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Nylon-3 Polymers with Selective Antifungal Activity

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Abstract

Host-defense peptides inhibit bacterial growth but show little toxicity toward mammalian cells. A variety of synthetic polymers have been reported to mimic this antibacterial selectivity; however, achieving comparable selectivity for fungi is more difficult because these pathogens are eukaryotes. Here, we report nylon-3 polymers based on a novel subunit that display potent antifungal activity (MIC = 3.1 $\mu\text{g}/\text{mL}$ for *C. albicans*) and favorable selectivity (IC₁₀ > 400 $\mu\text{g}/\text{mL}$ for 3T3 fibroblast toxicity; HC₁₀ > 400 $\mu\text{g}/\text{mL}$ for hemolysis).

Natural strategies to fend off microbial infection include production of relatively small peptides that manifest antimicrobial activity, part of the innate immune response.¹ These “host-defense peptides” have diverse sequences and bioactive conformations, and their biological effects appear to arise from multiple mechanisms.² Many host-defense peptides can adopt amphiphilic structures in which lipophilic and hydrophilic (usually cationic) side chains are segregated to distinct regions of the molecular surface.³ This global amphiphilicity is widely believed to underlie the ability of host-defense peptides to compromise bacterial membrane barrier function and thereby inhibit the growth of or kill prokaryotes.⁴ Numerous reports describe synthetic peptides or peptidomimetic oligomers designed to be globally amphiphilic that can serve as tools to elucidate the origins of host-defense peptide function and as candidates for therapeutic application.⁵ The evaluation of synthetic systems has recently expanded to include random copolymers that contain both hydrophilic and lipophilic subunits, which are much more readily prepared than are sequence-specific peptides or other oligomers.⁶

Antimicrobial agents have the highest potential for application when their deleterious effects are specific for microbial cells relative to human cells. Such selectivity has been achieved with a variety of compounds for bacterial growth inhibition vs. human cell destruction;^{6h,6m,7} the latter property is often assessed as lytic activity toward red blood cells (“hemolysis”).^{5e,8} Fundamental differences between prokaryotic and eukaryotic cellular membranes, including lipid composition and external surface charge density, seem to

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B.W. and S.H.G. are co-inventors on a patent application that covers the polymers described here.

Supporting Information

Experimental details for synthesis and characterization of nylon-3 polymers, antifungal and antibacterial assays, cytotoxicity on 3T3 fibroblasts and hemolysis on human RBCs. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

facilitate this selectivity.^{2,8b} In contrast, it is difficult to target fungal pathogens selectively relative to human cells, because fungi are eukaryotes.⁹ For example, many host-defense peptides are not effective inhibitors of fungal growth at physiological ionic strength,¹⁰ and only modest antifungal vs. hemolytic selectivity has been achieved with sequence-specific oligomers.¹¹ Here we describe a new family of nylon-3 polymers (poly- β -peptides) that display significant and selective toxicity toward the most common fungal pathogen among humans, *Candida albicans*.¹²

Nylon-3 materials are readily prepared via ring-opening polymerization of β -lactams,¹³ and we have previously reported that sequence-random co-polymers that contain a lipophilic and a cationic subunit can manifest significant antibacterial activity but low hemolytic activity if the subunit identities, lipophilic-cationic subunit proportion and other parameters are optimized.^{6h,6m,14} The co-polymer shown in Figure 1, for example, displays a particularly favorable antibacterial activity profile.^{6h} However, antifungal activity among previously reported nylon-3 copolymer families proved to be inseparable from hemolytic activity (unpublished). The present studies began with the preparation of a new β -lactam, **NM** (“no methyl”; Figure 2), which provides a cationic subunit at or below neutral pH. We were drawn to this subunit because it contains fewer saturated carbon atoms and therefore should have a lower hydrophobicity than previously examined cationic nylon-3 subunits derived from β -lactams **MM** and **DM** (“monomethyl” and “dimethyl”).^{6m} The synthesis of **NM** (Figure 3) involves cycloaddition of chlorosulfonylisocyanate to an alkene, as in previous cases, but this route differs from the precedents in that the side chain nitrogen is introduced after β -lactam formation.^{6h,13f,15} Although the yield of the iodo- β -lactam is only modest, this potentially versatile molecule can easily be prepared on a multi-gram scale.^{15–16} The β -lactam bearing a Boc-protected amino group in the side chain was readily incorporated into nylon-3 co-polymers via the base-catalyzed process we have previously employed, in which the N-terminal group on each polyamide chain is specified by the choice of polymerization co-initiator.^{13f} All polymers discussed below were prepared with 20-mer average length because previous work indicated that this size range is generally favorable in terms of maximizing antimicrobial activity and minimizing hemolytic activity.^{6m}

The antifungal activity of new **NM**-containing co-polymers (Figure 4) was evaluated with a clinically isolated strain of *C. albicans* (K1).¹⁷ The minimum inhibitory concentration (MIC) was measured using a protocol suggested by the Clinical and Laboratory Standard Institute (previously known as the National Committee for Clinical Laboratory Standard)¹⁸. In order to assess the effects of the new polymers on mammalian cells, we determined the concentration necessary for 10% lysis of human red blood cells (HC₁₀), and the concentration necessary to induce 10% cell death in NIH 3T3 fibroblasts (IC₁₀). Previously we have used the minimum hemolytic concentration (MHC) as a metric of red blood cell disruption, but we shifted to HC₁₀ for the present studies because it was sometimes difficult to identify the lowest polymer concentration that displayed a non-zero extent of hemolysis.^{6h,6m} The fibroblast assays provide an alternative measure, relative to hemolysis, of toxicity toward mammalian cells. Amphotericin B (AmpB), which is used clinically for *C. albicans* infections but associated with high toxicity toward mammalian cells, served as a positive control in these studies.¹⁹ Results are summarized in Table 1.

We began by examining random co-polymers (Figure 4) formed from new β -lactam **NM** and cyclohexyl β -lactam **CH**, because the latter had given rise to selective antibacterial copolymers when paired with the cationic subunit derived from **MM** (Figure 1).^{6h} All of the new polymers bore a *p*-*t*-butylbenzoyl group at the N-terminus, as in previous antibacterial examples. The maximum proportion of the cyclohexyl subunit that could be used without compromising aqueous solubility, 60:40 **CH**:**NM**, led to weak antifungal activity and weak hemolytic activity (MIC and HC₁₀ ~ 100 μ g/mL). Antifungal activity steadily increased

(i.e., MIC decreased) as the proportion of the lipophilic subunit declined, and no co-polymer containing > 50% of the cationic subunit manifested detectable hemolytic activity. Members of this polymer family were generally not toxic toward mouse fibroblasts. The activity levels observed for **CH:NM** co-polymers with 80% of the cationic subunit, on a $\mu\text{g/mL}$ basis, approached that of AmpB, but were accompanied by substantially less fibroblast cytotoxicity than AmpB. Replacing the *p-t*-butylbenzoyl end-group with an acetyl end-group did not alter the biological activity of poly-**NM**. The **NM** homopolymer displayed antifungal activity comparable to that of the most active **CH:NM** copolymers. Follow-up studies showed that poly-**NM** is fungicidal at the MIC, rather than merely inhibitory toward fungal growth.²⁰

The excellent activity profile observed for poly-**NM** contrasts with the behavior observed for two other cationic nylon-3 homopolymers, poly-**MM** and poly-**DM** (Table 1). Poly-**MM** shows very little antifungal activity, and this homopolymer is also not hemolytic or toxic toward 3T3 fibroblasts. Poly-**DM**, on the other hand, approximately matches poly-**NM** in activity against *C. albicans*, but poly-**DM** is hemolytic and moderately toxic toward 3T3 fibroblasts.

Poly-**NM** was evaluated for antibacterial activity against a panel of four species that we have previously used to assess poly-**MM** and poly-**DM** as well as cationic-hydrophobic copolymers (Table 2).^{6m} The antibacterial effects of poly-**NM** were generally comparable to those of the other two cationic nylon-3 homopolymers: significant activity was observed for *Bacillus subtilis*, which seems to be highly susceptible to a wide array of peptides and peptidomimetic oligomers and polymers, but all three homopolymers were considerably less active against *Escherichia coli*, *Enterococcus faecium* and *Staphylococcus aureus*. The generally low antibacterial activity of poly-**MM** and poly-**DM** has previously been rationalized in terms of their lack of hydrophobic subunits (e.g., the subunit derived from **CH**), which may limit their ability to disrupt bacterial membranes.^{6m,14} From this perspective, the relatively low antibacterial activity of poly-**NM** is not surprising. The potent antifungal activity of poly-**NM** is noteworthy in the context of this limited antibacterial activity.

The data we have presented show that nylon-3 polymers containing subunits derived from the new β -lactam **NM** display potent antifungal activity without a strong tendency to disrupt human red blood cell membranes or strong toxicity toward 3T3 fibroblasts. It is particularly intriguing that poly-**NM** displays such profound differences in biological activity relative to the structurally similar cationic nylon-3 homopolymers poly-**MM** and poly-**DM**. There are several differences among the subunits of these three polymers: (1) the added side-chain carbons in poly-**MM** and poly-**DM** relative to poly-**NM** cause a modest increase in hydrophobicity;²⁰ (2) the added carbons alter backbone flexibility; (3) the point of attachment of the aminomethyl side chain in **NM** differs from that in **MM** and **DM** (α -carbon vs. β -carbon). Further studies will be necessary to determine the mechanism by which these seemingly subtle molecular-level changes exert such a substantial influence on biological activity. We have previously proposed that nylon-3 copolymers exert antibacterial effects via disruption of prokaryotic cell membranes, and this hypothesis has been supported by studies of the 40:60 **CH:MM** co-polymer (Figure 1) with synthetic vesicles of varying lipid composition.¹⁴ However, our finding that maximal antifungal activity is manifested by poly-**NM**, the least hydrophobic nylon-3 polymer we have examined to date, raises the possibility that **NM**-containing polymers act via a mechanism that does not involve disturbance of lipid bilayers. The surprising biological activity profile discovered for **NM**-based nylon-3 suggests that antifungal applications of these new materials be pursued.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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20. Please see the supporting information.

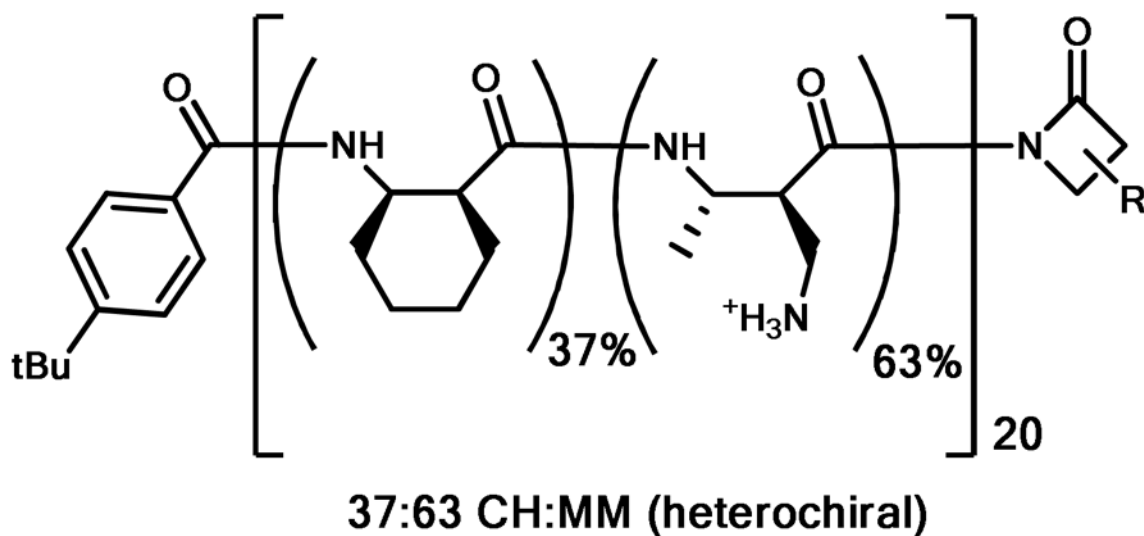


Figure 1. Representative sequence- and stereo-random nylon-3 co-polymer containing subunits derived from racemic *cis*-cyclohexyl β -lactam (**CH**) and racemic monomethyl aminomethyl β -lactam (**MM**). R represents the side chain group for either **CH** or **MM**. This co-polymer inhibits the growth of several bacterial species at relatively low concentrations but is only weakly hemolytic.^{6h}

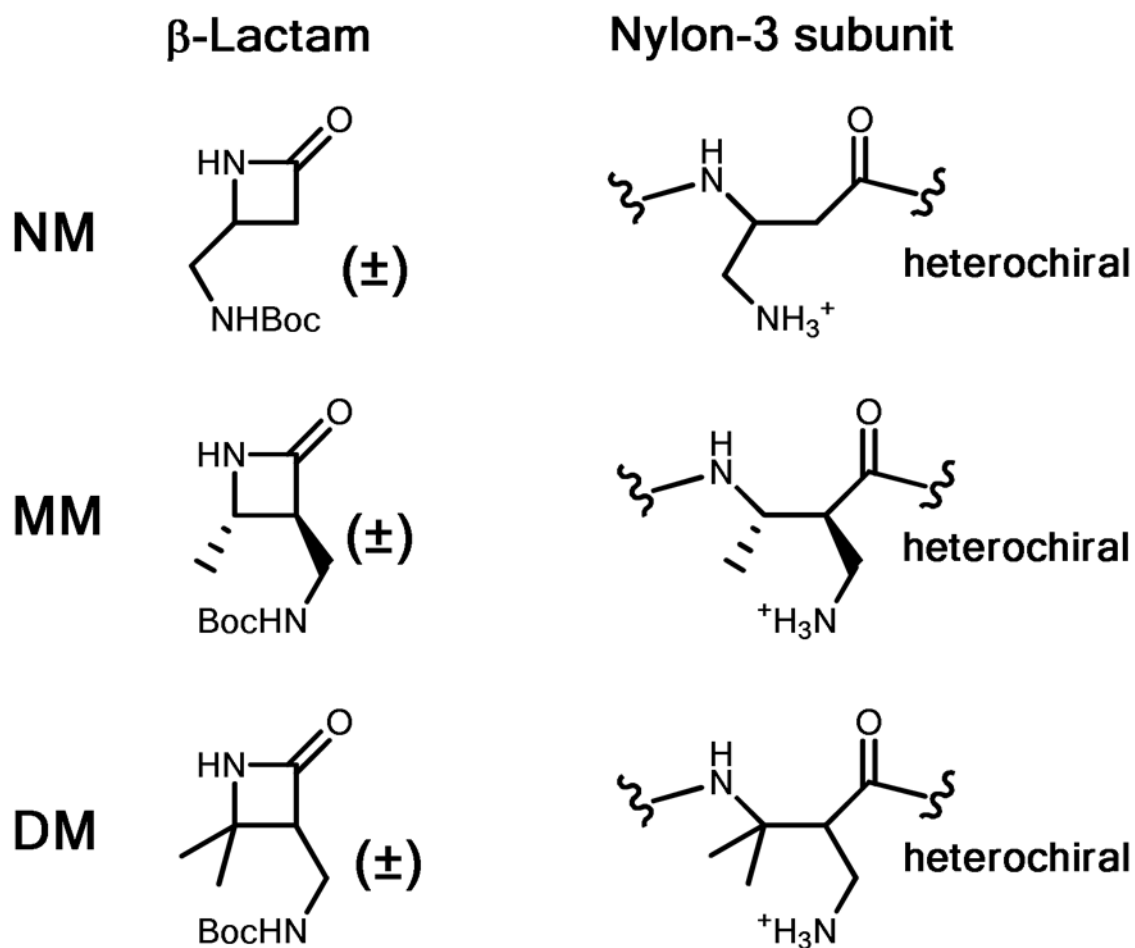


Figure 2. β -Lactams and corresponding hydrophilic (cationic) subunits within the nylon-3 polymer chain. All β -Lactams are racemic, and the resulting polymers are heterochiral.

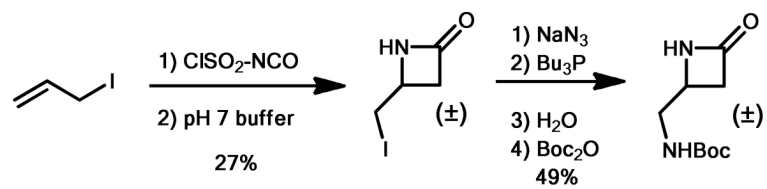


Figure 3.
The synthesis of racemic β-lactam **NM**.

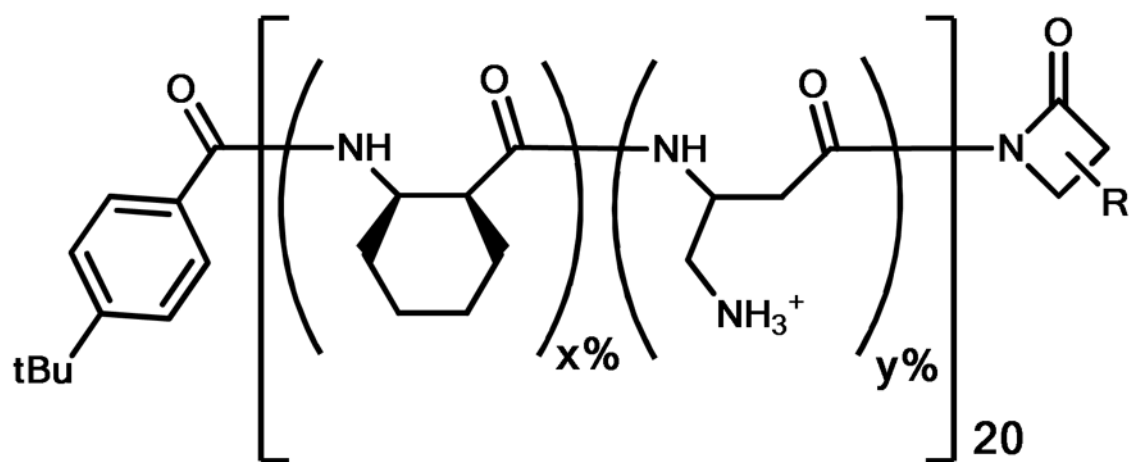


Figure 4.

The structure of **CH:NM** co-polymers. All copolymers are heterochiral and sequence-random. $x + y = 100$, $y = 40, 50, 60, 70, 80, \text{ or } 90$. R represents the side chain group of either **CH** or **NM**.

Table 1

Physical and biological properties of nylon-3 polymers

polymer composition	DP ^a	PDI ^b	MIC, µg/mL ^c	IC ₁₀ , µg/mL ^d	HC ₁₀ , µg/mL ^e
60:40 CH:NM	23	1.29	100	> 400	100–200
50:50 CH:NM	23	1.29	50	> 400	200
40:60 CH:NM	21	1.29	13	> 400	> 400
30:70 CH:NM	20	1.26	6.3	> 400	> 400
20:80 CH:NM	22	1.33	3.1	100–200	> 400
10:90 CH:NM	17	1.24	3.1	> 400	> 400
NM	20	1.13	3.1	> 400	> 400
MM	22	1.03	200	> 400	> 400
DM	18	1.13	6.3	50	3.1
Ampho. B ^f	N/A	N/A	0.78	< 1.5	ND

^aDP (degree of polymerization) indicates average polymer length (number of subunits).^bPDI is polydispersity index.^cMIC indicates the minimum inhibitory concentration for fungal growth as determined for *C. albicans* in the planktonic form.^dIC₁₀ indicates the concentration necessary to induce 10% cell death in NIH 3T3 fibroblasts.^eHC₁₀ indicates the concentration necessary for 10% lysis of human red blood cell.^fAmphotericin B was dissolved in 1:1 DMSO:water as the stock solution for bioassay. ND indicates hemolysis data were not obtained. All polymers have an N-terminal p-tbutylbenzoyl group.

Table 2

Antibacterial activities of cationic nylon-3 homopolymers

polymer	MIC, ^a $\mu\text{g/mL}$			
	<i>E. coli</i>	<i>B. subtilis</i>	<i>E. faecium</i>	<i>S. aureus</i>
NM	50	6.3	> 200	100
MM	> 200	6.3	> 200	100
DM	100	3.1	100	50

^aMIC is the minimum inhibitory concentration for bacterial growth.