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Structure–activity relationship studies on niphimycin, a guanidylpolyol macrolide antibiotic. Part 1: The role of the *N*-methyl-*N*′′-alkylguanidinium moiety

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Abstract—Several *N*-methyl-*N*′′-alkylguanidinium derivatives were synthesized and used as simplified analogues of niphimycin (NM), a guanidylpolyol macrolide, in structure–activity relationship studies. The C16-alkylated derivative exerted fungicidal activity by directly damaging the fungal plasma membrane and inducing oxidative stress in a manner similar to niphimycin. These results indicate that the *N*-methyl-*N*′′-alkylguanidinium moiety is required for antifungal activity by NM.

Niphimycin (NM), first isolated in 1967 from *Streptomyces hygroscopius* B-255,[1] is a guanidylpolyol macrolide[2] that possesses broad antimicrobial activity against Gram-positive bacteria, filamentous fungi, and yeasts.[3] Recent screening studies have revealed a strain of *Streptomyces* that secretes a fungicidal substance capable of drastically changing the permeability of the plasma membrane in *Saccharomyces cerevisiae*. Spectroscopic studies identified the active agent to be NM.[4] Despite its structural similarity to amphotericin B (AmB), a polyene macrolide antibiotic, the mode of action of NM is clearly distinguishable from that of AmB.[5] NM disrupts the plasma membrane by directly interacting with phospholipids such as phosphatidylcholine (PC), but it does not interact with ergosterol, a molecular target of AmB. The most striking feature of NM-induced cytotoxicity is the cellular production of reactive oxygen species (ROS) which are not generated when yeast cells are treated with AmB (Fig. 1). Previously, we proposed that a synergistic combination of direct plasma membrane damage and oxidative stress produces NM's potent antifungal activity.[5] Differences in the structures of NM and AmB suggest that NM's ability to damage the plasma membrane and/or generate ROS resides primarily in the alkyl side chain and terminal guanidine. As a first step toward elucidating the structure–activity relationship of NM, we here report the synthesis and biological evaluation of several *N*-methyl-*N*′′-alkylguanidinium derivatives.

The structures of the *N*-methyl-*N*′′-alkylguanidinium derivatives, and the strategies used for their synthesis, are shown in Schemes 1 and 2. Commercially available alkyl (C4, C8, C12, and C16) amines were coupled with *N*,*N*′′-bis-Boc-1-methyl-2-thiourea[6] using the $HgCl_2$-promoted guanylation process.[7] The yield of **4** was improved from 45% to 66% by using Ratcliffe's guanylating agent[8] in a slightly modified literature procedure.[9] 12-Aminododecanoic acid was converted to methyl ester **8**, which was then treated with $LiAlH_4$ in ether under reflux to give the corresponding ω-aminoalcohol **9** in 81% yield. Guanylation of **8** and **9** with Ratcliffe's agent provided bis-Boc-protected derivatives **10** and **11**, respectively, in good yields. Methyl ester **10** was hydrolyzed with KOH in methanol to afford **12** in 70% yield. Removal of the Boc moieties on **10**–**12** in TFA–CH_2Cl_2 (1:1) led to quantitative yields of the corresponding *N*-methyl-*N*′′-alkylguanidinium derivatives (**5**–**7**) with an oxygen functionality at the ω-position.

Keywords: Antifungal activity; Guanidylpolyol macrolides; Structure–activity relationship; Reactive oxygen species; Oxidative stress.
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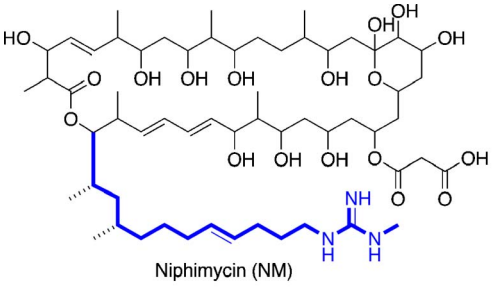
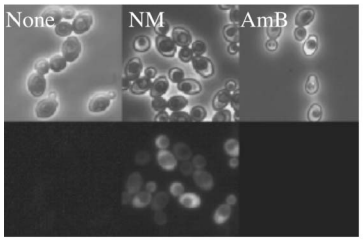




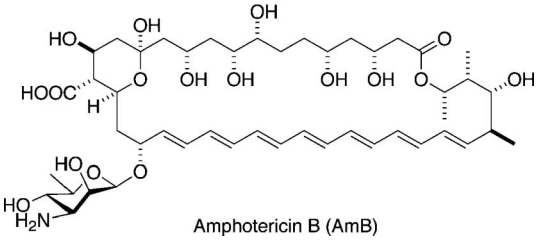
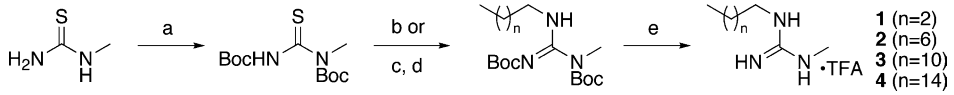


Figure 1. The effects of niphimycin (NM) and amphotericin B (AmB) on the generation of reactive oxygen species (ROS) in *S. cerevisiae* cells. After treatment with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), the cells were incubated either in YPD medium alone, or in YPD medium containing 10 μM NM or AmB at 30 °C for 30 min. Cells were observed using phase-contrast (top) and fluorescence (bottom) microscopies at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

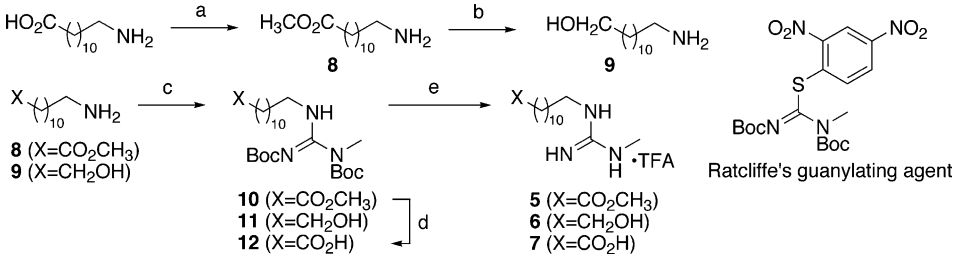
Compounds **1**–**7**[10] were evaluated for in vitro antimicrobial activity. Minimal inhibitory concentration (MIC) values were measured by the serial twofold broth dilution method[11] after 24-h cultivation in 3% nutrient broth at 37 °C for bacteria, and in YPD medium containing 1% yeast extract, 1% peptone, and 2% glucose at 25 °C for yeasts and filamentous fungi. The results are summarized in Table 1. Compounds **1** and **2** inhibited the growth of *Penicillium chrysogenum* at 100 μM, but did not inhibit the growth of the other microorganisms tested, even at concentrations up to 400 μM. Compound **3**, and especially compound **4**, resulted in good growth inhibition of the microorganisms tested except for *Micrococcus luteus*; neither compound was effective against *M. luteus*, even at 400 μM. C12-alkylated compounds with an oxygen functionality at the ω-position (compounds **5**–**7**) did not inhibit the growth of any of the microorganisms. The antimicrobial profiles of **4** and NM are similar, except for **4**'s higher MIC values against a strain of *S. cerevisiae* and against *Candida albicans*. These results suggest that the *N*-methylguanidinium moiety plus a hydrophobic structure (i.e., the C16 alkyl group) are both required for NM to exhibit antifungal activity. Compound **4** was further compared to NM in additional biological studies.

The effect of **4** on plasma membrane function was examined by analyzing the efflux of K^+ and UV-absorbing materials from *S. cerevisiae* X-2180 1A cells.[12] As shown in Figure 2, AmB caused a change in plasma membrane permeability, resulting in a dose-dependent K^+ efflux. However, AmB was less effective in promoting leakage of UV-absorbing materials, such as nucleotides, even at concentrations large enough to induce cell death. As with NM, treating yeast cells with **4** influenced plasma membrane function more dynamically than did AmB at doses approximately equivalent to the MIC value.

The ability of PC to protect the yeast plasma membrane against the dynamic action of **4** was examined. As shown in Figure 3, the addition of PC provided effective protection against **4**-induced growth inhibition.[13] In addition, plasma membrane damage was mitigated by PC, as reflected by the dose-dependent leakage of K^+ and UV-absorbing materials.[12] The protective effect of PC against plasma membrane damage induced by **4** strongly suggests that NM interacts with plasma membrane phospholipids via its alkyl side chain.



Scheme 1. Reagents and conditions: (a) NaH, $(Boc)_2O$/THF, 0 °C, rt, 3 h; (b) RNH_2, Et_3N, $HgCl_2$/DMF, 0 °C, 45–92%; (c) Sanger reagent (2,4-dinitro-1-fluorobenzene), K_2CO_3/CH_3CN; (d) RNH_2, *i*-Pr_2NEt/THF, rt, 66% for 2 steps; (e) 50% TFA/CH_2Cl_2, rt, 3 h, quantitative yield.



Scheme 2. Reagents and conditions: (a) $SOCl_2$/MeOH, −10 °C, rt, 86%; (b) $LiAlH_4/Et_2O$, 0 °C, reflux, 81%; (c) Ratcliffe's guanylating agent, *i*-Pr_2NEt/THF, rt, 99% for **10**, 92% for **11**; (d) aq KOH/MeOH, rt, 72%; (e) 50% TFA/CH_2Cl_2, rt, 3 h, quantitative yield.

Table 1. Antimicrobial activities of *N*-methyl-*N*′-alkylguanidine (**1**–**7**) and NM

Test organisms	MIC (μM)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	NM
Yeast								
Saccharomyces cerevisiae X-2180 1A	>400	>400	100	100	>400	>400	400	5
Saccharomyces cerevisiae IFO 0203	>400	>400	100	50	>400	>400	>400	—
Schizosaccharomyces pombe IFO 0342	>400	400	50	25	400	>400	200	—
Schizosaccharomyces pombe L972 h^-	>400	200	50	12.5	400	>400	200	2
Schizosaccharomyces pombe SA21 h^+	>400	400	50	12.5	400	>400	200	—
Candida albicans IFO 1061	>400	>400	400	200	>400	>400	400	2
Rhodotorula mucilaginosa IFO 0001	>400	>400	50	12.5	200	>400	200	2
Fungi								
Aspergillus niger ATCC 6275	>400	>400	50	25	200	>400	>400	2
Mucor mucedo IFO 7684	>400	>400	50	12.5	>400	>400	400	10
Penicillium chrysogenum IFO 4626	100	100	25	6.25	200	>400	400	1
Rhizopus oryzae IFO 4766	>400	>400	200	50	>400	>400	>400	1
Bacteria								
Escherichia coli IFO 2545	400	400	50	50	400	>400	400	—
Bacillus subtilis IFO 3007	>400	>400	25	12.5	400	>400	100	5
Micrococcus luteus IFO 3333	>400	>400	>400	>400	>400	>400	400	—

—, not tested.

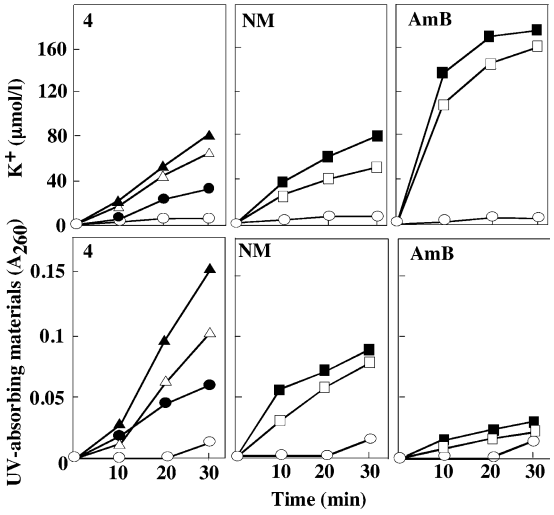


Figure 2. Leakage of UV-absorbing materials and K^+ from intact cells of *S. cerevisiae* caused by **4**, NM, and AmB. Cells (10^8/ml) were incubated in 50 mM succinate buffer (pH 6.0) containing **4** at 0 (○), 50 (●), 100 (△) or 200 μM (▲) at 30 °C. Cells were also incubated in buffer containing NM or AmB at 5 (□) or 10 μM (■) at 30 °C.

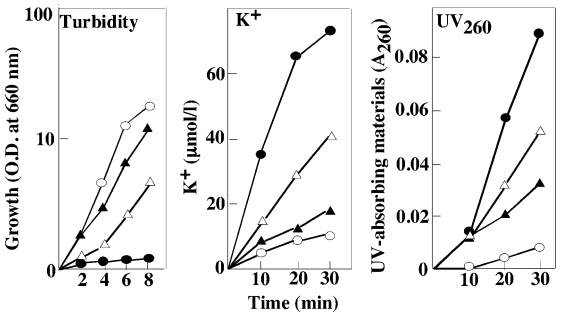


Figure 3. Protective effects of PC against **4**-induced growth inhibition, leakage of K^+, and leakage of UV-absorbing materials from *S. cerevisiae* cells. Cells were incubated in YPD medium containing **4** at 0 (○) or 100 μM (●) at 30 °C. In medium containing **4**, PC was added at 30 (△) or 80 μM (▲).

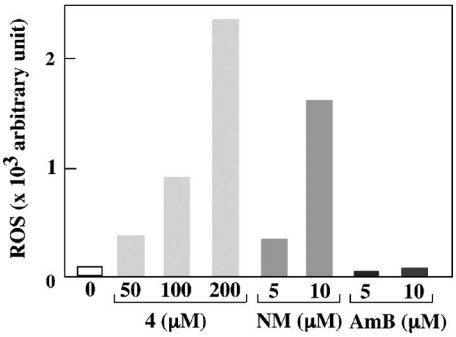


Figure 4. Promotion of ROS generation in *S. cerevisiae* cells by **4**, NM, and AmB. After DCFH-DA treatment, the cells were incubated at 30 °C for 60 min in YPD medium containing either **4**, NM, or AmB at the indicated concentrations.

Further similarities in the mode of action of **4** and NM were examined by comparing their abilities to promote cellular ROS production.[14] As shown in Figure 4, the level of cellular ROS production was elevated significantly when cells were incubated with lethal concentrations of **4** or NM, whereas ROS production was not observed when cells were incubated with AmB, even at a concentration of 10 μM. As with NM-treated cells, **4** caused a decrease in total glutathione content (Fig. 5),[15] indicating that glutathione peroxidase is not involved in the elimination of ROS in yeast cells treated with **4**.[16] Similarly, the co-addition of PC protected against ROS production induced by **4** and NM, indicating that the alkyl side chain of NM plays a mechanistic role in ROS production.

In conclusion, *N*-methyl-*N*′-alkyl-guanidine **4** exhibits fungicidal activity by disrupting the plasma membrane, most likely by targeting phosphatidylcholine. Thus, structural requirements for the antifungal activity of NM include the presence of an alkyl side chain with a

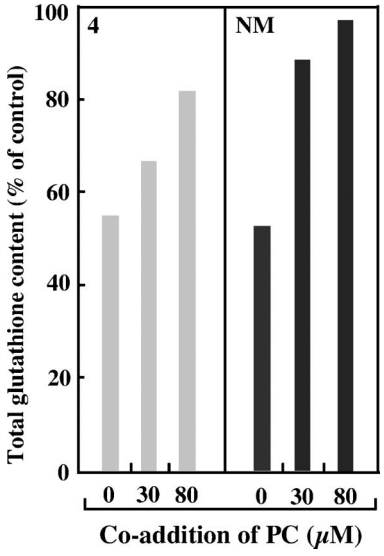


Figure 5. Decrease in total glutathione content in *S. cerevisiae* cells treated with **4** and NM, and the protective effect of PC. Cells were incubated at 30 °C for 60 min in YPD medium containing **4** (100 μm) or NM (10 μm), and PC at the concentrations indicated.

terminal guanidine, since the lactone ring structure of the polyol macrolide compound is analogous to that of AmB. The lower MIC values found for NM compared to those for **4** indicate that the polyol lactone ring augments the alkyl side chain's interaction with plasma membrane phospholipids.

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12. Cells from an overnight culture were harvested by centrifugation, washed with 50 mM succinate buffer (pH 6.0), and suspended in the buffer to give a density of 10^8 cells/ml. The cell suspension was shaken with or without each compound at 30 °C and the supernatant obtained after removing cells by centrifugation was assayed for its content of K^+ ions using a K^+ assay kit (HACH, Floriffoux, Bergium). The amount of UV-absorbing materials was expressed as a value of the absorption at a wavelength of 260 nm in cells of 1 cm light-path.
13. Cells from an overnight culture were inoculated into freshly prepared YPD medium to give an initial cell density of approximately 10^7 cells/ml. Cells were then grown with or without each compound with vigorous shaking and portions were withdrawn at various time intervals to measure the cell growth by the turbidity-dependent assay.
14. After preincubation of the yeast cells (10^7 cells/ml) in YPD medium with 40 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) at 30 °C for 60 min, the cell suspensions (1.0 ml) were withdrawn and further treated with each chemical for the indicated times, washed, and resuspended with 100 μl of phosphate-buffered saline. The fluorescence intensity of the suspension (100 μl) containing 10^7 cells was read using a Cytoflow 2300 fluorescence spectrophotometer with excitation at 480 nm and emission at 530 nm. Arbitrary units were directly given by the fluorescence intensity.
15. Cells from overnight cultures were further incubated in YPD medium (10^7 cells/ml) at 30 °C with or without each compound, collected, washed, resuspended in ice-cold 8 mM HCl containing 1.3% 5-sulfosalicylic acid, and broken with glass beads by repeated vortexing. The supernatant obtained after removing the beads and cell debris by centrifugation (5000*g*) for 15 min at 4 °C was used as the cytosol fraction. Glutathione was measured as the total glutathione content, i.e., GSH and GSSG, by the spectrophotometric monitoring of 2-nitro-5-thiobenzoate formation from 5,5-dithiobis(2-nitrobenzoic acid).
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