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Trivalent Ultrashort Lipopeptides are Potent pH Dependent Antifungal Agents

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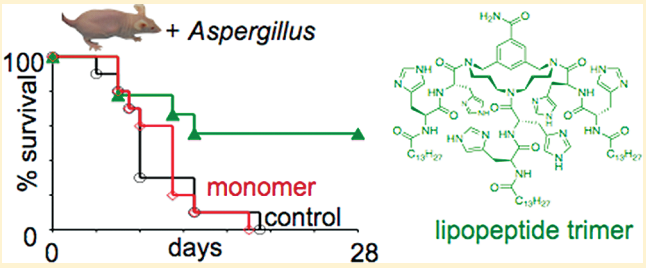
Supporting Information

ABSTRACT: The activity of antimicrobial peptides (AMPs) that contain a large proportion of histidine residues ($pK_a \sim 6$) depends on the physiological pH environment. Advantages of these AMPs include high activity in slightly acidic areas of the human body and relatively low toxicity in other areas. Also, many AMPs are highly active in a multivalent form, but this often increases toxicity. Here we designed pH dependent amphiphilic compounds consisting of multiple ultrashort histidine lipopeptides on a triazacyclophane scaffold, which showed high activity toward *Aspergillus fumigatus* and *Cryptococcus neoformans* at acidic pH, yet remained nontoxic. In vivo, treatment with a myristic acid conjugated trivalent histidine−histidine dipeptide resulted in 55% survival of mice ($n = 9$) in an otherwise lethal murine lung *Aspergillus* infection model. Fungal burden was assessed and showed completely sterile lungs in 80% of the mice ($n = 5$). At pH 5.5 and 7.5, differing peptide−membrane interactions and peptide nanostructures were observed. This study underscores the potential of unique AMPs to become the next generation of clinical antimicrobial therapy.



INTRODUCTION

Antimicrobial peptides (AMPs) are an integral part of the innate immune system and are used as a first line of defense against invading microorganisms.[1−5] Members of this group include peptides that contain a significant number of histidine residues, giving the peptide unique physical properties. These physical properties are mostly due to the pK_a of the histidyl imidazole ring, which lies within the physiological pH range in the body. Therefore, the overall positive charge of these peptides can vary depending on the acidity of the environment, which will affect the peptide's electrostatic interactions, hydrophobicity, and propensity to aggregate, which in turn can affect activity and toxicity.[6] This physical property change is physiologically relevant because various areas or organs of the human body have a slightly acidic pH, for example lung-lining fluids in cystic fibrosis and asthma[7] and also tumors.[8]

Native histidine-rich AMPs include the histatins[9,10] and clavanins.[11,12] Although known primarily for their antifungal properties, histatins have recently been shown to possess potent wound healing properties,[13,14] underlining the potential to use the unique properties of these peptides for many different applications. Designed peptides by substituting positively charged amino acids to histidine residues[15,16] have been reported, and amphipathic histidine-containing peptides based on the helical wheel[17] or LAH peptides[18] have shown antimicrobial activity and potential for use in other applications such as gene delivery.[19,20] In addition to pH effects, metal ions have also been shown to affect the biological activity of histidine containing peptides.[21] Previously, we reported innate-immunity-like peptides that contained multiple histidine residues and consisted of 12−15 amino acids. However, they were only slightly less toxic than the parental lysine-containing peptides and were lytic to numerous pathogens at slightly acidic environments[6] and also cancer cells.[22] The expectation that AMPs will yield novel and useful clinical therapies is growing, and the decreased toxicity of the histidine-containing peptides, combined with the pH dependent activity, makes them interesting candidates for development.

In this respect, the addition of a lipophilic alkyl chain is an effective method to increase the affinity of a peptide to the membrane, thus increasing the biological activity of certain peptide sequences.[23] Other innovative examples involve the incorporation of fluorinated residues, which have shown to increase activity through an increase of hydrophobicity in some cases and provide the opportunity to gain structural

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information through NMR.[24] Previously, we reported that linear ultrashort cationic lipopeptides even as short as 2−4 amino acids have potent antimicrobial and antifungal properties. The minimum peptide length and fatty acid length necessary for activity were described.[25,26] Also, we have reported that the multivalent display of AMPs drastically enhances membrane permeation activity,[27−29] however, the increase in potency is sometimes accompanied by a loss of selectivity. On the basis of these findings, we screened a small series of trimeric histidine−histidine dipeptides conjugated to fatty acids of differing lengths for biological activity. The triazacyclophane (TAC) scaffold was used because it has been proven to be a versatile platform for displaying multivalent peptides and provides an additional handle for future modifications.[30−34] Our data reveal that the presently described compounds exhibited in vitro pH dependent antifungal activity, especially against *Aspergillus fumigatus*. We focused on a lead compound for toxicity and in vivo experimentation: 55% of the mice infected with an otherwise lethal dose of *A. fumigatus* were rescued. In addition, different nanostructures were seen at pH 5.5 and 7.5, a physical property that could prove useful in future drug delivery strategies.[35]

RESULTS

Design and Synthesis. We utilized a molecular scaffold as a platform to design a histidine-containing amphiphilic compound that spatially segregates the charged components. The TAC-scaffold can conveniently accommodate up to four different molecular entities. Also, the three secondary amine groups and the carboxylic acid can be orthogonally protected,[36] making the scaffold well suited for solid-phase synthesis, and it has been employed to address a variety of biological issues.[30−34] We chose to couple a histidine−histidine dipeptide to the three amine groups on the TAC-scaffold, giving a theoretical potential for six positive charges at an acidic pH and leaving the carboxylic acid for attachment to the solid support. The histidine−histidine dipeptide triads were *N*-terminally capped with C_6, C_{14}, or C_{18} fatty acid acyl chains to vary the hydrophobicity (Figure 1). The compounds were synthesized

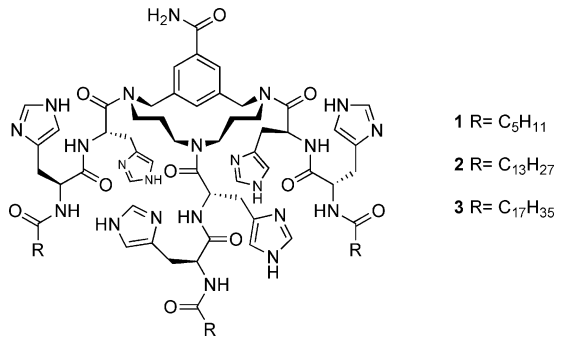

Figure 1. Structures of compounds **1**−**3**.

in four solid phase coupling steps using standard peptide synthesis protocols, cleaved from Rink amide resin under acidic conditions, which gave a *C*-terminal amide, purified using column chromatography, and identified using mass spectrometry.

The Trimer Is Highly Active at pH 5.5 against *Aspergillus fumigatus* and *Cryptococcus neoformans*. First, the compounds were screened for activity to obtain the minimum inhibitory concentrations (MICs) against Gram-positive and Gram-negative bacteria and against fungi. The compounds were observed to be inactive on *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* at pH 5.5 and at pH 7.5 and against *A. fumigatus* and *Cryptococcus neoformans* at pH 7.5 (Table S1, Supporting Information). However, trimeric compound **2** was highly active (3.12 μM) on both *A. fumigatus* and *C. neoformans* at pH 5.5 (Table 1). For comparison, the

Table 1. Minimum Inhibitory Concentration (MIC) Values of Compounds (μM)

	A. fumigatus ATCC 26430		*C. neoformans* ATCC MYA-422		*C. albicans* ATCC 10231	
	pH 5.5	pH 7.5	pH 5.5	pH 7.5	pH 5.5	pH 7.5
C_{14}−HH	50	>100	25	>100	>100	>100
1	>100	>100	>100	>100	>100	>100
2	3.12	>100	3.12	>100	>100	>100
3	>100	>100	25	>100	100	>100
AmB[a]	1	0.17	1.35	0.17	2.7	0.33

[a]AmB: amphotericin B.

dipeptide monomer C_{14}−HH showed moderate activity at 50 and 25 μM, respectively. Trimeric compound **2** was thus 16 and 8 times more active than the monomer C_{14}−HH. The effect of the scaffold was approximately 5 and 2.5 times because there are 3 histidine−histidine dipeptides on the scaffold, which is a moderate multivalency effect. The pH dependent activity is most likely due to the gain in amphiphilicity resulting from the positively charged histidine residues at pH 5.5. The reason that it was selectively active against fungi and not against bacteria is less clear.

Next, active compound **2** was tested on *A. fumigatus* with equivalent amounts of Cu(II), Ni(II), and Zn(II) because different metal ions have been shown to coordinate to histidine containing compounds[37,38] and may affect compound amphiphilicity by changing histidine charge and/or organization. Here the activity was completely abrogated for both pH 5.5 and 7.5. The charge and amphiphilicity of compound **2** was most likely reduced compared to the protonated **2**, resulting in a complete activity loss.

The Biocompatibility Is Excellent for Trimeric Compound 2. A safety profile was then obtained for compound **2**. In vitro, it was nonhemolytic at pH 7.5 and tested to a maximum concentration of 125 μM. Moreover, the compound was nontoxic to cultured murine macrophages up to the highest concentration tested (200 μM). Thus the biocompatibility was excellent for *Aspergillus*, with a LC_{50}/MIC ratio of 64. In vivo, immunocompromised ICR mice showed no observable toxicity effects with four treatments of 9 mg/kg administered intratracheally (i.t.) at pH 5.5 on days 1, 4, 7, and 11, a dose that corresponded to twice the treatment dose (n = 5).

Enzymatic degradation of the peptides using proteinase K revealed that whereas melittin was completely degraded after 2 h, 80% of the trivalent ultrashort lipopeptide remained uncleaved. Note that rapid degradation or full protection against degradation are a drawback for the use of membrane active peptides in therapy. We believe that the aggregation properties of these peptides play an important role in stabilizing them in vivo.

In Vivo Studies with Trimeric Compound 2. Trimeric compound **2** was tested in mice infected with a lethal dose of *A. fumigatus*. The lungs of immunocompromised ICR mice were infected, and treatment consisted of i.t. gavage with phosphate

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buffered saline (PBS) as a control, compared to the monomeric lipopeptide, and compound **2** at a dose of 4.5 mg/kg, on days 1, 4, 7, and 11 each at a pH of 5.5. Survival was assessed and showed that mice treated with compound **2** exhibited a survival of 55% (n = 9), whereas no treatment (PBS) or treatment with the monomer resulted in 0% survival (n = 10) (Figure 2).

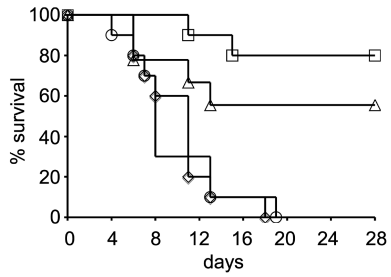

Figure 2. In vivo results. Survival of *A. fumigatus* infected mice treated i.t. with compound **2** (△), or C_{14}–HH (◇). Dose: 4.5 mg/kg. Control groups were amphotericin B (□) [1 mg/kg intraperitoneal (i.p.)] or saline (○) (n = 9–10).

Moreover, the fungal burden was assessed in the lungs, spleen, and kidneys and showed that 4/5 mice treated with compound **2** were completely devoid of fungus in the lungs, compared with 1/5 in the untreated group (Table 2). No *Aspergillus* could be detected in any organ in 3/5 treated mice.

pH Dependent Mode of Action. To obtain information on the mode of action of these constructs, the permeation of the membrane of *A. fumigatus* was probed using Sytox green, and depolarization was observed using the cationic cyanine dye $DiSC_3(5)$. Active trimeric compound **2** gave large fluorescence changes with $DiSC_3(5)$ at pH 5.5 but not at 7.5 (Figure 3). However, with Sytox green, no changes in fluorescence were observed (Figure S1, Supporting Information). This indicates that compound **2** causes membrane depolarization but does not cause massive membrane damage. All other peptides tested gave no signal changes with $DiSC_3(5)$ or Sytox green at both pH 5.5 and 7.5, which confirmed the inactivity observed in the MIC assay. Melittin was used as a positive control and showed large fluorescence increases.

Nanostructures Observed with Transmission Electron Microscopy (TEM). In view of the amphiphilic nature of the constructs and the supramolecular structures that are often observed for such constructs, which are likely to be relevant for medicinal applications, TEM images of solutions of compound **2** at the two pH values studied were obtained (Figure 4). For this, peptide solutions (50 μM) were observed at a magnification of 60000× at pH 5.5 and 7.5. At pH 7.5, sheets or ribbon-like nanostructures with variable widths were observed. At pH 5.5, however, small vesicles with radii of a few nanometers were seen. Larger magnification revealed that the vesicles had a size of ∼5–10 nm.

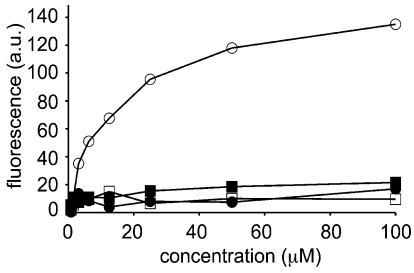

Figure 3. $DISC_3(5)$ fluorescence with *A. fumigatus* and peptides. Compound **2**: pH 5.5 (○), pH 7.5 (●). C_{14}–HH: pH 5.5 (□), pH 7.5 (■).

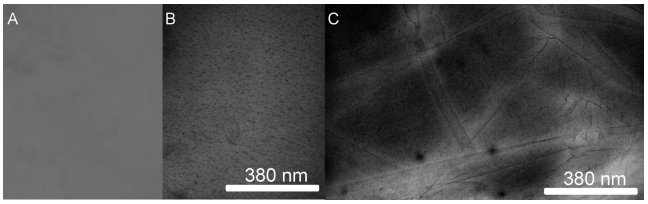

Figure 4. Transmission electron microscopy images of compound **2** at a concentration of 50 μM [(A) blank; (B) pH 5.5; (C) pH 7.5].

DISCUSSION AND CONCLUSIONS

Most of the current drugs that target *A. fumigatus* are based on targeting ergosterol in the fungal membrane or the ergosterol biosynthetic pathway.[39] Because there is a limited number of these drugs available, it is important to broaden the repertoire of antifungal agents by designing compounds that can target other components of the membrane. In general, compounds that target the membrane are advantageous in other diseases where the growing resistance to chemotherapeutic agents is a major concern.

Here we showed that a simple histidine lipopeptide, synthesized in its trivalent form on a molecular scaffold, was very active against *A. fumigatus* and *C. neoformans* at pH 5.5 but was inactive at pH 7.5. The other compounds with shorter and longer conjugated fatty acids were much less active, probably due to the differences in hydrophobicity, which lead to less optimal affinities for the membrane or undesirable self-association propensities.[40,41] Compound **2**, however, was active at low micromolar concentrations, which was similar to amphotericin B and other reported active antifungal peptides such as histatin-5.[42] The compound was biocompatible, being nontoxic to macrophages, giving a large therapeutic index, and being nontoxic in vivo. Potent antifungal in vivo activity was observed, rescuing 55% of mice infected with an otherwise lethal dose of *A. fumigatus*. In comparison, despite being

Table 2. CFUs of *Aspergillus fumigatus* Found in Organs in Infected Mice

	untreated				treated with compound **2**			
mouse	lung	spleen	kidney	kidney	lung	spleen	kidney	kidney
1	12920	40	30	30	0	0	0	0
2	4000	10	20	600	0	0	0	0
3	0	0	0	0	0	0	0	1111
4	80	70	60	5400	0	0	0	0
5	9200	140	230	110	40	20	70	4640

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administered at approximately 3 times the molar concentration as the trimer, the monomer remained inactive and emphasizes the in vivo importance of the multivalent effect. In other words, the trivalent construction may not only contribute to increased activity effects but may contribute to enhanced pharmacokinetic effects as well. Additional fungal burden experiments showed that lungs of 80% of the treated mice were completely devoid of *Aspergillus*.

Sytox green is a dye that cannot enter an intact cell unless there is severe membrane permeation or damage. Upon entering the compromised cell, binding to nucleic acids increases the fluorescence. In comparison, the fluorescence of the cationic cyanine dye $DiSC_3(5)$ changes when membranes undergo a shift from a hyperpolarized to a nonpolarized state. Therefore, these two methods shed light on peptide membrane interactions on living cells. Compound **2** dissipated the membrane potential of *A. fumigatus* in a pH dependent manner, but no massive membrane damage was observed. Absence of membrane damage was also apparent in the hemolysis and toxicity assays. Peptides were added at different growth stages in the fluorescence and MIC assays. For the fluorescence assays, spores of *A. fumigatus* were first grown for 24 h and then treated with the lipopeptides, which was different from the MIC assay, where the peptides were added immediately to the spores. The $DiSC_3(5)$ fluorescence began to increase at approximately 3 μM for compound **2** (Figure 3), which agreed with its MIC. In contrast, there was no fluorescence increase observed with the weakly active C_{14}–HH monomer even at twice the MIC. This may indicate that the activity of the monomer was limited to the conidia stage, whereas the trimeric form was active against both the *Aspergillus* conidia and the germinating conidia or hyphae. This is highly relevant because the immune system responds in different ways against invasive aspergillosis: the alveolar macrophages eliminate the conidia, and neutrophils clear the hyphae form. Because most cases of invasive aspergillosis occur in patients with dysfunctional neutrophils, it is important that the treatment would eliminate all forms of the fungus.[43−46]

Ribbon and sheet-like nanostructures were seen at pH 7.5, compared with micelles at pH 5.5. These transforming properties could be desirable for treatment of fungal infections in areas of the body that are slightly acidic, for example, the lungs of cancer patients or cystic fibrosis patients. Also, this difference in aggregation behavior as a function of the pH may explain in part the differences in activity of compound **2** at the two pH values. This strong self-association at pH 7.5 may prevent toxic effects on cells by decreasing the bulk concentration of the monomeric unit. For instance, the peptides could be safely delivered to a diseased or infected organ at pH 7.5, upon which the local low pH would transform the larger sheets into smaller active units. This localized activation can significantly limit systemic toxicity, and the small vesicles at lower pH values may facilitate penetration and interaction with pathogens. In addition, the nanostructures may also be more stable in vivo, and the pH-mediated transition from one state to the other may be exploited in drug delivery. In vivo, injection volumes are limited and usually require highly concentrated peptide solutions. For example, i.p. injection of a hypothetical peptide (MW = 2000 da) at a dose of 5 mg/kg in a murine model requires an injection concentration of ca. 300 μM, a concentration that may be many times higher than the effective in vitro dose. Thus the propensity of the test compound to form nanostructures, even at concentrations higher than the effective in vitro concentration, is an important consideration for designing a compound intended for medicinal use.

Taken together, these data indicate that this compound is uniquely active in vivo and warrants further investigation into the detailed mode of action using for example NMR[47] and further testing in other diseases where pH effects can be exploited for example cancer. The design of the TAC-scaffold offers the opportunity of adding an extra moiety for potential future modifications such as addition of a targeting or imaging component in other potential applications.[48] In general, the translation of AMPs from in vitro experimentation to in vivo activity has until now been difficult due to high systemic toxicity, instability, and degradation in vivo and inactivation by serum proteins. This study provides an example of an effective and safe de novo designed AMP and highlights the importance of unique AMPs as future clinical antimicrobial therapy.

EXPERIMENTAL SECTION

General. Rink amide PS-resin and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Calbiochem Novabiochem AG (Switzerland). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA; Sigma), piperidine (Merck), *N*,*N*-diisopropylethylamine (DIEA, Sigma), *N*-hydroxybenzotriazole hydrate (HOBT; Aldrich), and dimethylformamide (DMF, peptide synthesis grade; Biolab). All other reagents were of analytical grade. Buffers were prepared in double-distilled water. RPMI 1640 was purchased from Biological Industries (Beit Haemek, Israel). Compounds tested in vivo as acetic acid salts. Purity was determined to be >95% using HPLC. Values reported in in vitro assays are averages of duplicate experiments. Animal studies were performed in accordance with Tel Aviv University institutional policies.

Synthesis and Purification of TAC-Based Compounds 1, 2, and 3. The Fmoc-protecting groups of 0.5 g of PS S RAM resin (loading: 0.73 mmol/g) were removed using a 20% solution of piperidine in NMP (2 × 8 min, 4 mL each), followed by washing with NMP (2 × 2 min, 4 mL each) and DCM (2 × 2 min, 4 mL each). A positive Kaiser test[41] confirmed the presence of free amine groups. The TAC-scaffold HOC(O)-TAC(*o*-NBS)$_3$ (455 mg, 1.5 equiv) was coupled to the resin using BOP (242 mg; 1.5 equiv) and DIEA (190 μL, 3 equiv) in NMP (3 mL, 16 h), followed by washing (described above). Any possibly remaining amine groups were capped using a solution of 0.5 M Ac_2O, 0.125 M DIEA, and 0.015 M HOBt in NMP (2 × 10 min), followed by washing. The *o*-NBS-protecting groups were removed by washing the resin first with DMF (2 × 2 min, 4 mL each) then adding 2-mercaptoethanol (5.5 mL of a 1 M solution in DMF) and DBU (420 μL, 2.8 mmol) (2 × 30 min), followed by washing. The chloranil test[42] confirmed successful deprotection. The histidine residues were attached to the TAC-scaffold using Fmoc-L-His(Trt)-OH (2.7 g, 4.4 mmol) with BOP (1.9 g, 4.4 mmol) and DIEA (1.5 mL, 8.8 mmol) in NMP (8 mL) (3 h), followed by washing, Fmoc-removal, and washing. This procedure was repeated once in order to obtain the TAC-scaffold bound His(Trt)–His(Trt) dipeptides. The obtained batch of resin was divided in three equal portions, and for compound **1**, one portion was treated with hexanoic acid (2.3 equiv, 0.85 mmol, 88 μL), BOP (362 mg), DIEA (1.64 mmol, 283 μL) in NMP (3 mL) (16 h). For compound **2**, myristic acid (2.3 equiv, 0.85 mmol, 194 mg), BOP (362 mg), and DIEA (1.64 mmol; 283 μL) in DCE/NMP (3 mL, 1:1, v/v) (16 h), and for compound **3**, stearic acid (2.2 equiv, 0.82 mmol, 219 mg), BOP (362 mg), and DIEA (1.64 mmol, 283 μL) in DCE/NMP (3 mL, 1:1, v/v) (16 h) was used, followed by washing. Deprotection and cleavage of the constructs was performed with TFA/TIS/water 95:2.5:2.5 (v/v/v) (3 h) and compound **1** was precipitated in cold MTBE/*n*-hexanes 1:1 (v/v). For compounds **2** and **3**, the solutions were concentrated in vacuo. After lyophilization of the crude products, **1** was purified by preparative HPLC and **2** and **3** were purified by column

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chromatography (eluents: DCM to 10% MeOH in DCM gradient, both contained 1% Et_3N) to >95% purity (vide infra).

Synthesis of C_{14}–His–His (4). The Fmoc-protecting group of 0.1 g of PS S RAM resin (loading: 0.73 mmol/g) were removed using a 20% solution of piperidine in NMP (2 × 8 min, 1 mL each), followed by washing with NMP (2 × 2 min, 1 mL each) and DCM (2 × 2 min, 1 mL each). A positive Kaiser test confirmed the presence of free amine groups. The histidine residue was attached to the resin using Fmoc-L-His(Trt)-OH (179 mg, 292 μmol) with BOP (129 mg, 292 μmol) and DIEA (101 μL, 584 μmol) in NMP (1 mL) (3 h), followed by washing (described above), Fmoc-removal, and washing. This procedure was repeated once. After this, myristic acid (4 equiv, 194 mg, 292 μmol), BOP (129 mg, 292 μmol), and DIEA (101 μL, 584 μmol) in DCE/NMP (2 mL, 1:1, v/v) (16 h) was applied, followed by washing. Deprotection and cleavage was performed with TFA/TIS/water 95:2.5:2.5 (v/v/v) (3 h), and the cleaved products were precipitated in cold MTBE/*n*-hexanes 1:1 (v/v) and purified by preparative HPLC (vide infra).

Antifungal Activity. The antifungal activity of the lipopeptides was measured using the guidelines of Clinical Laboratory Standards document M27-A3 or M38-A2 for *Aspergillus*.[49,50] The peptides were examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 200 μL as follows: 100 μL of a suspension containing fungi at a concentration of 2×10^3 colony-forming units/mL in culture medium (RPMI 1640, 0.165 M MOPS, pH 7.5, or pH 5.5 with L-glutamine, without $NaHCO_3$ medium) was added to 100 μL of the peptide solution or the peptide solution containing an equimolar amount of $ZnCl_2$, $CuSO_4$, or $NiCl_2$, in serial 2-fold dilutions. The fungi were incubated at 35 °C for 48 h for *Aspergillus fumigatus* (ATCC 26430) and for *Candida albicans* (ATCC 10231) and for 72 h for *Cryptococcus neoformans* (ATCC MYA-422) using a Binder KB115 incubator. Growth inhibition was determined by eye, and antifungal activities were expressed as the minimal inhibitory concentration (MIC), the concentration at which no growth was observed.

Antibacterial Activity. The antibacterial activity was examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μL as follows: aliquots (50 μL) of a suspension containing bacteria at a concentration of 10^6 colony-forming units/mL in culture medium were added to 50 μL of water containing the peptide (prepared from a stock solution of 1 mg/mL peptide in water) in serial 2-fold dilutions in LB. Antibacterial activities were expressed as the MIC, the concentration at which no growth was observed after 18–20 h of incubation. The bacteria used were *E. coli* ATCC D21 and *S. aureus* ATCC 6538P.

Hemolysis of Human Red Blood Cells (hRBCs). The assay was done by using a final volume of 100 μL of PBS solution containing the peptides and hRBCs (final concentration 4%). Release of hemoglobin was monitored after an incubation time of 60 min at 37 °C by measuring the absorbance of the supernatant at 540 nm. Controls for 0% hemolysis (blank) and 100% hemolysis consisted of hRBCs suspended in PBS and 1% Triton, respectively.

Cell Culture. In vitro assays were performed on RAW264.7 murine macrophages (ATCC-TIB71). Cells were grown in DMEM supplemented with 10% FBS, L-glutamine, sodium pyruvate, nonessential amino acids, and antibiotics (Biological Industries, Beit Haemek, Israel). Incubator was set on 37 °C with a humidified atmosphere containing 5% CO_2. XTT cytotoxicity assay: 2×10^5 cells per well were grown overnight on a 96-well plate. The following day, the media were replaced with 100 μL culture medium containing different concentrations of the different peptides. Peptide concentrations ranged from 3.12 to 200 μM. The cells were then incubated for 16 h, and the media was replaced with 100 μL of fresh media and 50 μL of 2,3-bis-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) reaction solution (Biological Industries). The LC_{50} (the concentration at which 50% of the cells die) for each peptide was obtained from the dose-dependent cell viability curves.

In Vivo Toxicity Assay. Toxicity was tested using six-week-old female ICR mice immunocompromised by cortisone acetate injection. Cortisone acetate (150 mg/kg PBS with 0.1% Tween 80) was injected subcutaneously at 4 days prior to treatment with lipopeptide, on the day of first treatment, and 2 and 4 days postfirst treatment (n = 5). Compound **2** was administered i.t. at a dose of 9 mg/kg (in saline, pH 5.5, 50 μL) on days 1, 4, 7, and 11 and monitored for 28 days for survival, weight, overall appearance, and behavior. Weight was maintained, and no visible signs of toxicity were observed.

Enzymatic Degradation Assay. The trivalent ultrashort lipopeptides were dissolved in PBS at a concentration of 1 μg/μL, and proteinase K at a concentration of 5 μg/μL (2% DMSO final concentration). A solution of the enzyme (8 μL) was then added to a solution of a peptide (400 μL), and the amount of the nondegraded peptide was evaluated by using RP-HPLC (analytical Vydac C4 column (Grace Discovery Sciences, Deerfield, IL), solvent A is 0.1% TFA in water and solvent B 0.1% TFA in acetonitrile). The gradient used was 10–90% of solvent B during 40 min. A sample of a peptide was injected to the column, as a reference for the evaluation of the total peptide, prior to the addition of the enzyme. Melittin was used as a control to test the activity of the enzyme.

Treatment of Immunocompromised *A. fumigatus* Infected Mice. Six-week-old female ICR mice were immunocompromised by cortisone acetate injection. Cortisone acetate (150 mg/kg PBS with 0.1% Tween 80) was injected subcutaneously at 4 days prior to treatment with lipopeptide, on the day of first treatment, and 2 and 4 days postfirst treatment. Lipopeptides were administered i.t. at 4.5 mg/kg (50 μL) on days 1, 4, 7, and 11. Mice were infected with *Aspergillus* on day 1, and survival was assessed. Mice were monitored for 28 days (n = 10). Note: n = 9 for compound **2** treatment due to death during anesthetic before infection.

Determination of Fungal Loads; CFU Determination. Six-week-old female ICR mice were immunocompromised by cortisone acetate injection. Cortisone acetate (300 mg/kg PBS with 0.1% Tween 80) was injected subcutaneously at 3 days prior to conidial infection, on the day of infection, and 2 days postinfection. Then 2.5×10^5 conidia/mouse of *A. fumigatus* (AF293) strain were administered intranasally on the day of infection. Compound **2** was administered i.t. at 4.5 mg/kg on days 1 and 4 following infection. Mice were sacrificed 5 days postinfection and lung, spleen, and kidneys were used for CFU determination. Experimental layout as follows. Group 1: 5 animals, immunocompromised, infected. Group 2: 5 animals, immunocompromised, infected, treated with compound **2**. CFU determination: organ samples were removed and placed in a sterile tube containing 1 mL of sterile saline. Homogenates were prepared in saline using a tissue raptor homogenizer (Qiagen). After that, 10 or 100 μL of homogenates were spread onto YAG plates (containing chloramphenicol) and incubated at 37 °C for 24–48 h before *A. fumigatus* colonies were counted.

Membrane Permeation Studies with *A. fumigatus*. A 100 μL suspension containing *A. fumigatus* at a concentration of 2×10^3 colony-forming units/mL in culture medium (RPMI 1640, 0.165 M MOPS, pH 7.4, or 5.5 with L-glutamine, without $NaHCO_3$ medium) was added to a sterile black 96-well plate and grown for 20 h at 35 °C. Sytox green or $DiSC_3(5)$ was added in a volume of 50 μL (sodium phosphate buffer (SPB), pH 5.5 or 7.5) for a final concentration of 0.2 μM and incubated for 15 min. Peptides were added in 50 μL (SPB, pH 5.5 or 7.5) for a final concentration range of 100–1.56 μM. Fluorescence was measured after 1 h with excitation 485 nm, emission 530 nm for Sytox green, and excitation 640 nm, emission 660 nm for $DiSC_3(5)$.

Visualization of Nanostructures using Transmission Electron Microscopy. The compounds were dissolved in growth medium at a concentration of 50 μM. A drop containing the compounds was deposited onto a carbon-coated grid and negatively stained with 2% uranyl acetate. Grids were examined using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic details including scheme, purity (HPLC), and characterization (HR-MS). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AMP, antimicrobial peptide; TAC, triazacyclophane; TEM, transmission electron microscopy

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