8	4	8	4	4	>64	0.25	0.25	>64
L. *C. glabrata* 195[b]	4	4	4	4	8	8	>64	0.25	0.5	>64
M. *C. albicans* ATCC 96901	4	4	4	4	4	8	>64	0.125	1	>64
N. *C. albicans* ATCC 64124	4	4	4	4	4	8	>64	0.25	0.5	>64
O. *C. albicans* ATCC 11651	8	8	8	8	8	8	>64	0.125	0.5	>64
P. *C. albicans* Clinical isolate 2066[b]	8	8	8	4	8	4	1	1	64	>64
Q. *C. albicans* Clinical isolate 2068[b]	8	8	8	8	8	8	0.5	1	64	>64
R. *C. neoformans* ATCC 32045	2	4	4	2	4	4	0.5	0.25	8	>64
S. *C. neoformans* ATCC 66031	2	4	4	2	4	4	0.25	0.25	8	>64

[a]All MICs were determined using the broth double-dilution method. The highest concentration tested was 64 μg/mL. Cells were grown in RPMI 1640 medium at 37 °C under 5% CO_2. The experimental duration was 24 to 48 h for *Candida* strains and 72 h for the *C. neoformans* strains. Each concentration was tested in triplicate, and results were reproduced in at least two independent experiments. [b]Strains received from Prof. J. Berman, George S. Wise Faculty of Life Sciences, Tel Aviv University.

synthesized a collection of cationic amphiphiles derived from the AG tobramycin (TOB) differing in the degree of unsaturation and the double-bond configuration in their lipid segment and evaluated their antifungal activity and effects on mammalian cells.

RESULTS AND DISCUSSION

Synthesis of tobramycin-derived cationic amphiphiles containing unsaturated fatty acids. We synthesized a collection of six cationic amphiphiles derived from the AG antibiotic TOB, which served as the positively charged headgroup, and a fatty acid containing a C_{18} chain. Six fatty acids differing in the degree of unsaturation were chosen (Scheme 1): the fully saturated octadecanoic acid known as stearic acid (compound **1**), the monounsaturated ω-9 elaidic acid with a single *trans* double bond (compound **2**), the monounsaturated ω-9 oleic acid with one *cis* double bond (compound **3**), the diunsaturated ω-6 linolelaidic acid with two *trans* double bonds positioned at C-9 and C-12 (compound **4**), the diunsaturated ω-6 linoleic acid with two *cis* double bonds positioned at C-9 and C-12 (compound **5**), and the ω-3 linolenic acid with three *cis* double bonds positioned at the C-9, C-12, and C-15 of the fatty acid carbon chain (compound **6**).

Synthesis was accomplished by forming an amide bond between the free fatty acids and the 6′-amine of the TOB-derived precursor **1a**[36,37] using HATU to afford the Boc-protected compounds in 57−79% isolated yields. These were deprotected by treatment with TFA to yield the desired compounds **1**−**6** in quantitative yields as penta-TFA salts (Scheme 1). The purity of compounds **1**−**6** was >95% as determined by ultraliquid chromatography−mass spectroscopy (sections 1−3, Supporting Information).

Antifungal activity. The antifungal activity of cationic amphiphiles **1**−**6** was evaluated using the broth double-dilution assay[38] against a panel of *Candida spp.* and *Cryptococcus neoformans* strains (Table 1). *C. neoformans* causes a severe form of meningitis and meningoencephalitis in those with compromised immune systems.[39] These fungal pathogens are among the most common causes of opportunistic fungal infections worldwide.[40]

Of the 19 tested strains, eight (strains **H**−**O**) are resistant to the commonly used azole drug FLC (for information about the strains, also see Table S3, Supporting Information). Two strains (**P** and **Q**) are highly resistant to the echinocandin drug CASP; this resistance is conferred by a mutation in the *FKS1* gene.[41] FLC, CASP, and the fungal membrane disrupting polyene AmB were used as controls. Notably, the degree of *cis* unsaturation had little to no effect on the antifungal activity of cationic amphiphiles **1**−**6** against the tested panel of fungal pathogens. MIC values ranged from 2 to 8 μg/mL (Table 1).

Next we evaluated the antibacterial activities of compound **1**, which has a fully saturated lipid segment, and **6**, which has the highest degree of unsaturation in its lipid segment of the derivatives tested against three Gram positive and three Gram negative pathogens. Both compounds displayed poor antibacterial activities against the tested strains (MIC range of 32 μg/mL to >64 μg/mL, see Table S4 in the Supporting Information). These results indicate that the reported cationic amphiphiles are selective antifungal agents.

Addition of double bonds to a lipid chain results in π-bonded electrons that are less tightly held by the nucleus and are therefore potentially available as hydrogen bond donors. The calculated log D values of the free base forms of compounds **1**−**6** (Table 2) revealed that each addition of a double bond to the lipid incrementally decreased the log D value. For the TOB-derived cationic amphiphiles in this study, there was no correlation between log D value and antifungal activity.

Effect of the degree of unsaturation and double bond configuration on membranes of mammalian red blood cells. One of the major drawbacks in considering cationic

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Table 2. Partition Coefficients of Compounds 1−6[a]

Compound	Log D (pH = 7.4)
1	−6.55
2	−6.91
3	−6.91
4	−7.27
5	−7.27
6	−7.63

[a]Log D values were calculated in MarvinSketch software (version 6.3.1) with default parameters. Electrolyte concentration: $[Cl^-] = [Na^+] = [K^+] = 0.1$ M.

amphiphiles for systemic antimicrobial treatment is that these compounds nonselectively damage mammalian cell membranes. Evaluation of RBC hemolysis enables quantitative evaluation of mammalian cell membrane permeabilization activity and has therefore become the most commonly applied method for determination of the membrane selectivity of antimicrobial cationic amphiphiles.[42] The effect of the degree of lipid unsaturation and double bond configuration of cationic amphiphiles **1**−**6** on RBC hemolysis was evaluated using RBCs isolated from laboratory rats. The concentrations at which 50% of RBCs were lysed (HC_{50}) by TOB-derived cationic amphiphiles are summarized in Figure 2.

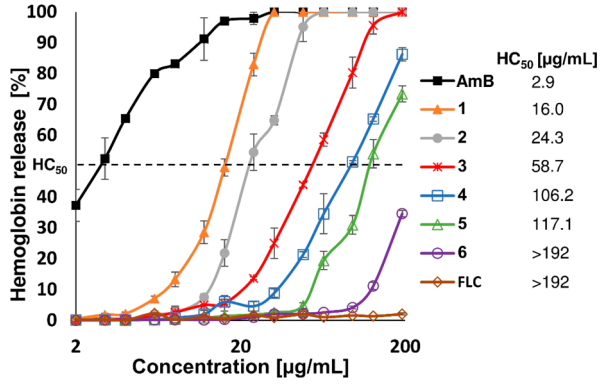

Figure 2. Hemolysis of RBCs. Rat erythrocytes were incubated with AmB (black), **1** (orange), **2** (gray), **3** (red), **4** (blue), **5** (green), **6** (purple), and FLC (brown) for 1 h at 37 °C. Released hemoglobin absorbance was measured at 550 nm. Results are expressed as the percentage of hemoglobin released relative to Triton X100 (100% hemolysis). HC_{50} values are given in the legend. Each concentration was tested in triplicate, and the results are expressed as means ± standard error from two independent experiments.

The potent and last resort antifungal drug AmB, which is known to cause extensive RBCs hemolysis,[5] was used as a positive control, and the azole antifungal FLC, which does not directly permeabilize the yeast cell membrane, was used as a negative control.[6] Whereas the degree of unsaturation and double bond configuration in the lipids of cationic amphiphiles **1**−**6** had no significant influence on antifungal activity against the tested panel of yeast strains, a significant differential effect on rat RBC hemolysis was observed ($p < 0.0004$−0.03 for compound **6** in comparison to each of compounds **1**−**4**, Student's t test). Of the cationic amphiphiles **1**−**6**, compound **1**, derived from the fully saturated stearic acid, was the most hemolytic, and the ω-3 linolenic acid-derived cationic amphiphile **6** with the highest degree of *cis* unsaturation was more than 1 order of magnitude less hemolytic than **1** (Figure 2). Notably, the HC_{50} of cationic amphiphile **6**, the least hemolytic of the cationic amphiphiles in this study, was at least 2 orders of magnitude higher than that of AmB. Furthermore, the configuration of the double bond in the lipid also had a substantial effect on the hemolysis. The HC_{50} of compound **2**, containing a single *trans* double bond in its lipid, was approximately 2.4-fold lower than that of compound **3**, which contains the *cis* double bond isomer. Comparison between the HC_{50} values of compound **4**, composed of the diunsaturated ω-6 linolelaidic acid with two *trans* double bonds positioned at C-9 and C-12, and compound **5**, with the corresponding diunsaturated ω-6 linoleic acid with two *cis* double bonds, revealed that the latter was less hemolytic. With this pair, the double bond configuration effect was less pronounced than when compounds **2** and **3** were compared (Figure 2). Our results are in agreement with the previously reported demonstrations that introduction of an unsaturated lipid chain in lipo-γ-AA peptides and in lysine-based cationic amphiphiles significantly decreases their hemolytic activity.[43,44]

We calculated the selectivity index between RBC hemolysis and antifungal MIC (HC_{50} divided by the highest MIC value measured for the tested panel of fungi) for compound **1** with a fully saturated fatty acid and compound **6** with the highest degree of *cis* unsaturation. Comparison between the selectivity index values revealed that the increase in unsaturation resulted in a 12-fold increase in the selectivity index of compound **6** compared to that of compound **1**: For compound **1** the selectivity index was 2, and for compound **6** the index was 24.

The results of the hemolysis tests indicated that an increase in the degree of *cis* unsaturation of the lipid segment reduced undesired mammalian cell membrane damage without a significant effect on the ability of the cationic amphiphiles to disrupt yeast cell membranes. Thus, in designing antifungal cationic amphiphiles, introduction of lipids carrying a high degree of *cis* unsaturation can lead to a significant increase in the specificity to the fungal cell membrane and reduce severe RBC membrane damage that can lead to side effects such as hemolytic anemia.

Effects on the viability of immortalized and primary mammalian cells. Mammalian RBC hemolysis serves as an indication of mammalian membrane damage. However, cationic amphiphiles may have additional toxic effects on mammalian cells that reduce their viability. To further assess the potential toxicity of cationic amphihiles **1**−**6** toward mammalian cells, effects on cell viability were tested on three nucleated mammalian cells lines. The chosen lines were HepG2 cells, an immortalized human liver hepatocellular carcinoma cell line, 3T9MEFs, an immortalized mouse embryonic fibroblast cell line, and HEK 293 cells, an immortalized human embryonic kidney cell line. These cell lines were treated with each of the cationic amphiphiles at concentrations up to 256 μg/mL for 24 h, after which cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 3).

Since transformed and immortalized cell lines often differ genetically and phenotypically from their tissues of origin, we also evaluated the impact of cationic amphiphiles **1**−**6** on the viability of primary dermal fibroblasts obtained from newborn mice. Similar to the results of the hemolysis assay, there was a correlation between the degree of *cis* unsaturation and the ability of the compounds to reduce cell viability (as indicated by the MTT cell viability assay). The highest reduction in viability was displayed by the fully saturated stearic acid-derived cationic

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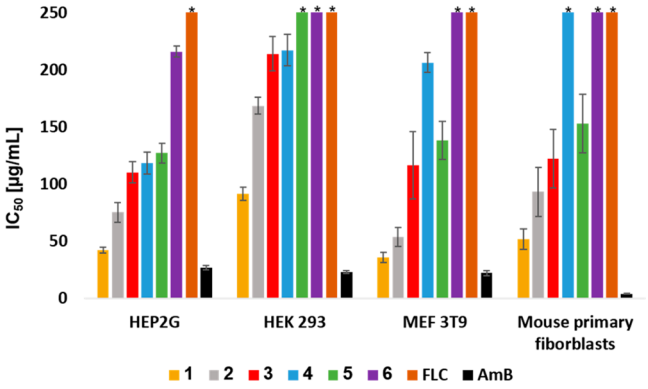

Figure 3. Effects of compounds **1**–**6** on the viability of mammalian cells. Cells were plated in 96-well microtiter plates. After 24 h, the tested compounds (at concentrations up to 256 μg/mL) were added. After an additional 24 h, cell viability was determined by MTT assay. The percentage of viability was determined relative to control untreated cells. Each concentration was tested in triplicate, and the results are expressed as means ± SD of the IC_{50} values from at least three independent experiments. In columns marked with asterisks, the decrease in viability did not reach 50% even at the highest concentration tested, 256 μg/mL.

amphiphile **1**, and the lowest reduction was displayed by the linolenic acid-derived cationic amphiphile **6** with three *cis* double bonds in its lipid chain. The concentration of compound **1** that led to a 50% reduction in cell viability (IC_{50}) ranged between approximately 36 μg/mL for 3T9MEFs to about 90 μg/mL for HEK 293 cells, whereas the IC_{50} values of compound **6** were approximately 216 μg/mL for HepG2 cells and above the maximal concentration tested (256 μg/mL) for the other cell types (Table S6, Supporting Information). Hence, at concentrations significantly higher than the MIC range against the tested yeast strains, compound **6** did not reduce the viability of immortalized and primary mammalian cell lines. Notably, the extent of the effects of the lipid double bond configuration on the nucleated mammalian cells viability was cell-type specific. For example, in 3T9MEFs, the IC_{50} of compound **2**, which is substituted with elaidic acid with a single *trans* double bond, was about 2.2-fold lower than that of compound **3**, which is composed of oleic acid with a *cis* double-bonded isomer. In the other tested cell lines, however, the differences in IC_{50} values were more modest.

We then calculated the selectivity index between antifungal activity and the effect on mammalian cell viability (lowest IC_{50} value measured for the tested cell lines divided by the highest MIC value measured for a tested panel of fungi) for compounds **1** and **6**. The selectivity index values were 4.5 for compound **1** and 27 for compound **6**. Compound **6** with the unsaturated lipid had a 6-fold higher selectivity index than compound **1** with the fully saturated fatty acid segment. The results of the mammalian cell viability assay and the selectivity index calculations indicated that, as observed for hemolytic activity, an increase in the degree of unsaturation reduces the toxicity of antifungal cationic amphiphiles.

Inhibition of the growth of *C. albicans* in a coculture with mammalian cells. To date, the antimicrobial activity of synthetic antifungal cationic amphiphiles has not been evaluated in cultures containing both the pathogen and mammalian cells. The efficacy of antimicrobial cationic amphiphiles is reduced due to nonspecific interactions with numerous lipophilic and anionic moieties found in the cell culture.[45−48] We therefore investigated the antifungal activity and cell-selectivity of cationic amphiphiles **1** and **6** in a

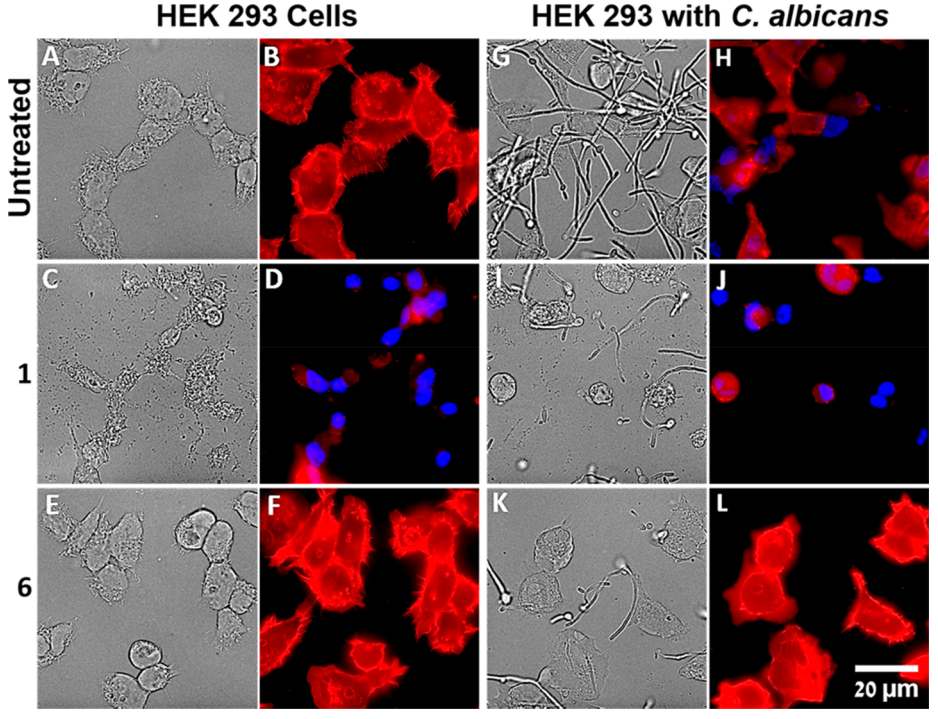

Figure 4. Effect of compounds **1** and **6** on a coculture of HEK 293 cells with *C. albicans* SC5314. HEK 293 cells alone (**A**–**F**) and HEK 293 cells cocultured with *C. albicans* (**G**–**L**) were left untreated (**A**, **B**, **G**, **H**) or treated with compounds **1** (**C**, **D**, **I**, **J**) or **6** (**E**, **F**, **K**, **L**). Bright field images and images of samples stained with phalloidin (red) and Hoechst dye (blue) were visualized with an Olympus motorized inverted fluorescent microscope.

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coculture of both pathogen and mammalian cells (Figure 4). To form the coculture, *C. albicans* SC5314 cells were added to a culture of HEK 293 cells at a multiplicity of infection of 2. After 30 min of incubation, cells were treated with 128 μg/mL of compound **1** or **6** or untreated. Notably, this concentration is 32 and 16 times higher than the MIC values of **1** and **6** against *C. albicans* SC5314, respectively (Table 1). The progress of the experiment was monitored by light microscopy. After 4 h of incubation, a thick network of *Candida* cells (hyphae form) and highly damaged mammalian cells appeared in untreated control wells, and the experiments were therefore terminated. Cells were then stained with Hoechst 33258, a DNA binding fluorescent dye that labels the nuclei of dead cells with damaged plasma membrane but not of healthy cells. Next, the cells were fixed and stained with phalloidin, which fluorescently labels F-actin fibers, a major constituent of the cell cytoskeleton. This staining paradigm enabled visualization of HEK 293 cell viability as reflected by cell morphology, exclusion of Hoechst 33258 from healthy cells, and cytoskeleton integrity.

As shown in Figure 4A and 4B, in control cultures consisting of HEK 293 cells alone, the untreated cells were alive and healthy, as indicated by their morphology, their phalloidin staining pattern, and the exclusion of Hoechst 33258 dye. Treatment with compound **1** led to substantial HEK 293 cell death, as indicated by damaged cytoskeletons, abnormal morphologies, and Hoechst 33258-stained nuclei (Figure 4C and 4D). These results are in line with the results obtained when cell viability was assessed by the MTT assay (Figure 3). In contrast to compound **1**, compound **6** did not cause any apparent cytotoxic effect on HEK 293 cells (Figures 4E and 4F). Co-culturing HEK 293 cells with *C. albicans* resulted in the appearance of a network of fungal cells and damaged HEK 293 cells (Figure 4G and 4H). Treatment of the cocultures with compound **1** or **6** substantially reduced the numbers of fungal cells (Figures 4I and 4K, respectively). This reduction was accompanied by extensive damage of compound **1**-treated (Figures 4I and 4J), but not compound **6**-treated, HEK 293 cells (Figures 4K and 4L).

Taken together these results indicate that compound **6** targets the fungal cells selectively without affecting the viability of mammalian cells. These observations are in agreement with our findings that of the six antimicrobial cationic amphiphiles synthesized, compound **1**, composed of the saturated stearic acid, displayed potent antifungal activity but was also the most hemolytic and cytotoxic to the tested mammalian cells. In contrast, compound **6**, composed of linolenic acid with the highest degree of *cis* unsaturation evaluated in this study, displayed potent antifungal activity against the tested yeast strains as well as the lowest levels of hemolysis and mammalian cell cytotoxicity.

Cell membrane permeabilization activity. The intriguing observation that raising the degree of *cis* unsaturation in the lipid segment of the antifungal cationic amphiphiles did not affect antifungal potency but significantly reduced the toxicity toward mammalian cells raised the question of whether or not, like other cationic amphiphiles, compound **6** acts through disruption of the fungal membrane. The cell permeabilizing effects of compounds **1** and **6** were therefore evaluated using a propidium iodide (PI) dye exclusion assay with the number of PI-stained membrane-damaged cells quantified by flow cytometry as previously described.[49,50]

Since the antimicrobial activity of cationic amphiphiles is affected by the inoculum,[51] we initially determined the MIC values of cationic amphiphiles **1** and **6** and of the control antifungal agents at the inoculum used for the flow cytometry analysis, which was 100-fold higher than that used for the MIC experiment (Table S7, Supporting Information). We incubated *C. glabrata* ATCC 66032 (Strain F, Table 1), which exists only in the yeast form, with compounds **1** or **6** at the MIC under these conditions and monitored yeast cell membrane integrity every 60 min for 6 h. At each time point, a sample was withdrawn from the culture, stained with PI, and analyzed by flow cytometry. From the obtained graphs of percentage of membrane damaged cells vs time, we extracted $T_{1/2}$ values, which were defined as the time at which the membranes of 50% of the cells were damaged (Figure 5). As positive controls we

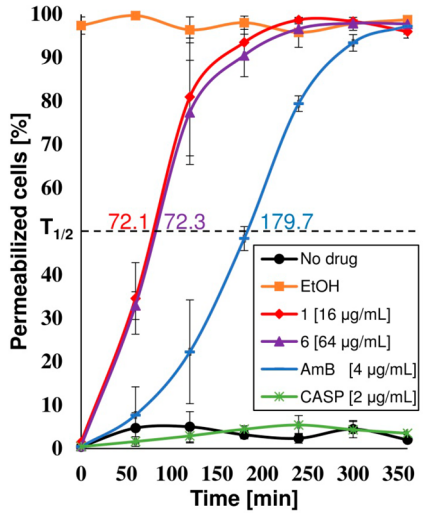

Figure 5. *C. glabrata* ATCC 66032 cell cultures in stationary phase were incubated with compound **1** or **6** for 6 h. Samples were removed every 60 min, stained with PI, and analyzed using flow cytometry. Each data point is based on analysis of 10,000−20,000 cells. Results shown are averages of two independent experiments.

used ethanol (100% permeability), and the fungal cell membrane disrupting compound AmB. As negative controls we sampled untreated culture and cultures treated with CASP, which does not directly affect the fungal cell membrane.

As expected, membrane integrity was not altered in samples treated with CASP, whereas AmB rapidly permeabilized yeast cell membranes (Figure 5, $T_{1/2}$ value of 179.7 min). The $T_{1/2}$ values of compounds **1** and **6** were almost identical (72.1 and 72.3 min, respectively); thus, the results of the PI assay support our hypothesis that, at MIC, both cationic amphiphiles **1** and **6** exert their antifungal activity by permeabilizing the fungal cell membrane and that the degree of lipid unsaturation does not have an effect on the mode of action or the kinetics of the *in vitro* antifungal activity. Notably, compound **6** disrupted the yeast cell membranes about 2.5-fold more rapidly than clinically used AmB.

Finally, the results of the PI assay suggest that the reason for the improved selectivity of antifungal cationic amphiphile **6**, with the highest degree of *cis*-unsaturation in its fatty acid segment, relative to compound **1** does not result from a difference in mode of action on fungi. The observed selectivity presumably stems from the fundamental differences in compositions of the plasma membranes of fungal and mammalian cells.[24] The mammalian cell plasma membrane contains cholesterol, whereas that of the fungal cell contains

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ergosterol.[31] The percentage of negatively charged lipids such as phosphatidylinositol, which are known to interact with antimicrobial cationic peptides, is higher in the plasma membrane of fungal cells than in the membrane of mammalian cells.[31,52,53] These fundamental differences in membrane composition may be the cause for the observed improvement in the selective antifungal activity of compound **6**.

CONCLUSIONS

The structures of antifungal tobramycin-derived cationic amphiphiles were systematically altered by the attachment of fatty acid chains differing in the degree of unsaturation and/or double-bond configuration. We demonstrated that the degree of unsaturation and double bond configuration had no significant effect on antifungal activity. As the degree of unsaturation increased, however, there was a significant reduction in hemolysis of mammalian red blood cells and in the harmful effect on nucleated immortalized and primary mammalian cells' viability. We showed that the cationic amphiphile composed of a fatty acid with *cis* double bonds caused less hemolysis than the corresponding cationic amphiphiles composed of a fatty acid with the corresponding *trans* double bonds.

We demonstrated the efficacy of antifungal cationic amphiphiles in a coculture of *C. albicans* and mammalian cells (HEK 293). Notably, at a concentration 16-fold higher than its MIC against the *C. albicans* strain used for the coculture experiment, cationic amphiphile **6** effectively protected the mammalian cells from damage by *Candida* cells and caused lysis of the fungal pathogen cells. Quantitative evaluation of the membrane integrity of *Candida* cells treated with the reported cationic amphiphiles revealed that these compounds rapidly permeabilized the yeast cells and that increased degree of lipid unsaturation did not alter the rapid membrane permeabilizing activity of this series of compounds. The results reported herein demonstrate that by optimizing the degree of unsaturation and the configuration of the double bond in the lipid segment of cationic amphiphiles it is possible to retain potent and broad spectrum antifungal activity while reducing significantly the membrane damage and cytotoxic effects toward mammalian cells. We expect that these finding will lead to identification of clinically useful broad spectrum antifungal cationic amphiphiles.

EXPERIMENTAL SECTION

General chemistry methods. *Instrumentation and reagents.* ^{1}H NMR and 1D-TOCSY experiments were recorded on Bruker Avance 400 or 500 MHz spectrometers, and chemical shifts (reported in ppm) were calibrated to D_2O or CD_3OD (d = 4.79 and 3.31 ppm, respectively). ^{13}C NMR experiments were recorded on Bruker Avance 400 or 500 MHz spectrometers at 100 or 125 MHz. Multiplicities are reported using the following abbreviations: br. = broad, s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, q = quartet, m = multiplet. Coupling constants (J) are given in Hertz. High-resolution electrospray ionization (HRESI) mass spectra were measured on a Waters Synapt instrument. Chemical reactions were monitored by TLC (Merck, Silica gel 60 F254). Visualization was achieved using a cerium molybdate stain [$(NH_4)_2Ce(NO_3)_6$ (5 g), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (120 g), H_2SO_4 (80 mL), H_2O (720 mL)]. All reactions were carried out in an argon atmosphere with anhydrous solvents unless otherwise noted. All chemicals were obtained from commercial sources. Compounds were purified using flash chromatography on silica gel columns (Merck, Kieselgel 60). The purity of compounds **1**−**6** was ≥95% as determined by ULC-ESI-MS. The chromatographic separation was achieved by using a Waters e2695 HPLC Separation Module and an XBridge Amide, 3.5 μm, 3.0 × 100 mm column. Sample aliquots of 10 μL were injected onto the column at a flow rate of 300 μL/min. HPLC separation conditions, and the MS data are described in the Supporting Information.

Compound 1. Stearic acid (47 mg, 0.16 mmol), HATU (78.6 mg, 0.21 mmol), and DIPEA (85 μL, 0.47 mmol) were dissolved in DMF (2 mL) under argon atmosphere, stirred for 15 min at ambient temperature, and cooled in an ice bath. Compound **1a** (100 mg, 0.10 mmol) was then added to the cold mixture, and upon dissolution the temperature was allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether, 1:1). Upon completion the reaction mixture was diluted with EtOAc (20 mL). The organic layer was washed with 1.0 M HCl (2 × 15 mL), saturated $NaHCO_3$ (2 × 15 mL), and brine (2 × 15 mL) then dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography (SiO_2 using EtOAc/petroleum ether) gave the corresponding NHBoc-protected product. TFA (0.5 mL) was added at ambient temperature and, after 3 min, was removed under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford compound **1** as a white solid (80.0 mg, 62.7%). HRESI-MS, *m*/*z* calculated for $C_{36}H_{73}N_6O_9^+$, 733.5439; found for $[M + H]^+$, 733.5432. ^{1}H NMR (500 MHz, D_2O) δ 5.84 (d, J = 2.9 Hz, H-1′, 1H), 5.06 (d, J = 2.3 Hz, H-1″, 1H), 4.03 (dd, J = 9.6 Hz, H-4, 1H), 3.97−3.92 (m, H-5′, H-5″, H-2″, 3H), 3.85 (dd, J = 9.0 Hz, H-6, 1H), 3.79 (dd, J = 9.5 Hz, H-5, 1H), 3.72−3.39 (m, H-1, H-3, H-2′, H-4′, H-6′, H-3″, H-4″, H-6″ (2H), 9H), 3.21 (dd, J = 13.4, 7.56 Hz, H-6′, 1H), 2.56 (m, H-2eq, 1H), 2.33−2.23 (m, stearyl chain (2H), H-3′eq, 3H), 2.06 (ddd, J = 11.3 Hz, H-3′ax, 1H) 1.98 (ddd, J = 12.5 Hz, H-2ax, 1H), 1.56 (m, stearyl chain, 2H), 1.28 (m, stearyl chain, 28H), 0.88 (t, J = 6.4 Hz, stearyl chain, 3H). ^{13}C NMR (100 MHz, D_2O) δ 178.9, 164.2 (q, J_{CF} = 35.4 Hz, $CF_3\underline{C}O_2H$), 117.7 (q, J_{CF} = 290.1 Hz, $\underline{C}F_3CO_2H$), 102.3, 95.2, 84.9, 78.6, 75.6, 72.5, 71.8, 69.5, 67.8, 66.0, 55.9, 51.2, 49.8, 49.2, 41.4, 40.3, 37.2, 33.0, 30.8, 30.5, 30.4, 30.3, 30.2, 29.1, 26.9, 23.8, 15.0.

Compound 2. Elaidic acid (47 mg, 0.16 mmol), HATU (71.0 mg, 0.19 mmol), and DIPEA (72 μL, 0.41 mmol) were dissolved in DMF (2 mL) under argon atmosphere, stirred for 15 min at ambient temperature, and cooled in an ice bath. Compound **1a** (100 mg, 0.10 mmol) was then added to the cold mixture, and upon dissolution the temperature was allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether, 1:1). Upon completion the reaction mixture was diluted with EtOAc (20 mL). The organic layer was washed with 1.0 M HCl (2 × 15 mL), saturated $NaHCO_3$ (2 × 15 mL), and brine (2 × 15 mL) then dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on SiO_2 using EtOAc/petroleum ether (4:10) as eluent gave the corresponding NHBoc-protected product. TFA (0.5 mL) was added at ambient temperature and, after 3 min, was removed under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford compound **2** as a white solid (79.6 mg, 62.5%). HRESI-MS, *m*/*z* calculated for

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$C_{36}H_{71}N_6O_9^+$, 731.5283; found for $[M + H]^+$, 731.5286. ^1H NMR (500 MHz, D_2O) δ 5.84 (d, J = 2.0 Hz, H-1′, 1H), 5.42 (m, elaidyl chain, 2H), 5.07 (br. s, H-1”, 1H), 4.04 (dd, J = 9.5 Hz, H-4, 1H), 3.97−3.92 (m, H-5′, H-5′′, H-2”, 3H), 3.85 (dd, J = 8.8 Hz, H-6, 1H), 3.79 (dd, J = 9.7 Hz, H-5, 1H), 3.72−3.38 (m, H-1, H-3, H-2′, H-4′, H-6′, H-3′′, H-4”, H-6” (2H), 9H), 3.21 (dd, J = 13.3, 7.55 Hz, H-6′, 1H), 2.56 (m, H-2eq, 1H), 2.32−2.23 (m, elaidyl chain (2H), H-3′eq, 3H), 2.06 (ddd, J = 11.7 Hz, H-3′ax, 1H) 1.97 (m, elaidyl chain (4H), H-2ax, 5H), 1.56 (m, elaidyl chain, 2H), 1.28 (m, elaidyl chain, 20H), 0.87 (t, J = 6.7 Hz, elaidyl chain, 3H). ^{13}C NMR (125 MHz, D_2O) δ 178.8, 164.2 (q, J_{CF} = 35.0 Hz, $CF_3\underline{C}O_2H$), 131.8, 131.7, 117.8 (q, J_{CF} = 290.1 Hz, $\underline{C}F_3CO_2H$), 102.3, 95.3, 84.9, 78.7, 75.6, 72.6, 71.9, 69.5, 67.8, 66.1, 56.0, 51.2, 49.8, 49.2, 41.4, 40.2, 37.2, 33.7, 33.1, 32.7, 31.1, 30.7, 30.6, 30.5, 30.3, 30.2, 30.1, 29.1, 26.9, 23.8, 15.1.

Compound 3. Oleic acid (47 mg, 0.16 mmol), HATU (78.6 mg, 0.21 mmol), and DIPEA (85 μL, 0.47 mmol) were dissolved in DMF (2 mL) under argon atmosphere, stirred for 15 min at ambient temperature, and cooled in an ice bath. Compound **1a** (100 mg, 0.10 mmol) was then added to the cold mixture, and upon dissolution the temperature was allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether, 1:1). Upon completion the reaction mixture was diluted with EtOAc (20 mL). The organic layer was washed with 1.0 M HCl (2 × 15 mL), saturated $NaHCO_3$ (2 × 15 mL), and brine (2 × 15 mL) then dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on SiO_2 using EtOAc/petroleum ether (7:10) as eluent gave the corresponding NHBoc-protected product. TFA (0.5 mL) was added at ambient temperature and, after 3 min, was removed under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford compound **3** as a white solid (73.0 mg, 57.4%). HRESI-MS, m/z calculated for $C_{36}H_{71}N_6O_9^+$, 731.5283; found for $[M + H]^+$, 731.5285. ^1H NMR (400 MHz, D_2O) δ 5.81 (d, J = 3.3 Hz, H-1′, 1H), 5.47 (m, oleyl chain, 2H), 5.06 (d, J = 3.8 Hz, H-1”, 1H), 4.00−3.89 (m, H-4, H-5′, H-2”, H-5′′, 4H), 3.84 (dd, J = 9.0 Hz, H-6, 1H), 3.78−3.64 (m, H-5, H-2′, H-4′, H-6”, 4H), 3.62−3.41 (m, H-1, H-3, H-6′, H-4”, H-6”, 6H), 3.28 (dd, J = 13.7, 6.9 Hz, H-6′, 1H), 2.54 (ddd, J = 12.7, 3.9 Hz, H-2eq, 1H), 2.34−2.25 (m, oleyl chain (2H), H-3′eq, 3H), 2.08−1.99 (m, oleyl chain (4H), H-3′ax, 5H), 1.92 (ddd, J = 12.7 Hz, H-2ax, 1H) 1.58 (m, oleyl chain, 2H), 1.29 (m, oleyl chain, 20H), 0.86 (t, J = 6.8 Hz, oleyl chain, 3H). ^{13}C NMR (100 MHz, D_2O) δ 178.8, 164.2 (q, J_{CF} = 35.2 Hz, $CF_3\underline{C}O_2H$), 131.4, 131.3, 117.8 (q, J_{CF} = 290.2 Hz, $\underline{C}F_3CO_2H$), 102.3, 95.3, 84.9, 78.7, 75.6, 72.6, 71.9, 69.5, 67.8, 66.1, 56.0, 51.2, 49.8, 49.2, 41.4, 40.2, 37.2, 33.1, 32.7, 30.9, 30.8, 30.7, 30.6, 30.5, 30.4, 30.3, 30.2, 29.2, 28.4, 28.3, 26.9, 23.8, 15.1.

Compound 4. Linolelaidic acid (48 μL, 0.12 mmol), HATU (47.2 mg, 0.12 mmol), and DIPEA (72 μL, 0.41 mmol) were dissolved in DMF (1 mL) under argon atmosphere, stirred for 15 min at ambient temperature, and cooled in an ice bath. Compound **1a** (100 mg, 0.10 mmol) was then added to the cold mixture, and upon dissolution the temperature was allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (DCM/MeOH, 95:5). Upon completion the reaction mixture was diluted with EtOAc (20 mL). The organic layer was washed with 1.0 M HCl (2 × 15 mL), saturated $NaHCO_3$ (2 × 15 mL), and brine (2 × 15 mL) then dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on SiO_2 using DCM/MeOH (97:3) as eluent gave the corresponding NHBoc-protected product. TFA (0.5 mL) was added at ambient temperature and, after 3 min, was removed under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford compound **4** as a white solid (99.0 mg, 78.7%). HRESI-MS, m/z calculated for $C_{36}H_{69}N_6O_9^+$, 729.5126; found for $[M + H]^+$, 729.5136. ^1H NMR (400 MHz, D_2O) δ 5.68 (d, J = 2.9 Hz, H-1′, 1H), 5.40 (m, linolelaidyl chain, 4H), 4.95 (d, J = 2.7 Hz, H-1”, 1H), 3.87−3.75 (m, H-4, H-5′, H-2”, H-5′′, H-6”, 5H), 3.71 (dd, J = 8.9 Hz, H-5, 1H), 3.67−3.48 (m, H-6, H-4′, H-2’ 3H), 3.47−3.28 (m, H-1, H-3, H-6′, H-3′′, H-4”, H-6”, 6H), 3.14 (dd, J = 6.3 Hz, H-6′, 1H), 2.57 (m, linolelaidyl chain, 2H), 2.36 (ddd, J = 12.1, 3.7 Hz, H-2eq, 1H), 2.23−2.11 (m, linolelaidyl chain (2H), H-3′eq, 3H), 1.96−1.85 (m, linolelaidyl chain (4H), H-3′ax, 5H),1.74 (ddd, J = 12.4 Hz, H-2ax, 1H), 1.47 (m, linolelaidyl chain, 2H), 1.18 (m, linolelaidyl chain, 14H), 0.75 (t, J = 6.6 Hz, linolelaidyl chain, 3H). ^{13}C NMR (100 MHz, D_2O) δ 179.4, 164.3 (q, J_{CF} = 34.4 Hz, $CF_3\underline{C}O_2H$), 133.2, 133.1, 130.4, 130.3, 117.8 (q, J_{CF} = 292.6 Hz, $\underline{C}F_3CO_2H$), 102.2, 95.5, 85.4, 79.9, 75.7, 72.4, 71.6, 69.6, 68.1, 66.0, 57.9, 56.1, 51.4, 50.3, 49.9, 49.3, 41.4, 40.4, 37.2, 36.3, 33.2, 32.1, 31.0, 30.1, 29.9, 29.8, 29.7, 29.6, 26.8, 23.4, 14.9.

Compound 5. Linoleic acid (70 mg, 0.25 mmol), HATU (94.4 mg, 0.25 mmol), and DIPEA (144 μL, 0.83 mmol) were dissolved in DMF (3 mL) under argon atmosphere, stirred for 15 min at ambient temperature, and cooled in an ice bath. Compound **1a** (200 mg, 0.21 mmol) was then added to the cold mixture, and upon dissolution the temperature was allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether, 1:1). Upon completion the reaction mixture was diluted with EtOAc (20 mL). The organic layer was washed with 1.0 M HCl (2 × 15 mL), saturated $NaHCO_3$ (2 × 15 mL), and brine (2 × 15 mL) then dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on SiO_2 using EtOAc/petroleum ether (7:10) as eluent gave the corresponding NHBoc-protected product. TFA (0.5 mL) was added at ambient temperature and, after 3 min, was removed under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford compound **5** as a white solid (144.8 mg, 56.9%). HRESI-MS, m/z calculated for $C_{36}H_{69}N_6O_9^+$, 729.5126; found for $[M + H]^+$, 729.5115. ^1H NMR (400 MHz, CD_3OD) δ 5.86 (d, J = 3.2 Hz, H-1′, 1H), 5.34 (m, linoleyl chain, 4H), 5.03 (d, J = 3.6 Hz, H-1”, 1H), 4.00−3.88 (m, H-4, H-5′, H-5′′, 3H), 3.80−3.74 (m, H-2”, H-6, 2H), 3.70 (dd, J = 9.5 Hz, H-5, 1H), 3.64 (dd, J = 14.4, 3.4 Hz, H-6”, 1H), 3.59−3.50 (m, H-2′, H-4′, 2H), 3.49−3.35 (m, H-1, H-3, H-6′, H-3′′, H-4”, H-6”, 6H), 3.07 (dd, J = 13.3, 8.4 Hz, H-6′, 1H), 2.77 (dd, J = 6.3 Hz, linoleyl chain, 2H), 2.46 (ddd, J = 12.4, 4.1 Hz, H-2eq, 1H), 2.29−2.19 (m, linoleyl chain (2H), H-3′eq, 3H), 2.11−1.96 (m, linoleyl chain (4H), H-2ax, H-3′ax, 6H), 1.61 (m, linoleyl chain, 2H), 1.34 (m, linoleyl chain, 14H), 0.91 (t, J = 7.0 Hz, linoleyl chain, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 177.8, 163.3 (q, J_{CF} = 34.4 Hz, $CF_3\underline{C}O_2H$), 131.0, 130.8, 129.1, 129.0, 118.1(q, J_{CF} = 290.7 Hz, $\underline{C}F_3CO_2H$), 102.4, 95.4, 85.6, 78.9, 76.1, 73.1, 71.7, 70.2, 68.8, 66.8, 56.3, 51.4, 50.0, 41.8, 40.8, 37.0, 32.6, 31.4, 30.8, 30.5, 30.4, 30.37, 30.3, 29.5, 28.2, 27.1, 26.5, 23.6, 14.4.

Compound 6. Linolenic acid (70 mg, 0.25 mmol), HATU (94.4 mg, 0.25 mmol) and DIPEA (144 μL, 0.83 mmol) were dissolved in DMF (3 mL) under argon atmosphere, stirred for

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15 min at ambient temperature, and cooled in an ice bath. Compound **1a** (200 mg, 0.21 mmol) was then added to the cold mixture, and upon dissolution the temperature was allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether, 1:1). Upon completion the reaction mixture was diluted with EtOAc (20 mL). The organic layer was washed with 1.0 M HCl (2 × 15 mL), saturated $NaHCO_3$ (2 × 15 mL), and brine (2 × 15 mL) then dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on SiO_2 using EtOAc/petroleum ether (7:10) as eluent gave the corresponding NHBoc-protected product. TFA (0.5 mL) was added at ambient temperature and, after 3 min, was removed under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford compound **6** as a white solid (173.0 mg, 68.1%). HRESI-MS, *m/z* calculated for $C_{36}H_{67}N_6O_9^+$, 727.4970; found for $[M + H]^+$, 727.4964. ^1H NMR (400 MHz, CD_3OD) δ 5.90 (d, J = 3.5 Hz, H-1′, 1H), 5.34 (m, linolenyl chain, 6H), 5.03 (d, J = 3.6 Hz, H-1″, 1H), 4.09 (dd, J = 9.1, 10.0 Hz, H-4, 1H), 3.97−3.88 (m, H-5′, H-5″, 2H), 3.81−3.77 (m, H-2″, H-6, 2H), 3.72 (dd, J = 9.8 Hz, H-5, 1H), 3.64 (dd, J = 14.6, 3.3 Hz, H-6″, 1H), 3.59−3.46 (m, H-1, H-3, H-2′, H-4′, 4H), 3.43−3.35 (m, H-6′, H-3″, H-4″, H-6″, 4H), 3.08 (dd, J = 13.3, 8.4 Hz, H-6′, 1H), 2.82−2.79 (m, linolenyl chain, 4H), 2.52 (ddd, J = 12.5, 4.2 Hz, H-2eq, 1H), 2.25−2.19 (m, linolenyl chain (2H), H-3′eq, 3H), 2.16−2.05 (m, linolenyl chain (4H), H-2ax, H-3′ax, 6H), 1.60 (m, linoleyl chain, 2H), 1.33 (m, linolenyl chain, 8H), 0.97 (t, J = 7.5 Hz, linolenyl chain, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 177.8, 163.3 (q, J_{CF} = 34.5 Hz, $CF_3\underline{C}O_2H$), 132.7, 131.0, 129.2, 129.1, 128.9, 128.2, 118.1(q, J_{CF} = 290.8 Hz, $\underline{C}F_3CO_2H$), 102.4, 95.4, 85.5, 78.8, 76.1, 73.1, 71.8, 70.2, 68.7, 66.7, 56.3, 51.4, 50.0, 41.8, 40.8, 37.0, 31.3, 30.7, 30.4, 30.3, 30.2, 29.4, 28.2, 27.1, 26.5, 26.4, 21.5, 14.6.

General biological evaluation methods. *Preparation of test compound stock solutions.* The antifungal agents FLC, CASP, and AmB were purchased from Sigma-Aldrich and were dissolved in anhydrous absolute ethanol, H_2O (18 MΩ), and DMSO, respectively, to give final concentrations of 5 mg/mL. Compounds **1**-**6** were dissolved in H_2O (18 MΩ) to give final concentrations of 5 mg/mL.

Fungal strains. The yeast strains (Table 1) *C. albicans* ATCC 90028 (A), *C. albicans* ATCC 24433 (B), *C. parapsilosis* ATCC 90018 (D), *C. glabrata* ATCC 66032 (I), *C. albicans* ATCC 96901 (M), *C. albicans* ATCC 64124 (N), *C. albicans* ATCC 11651 (O), *C. neoformans* ATCC 32045 (R), and *C. neoformans* ATCC 66031 (S), were purchased from the ATCC. The yeast strains *C. albicans* ATCC SC5314 (C), *C. tropicalis* clinical isolate 660 (E), *C. dubliniensis* Wu284 (F), *C. guilliermondii* B3163 (G), *C. albicans* clinical isolate 280 (H), *C. glabrata* clinical isolate 50 (J), *C. glabrata* ATCC 2001 (K), *C. glabrata* 195 (L), *C. albicans* clinical isolate 2066 (P), and *C. albicans* clinical isolate 2068 (Q) were provided by Prof. Judith Berman from the George S. Wise Faculty of Life Sciences, Tel Aviv University.

Minimal inhibitory concentration test for antifungal activity. Compounds were tested using the broth double-dilution method in 96-well plates (Corning). For inoculum preparation, starter cultures of yeast strains, *Candida* or *C. neoformans spp.*, were incubated in RPMI 1640 for 24 or 72 h respectively (37 °C, 5% CO_2, aerobic conditions) and then diluted 1:100 into fresh medium.

Tested compounds were added to RPMI 1640 media to form the mother liquors (32 μL of stock solution in 1218 μL of RPMI 1640) at a concentration of 128 μg/mL. Next, 100 μL serial double dilutions of the tested compounds (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg/mL) were prepared in RPMI 1640 in flat-bottomed 96-well microplates.

An equal volume (100 μL) of yeast cell suspension in RPMI 1640 was added to each well for a final volume of 200 μL. Final inoculum was typically between 5×10^4 and 5×10^5 CFU/mL. Control wells containing only RPMI 1640 media (blanks) or only fungal cells in RPMI 1640 media were also prepared. After incubation at 37 °C in 5% CO_2 (24 h for *Candida* species and 72 h for *C. neoformans*), MTT (50 μL of a 1 mg/mL solution in H_2O) was added to each well followed by additional incubation at 37 °C for 2 h. The MIC value was defined as the lowest concentration of compound in which no fungal growth was observed. Results were obtained from two independent experiments, and each drug concentration was tested in triplicate in each experiment.

Minimal inhibitory concentration test for antibacterial activity. MICs were determined for compounds **1** and **6** against *Staphylococcus epidermidis* ATCC 12228 (A), *S. aureus* ATCC 9144 (B), *Enterococcus faecalis* ATCC 29212 (C), *Pseudomonas aeruginosa Migula* ATCC 47085 (D), *Acinetobacter baumannii* ATCC 19606 (E), and *E. coli* ATCC 25922 (F) (Table S4, Supporting Information). Compounds were tested using the broth double-dilution method in 96-well plates (Corning). For inoculum preparation, starter cultures were incubated for 24 h (37 C, 5% CO_2, aerobic conditions) and then diluted in fresh Lysogeny Broth (LB) to obtain an optical density of 0.008 (OD_{600}).

Tested compounds were added to LB to form mother liquors (32 μL of stock solution in 1218 μL of LB) at a concentration of 128 μg/mL. Next, 100 μL serial double dilutions of the tested compounds (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg/mL) were prepared in LB in flat-bottomed 96-well microplates. An equal volume (100 μL) of bacterial suspension in LB was added to each well for a final volume of 200 μL and a final OD_{600} of 0.004. Control wells containing only LB (blanks) or only bacteria in LB were also prepared. After 24 h, MTT (50 μL of a 1 mg/mL solution in H_2O) was added to each well followed by additional incubation at 37 °C for 2 h. The MIC value was defined as the lowest concentration of compound in which no bacterial growth was observed. Results were obtained from two independent experiments, and each drug concentration was tested in triplicate in each experiment.

Erythrocyte hemolysis assay. Laboratory rat RBCs (2% w/w) were incubated with each dose of the tested compounds for 1 h at 37 °C in 5% CO_2. Compounds were tested in 96-well plates (Corning) using the double-dilution method starting at a concentration of 192 or 128 μg/mL. The negative control was PBS (without compounds), and the positive control was a 1% v/v solution of Triton X100 (100% hemolysis). Following centrifugation (2000 rpm, 10 min, 10 °C), 100 μL of the supernatant was transferred into a 96-well plate, and absorbance at 550 nm was measured using a microplate reader (Genios, TECAN). The results are expressed as percentage of hemoglobin released relative to the positive control (Triton X100). Each concentration was tested in triplicate, and the results are expressed as means ± standard error from two independent experiments. Each independent experiment was performed on a blood sample taken from a different laboratory rat.

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Mammalian cell viability assay. Cell lines (HEK 293, HepG2, and 3T9MEF) and primary cells (mouse primary dermal fibroblasts) were grown in DMEM supplemented with 10% FCS and 1% penicillin−streptomycin in a humidified chamber of 95% air, 5% CO_2 at 37 °C. For the MTT assay, cells were seeded into 96-well microtiter plates after calibrating the amount of cells which yield subconfluent culture and an appropriate MTT signal. Accordingly 48×10^3 or 24×10^3 cells per well for cell lines or primary cells, respectively, were seeded in 200 μL/well of medium and allowed to adhere for 24 h. Thereafter, solutions containing the tested compounds (10 μL per well) were added to the cells at final concentrations ranging from 2 to 256 μg/mL, and cultures were incubated for an additional 24 h. Cell viability was determined by the MTT assay as follows: MTT was added to the medium at a final concentration of 0.25 mg/mL, followed by a 2-h incubation. The medium was then removed by aspiration, and DMSO (100 μL per well) was added. Absorbance at 570 nm in each well was determined using a Tecan Spectra Fluor microplate reader with a reference wavelength of 630 nm. Each compound was tested in triplicate, and the results are expressed as means ± standard error from at least three independent experiments.

Coculture of HEK 293 and *C. albicans* cells.

A *Coating microscope cover glass with poly-D-lysine (PDL).* Microscope coverslips (18 mm diameter and 0.13−0.17 mm thickness) were placed in a 12-well plate (one slide in each well), covered with PDL solution (1 mL/well, 50 mg/mL), and incubated for 45 min. The PDL was then removed by aspiration, and the coverslips were sterilized and dried under UV light for an additional 20 min. Finally coverslips were rinsed with PBS (1 mL/well).

B *Fungal cell growth conditions. C. albicans* SC5314 cells were grown in the yeast form on YPAD agar plates (yeast peptone dextrose plus 40 mg/L adenine and 80 mg/L uridine) at 30 °C overnight. An aliquot of 1 mL DMEM was then inoculated with a loopful of cells from the agar plate, and number of cells was determined by measuring OD_{600} (OD_{600} value of 1 equals 3×10^7 cells/mL).

C *Mammalian cell growth conditions.* HEK 293 cells were grown on PDL-coated microscope coverslips (6×10^5 cells per well) in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin in a humidified chamber of 95% air, 5% CO_2 at 37 °C. The HEK 293 cells were allowed to adhere for 24 h.

D *Incubation of mammalian cells with fungi cells.* After the adherence of the HEK 293 cells to the coverslips, *C. albicans* SC5314 cells at a multiplicity of infection of 2 (i.e., 12×10^5 cells per well) were added on top of the HEK 293 cells. After 30 min of incubation the coculture was treated with compound **1** or **6** or left untreated and was further incubated for 4 h.

E *Cell staining.* Hoechst 33258 was added to each well at a final concentration of 1 μg/mL, and the plate was incubated for 10 min at 37 °C. Medium was removed by aspiration, and wells were gently rinsed with PBS. Paraformaldehyde (4%, 0.5 mL) was then added to each well, and after 30 min of incubation at room temperature it was removed, and wells were washed twice with Buffer A (TBS X1, 2 mM $CaCl_2$). Thereafter, TritonX 0.1% in buffer A (0.5 mL) was added, and after 10 min of incubation at room temperature wells were washed twice with Buffer A. Finally phalloidin was added (1 μg/mL, 300 μL/well). After incubation for 30 min at room temperature, wells were washed twice with Buffer A. The coverslips were then taken out of the wells and mounted onto microscope slides using ProLong Gold Antifade Mountant. The cells were photographed with an Olympus motorized inverted research microscope Model IX81 (60× magnification).

Propidium iodide-based membrane permeabilization assay. *C. glabrata* ATCC 66032 cells were grown in RPMI at 37 °C, 5% CO_2 overnight. Tested drugs and synthesized compounds were added to 500 μL of cell culture at final concentrations of 2, 4, 16, and 64 μg/mL for CASP, Amp B, compound **1**, and compound **6**, respectively. Cultures were incubated with the compounds for an additional 6 h at 37 °C. An aliquot of 10 μL was collected from each of the culture tubes every 60 min and added to 80 μL of TE 50:50 buffer (50 mM Tris HCl pH 8.0, 50 mM EDTA) and 10 μL of propidium iodide stain at a final PI concentration of 1 mg/mL. Cells treated with ethanol (which results in 100% cell permeability) were prepared as follows: 500 μL of the cell suspension were centrifuged for 4 min at 10,000 rpm (Eppendorf, Centrifuge 5424). The supernatant was removed and resuspended in 100 μL of 96% ethanol. After 5 min, the ethanol suspension was centrifuged for 4 min at 10,000 rpm. The supernatant (ethanol) was removed, and the pellet was resuspended in 500 μL of RPMI. Flow cytometry data were collected from 10,000 to 20,000 cells per time point using B2 laser excitation (excitation at 488 nm and emission at 614/50 nm) on a MACSQuant flow cytometer. Analysis was performed using FlowJo 8.7 software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00272.

^1H and ^{13}C NMR spectra, ULC-MS traces, compound purity information, HC_{50} and IC_{50} values with SD, antibacterial activity data, inoculum dependent MIC data and mammalian cell viability curves are provided as Supporting Information. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone +972 3 6408687; E-mail: mfridman@post.tau.ac.il.

ORCID

Micha Fridman: 0000-0002-2009-7490

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AG, aminoglycoside; AmB, amphotericin B; CASP, caspofungin; FLC, fluconazole; TIBSCl, 2,4,6-triisopropylbenzenesulfonyl chloride; HC_{50}, concentrations at which 50% of RBCs were lysed; IC_{50}, concentration toxic to 50% of cells; log P, partition coefficient; MIC, minimal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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bromide; PI, propidium iodide; RBC, red blood cells; TOB, tobramycin; $T_{1/2}$, time at which the membranes of 50% of the cells were damaged.

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