Helical Foldamers

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Consequences of Isostructural Main-Chain Modifications for the Design of Antimicrobial Foldamers: Helical Mimics of Host-Defense Peptides Based on a Heterogeneous Amide/Urea Backbone**

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The design of unnatural oligomers with predictable folding patterns (i.e. foldamers) and function has attracted considerable attention over the last ten years.[1] Applications in biology include the development of antimicrobial agents, cell-penetrating agents, and inhibitors of protein–protein interactions.[1a,2] Non-natural oligoamides built from α-amino acid residues (e.g. peptoids) and higher homologues (e.g. β-, γ-, and δ-peptides) are the epitomes of peptidomimetic foldamers.[1,2] A substantial increase in the number of backbones with folding propensity came from the exploration of the β- and γ-peptide families (i.e. by introduction of isosteric or isoelectronic backbone modifications).[1d] The substitution of a urea moiety for the CH_2-CO-NH units in the γ^4-peptide backbone is an interesting case of quasi-isostructural replacement.[3] High-resolution structural studies in solution[3] and in the solid state[4] have shown that the oligourea backbone adopts a remarkably stable helical fold reminiscent of the 14-helix described for the cognate γ-peptides (Figure 1).[5,6]

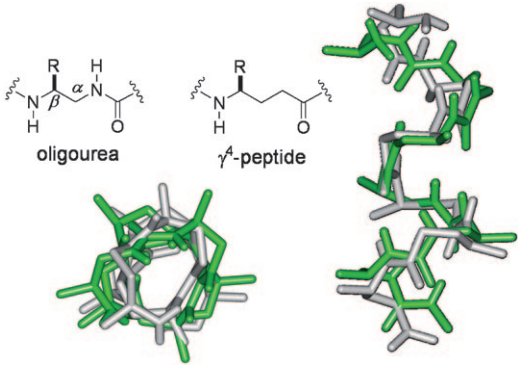

Figure 1. Superimposition of helical structures formed by oligoureas (green)[4] and γ^4-peptides (gray).[5]

How such quasi-isostructural backbones[6] compare in molecular recognition is presently unknown. We reported previously that oligoureas designed to mimic globally amphiphilic α-helical host-defense peptides display broad antibacterial activity with selectivity for prokaryotic versus mammalian red blood cell membranes.[7] To better understand structure–property relationships and to gain insight into the mechanisms of membrane disruption, we have now undertaken detailed comparative studies of oligoureas, their γ-peptide counterparts, and various mixed amide/urea congeners (**1**–**8**) bearing identical side chains (Scheme 1).

Oligourea **1** was found previously to display both 1) a strong helix propensity in a lipidic environment, and 2) significant antibacterial activity against Gram negative and Gram positive bacteria.[7] However, further development of **1** was limited by synthetic hurdles. Stepwise elongation of **1** on a Rink resin using monomers protected with *N*-fluorenylmethoxycarbonyl (Fmoc) groups[3e,7,8] resulted in poor overall yields. The purity of crude product based on C_{18} reverse-phase HPLC was only 17%.[9] Although chemistry using building blocks protected with the *N*-*tert*-butoxycarbonyl (Boc) group is more robust, it was found that the urea linkage formed by anchoring the first residue on a 4-methylbenzhydrylamine

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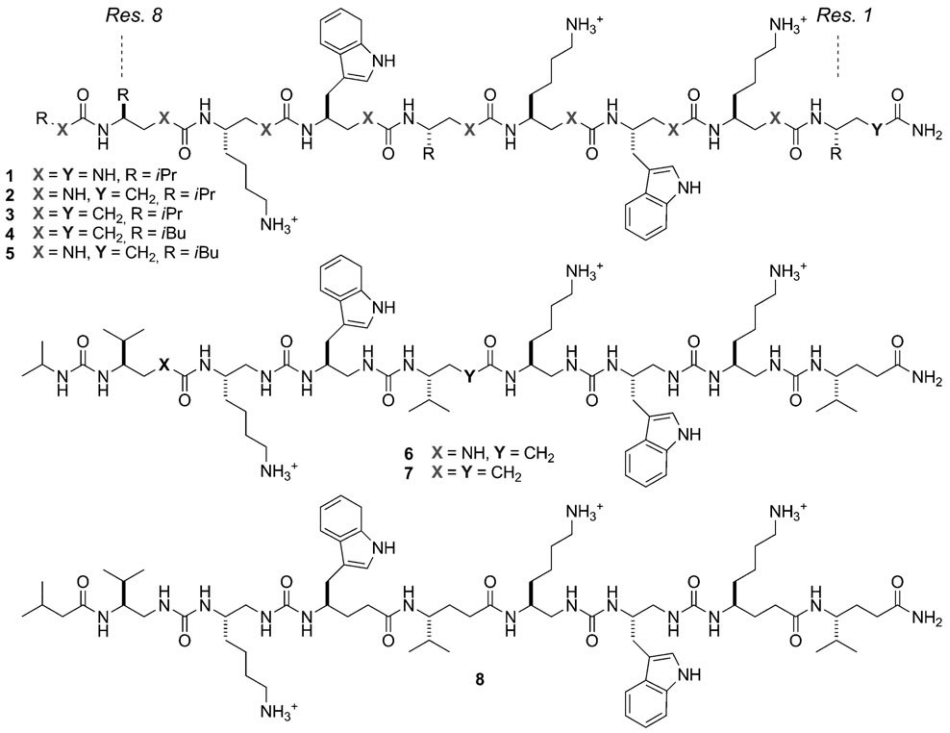

Scheme 1. Globally amphiphilic oligoureas, γ^4-peptides, and related hybrids **1**–**8**.

(MBHA) resin or the less acid-sensitive benzhydrylamine (BHA) resin does not resist the acidic conditions (trifluoroacetic acid or 3 N HCl in dioxane) required to cleave the Boc group. To overcome this difficulty, we envisaged the preparation of oligoureas bearing a stable terminal amide group by direct attachment of an isosteric γ^4-amino acid on MBHA resin. This strategy, together with the use of *N*-Boc-protected monomers, proved to be very effective, with purities of crude **2** routinely over 60% (Figure S1 in the Supporting Information).[9] It is noteworthy that neither 2.5-helix propensity[3d] (Figure S2 and S3 in the Supporting Information)[9] nor antibacterial activity were affected by this point mutation. The minimum inhibitory concentrations (MICs) obtained for **2** were very similar to those found for melittin, a honeybee toxin, and omiganan,[10] a cationic peptide currently in phase III clinical trials for the prevention of catheter-related bloodstream infections (Table 1). Whereas omiganan exhibits weak bactericidal activity (MBCs $> 256\ \mu g\,mL^{-1}$), the minimum bactericidal concentrations (MBCs) measured for **2** were equal to or twice as large as corresponding MICs. Moreover, **2** was found to be equally potent on methicillin-sensitive (ATCC 25923) and methicillin-resistant (clinical IBS 45-501) *S. aureus* strains.

To determine whether the urea backbone is critical for antibacterial activity, we synthesized **3**, the γ^4-peptide analogue of **2**. Antibacterial activity of γ-peptide **3** was determined against *E. coli*, *P. aeruginosa*, and *S. aureus*. γ-Peptide **3**, although intrinsically less polar than **2**, was virtually inactive (MIC and MBC $> 256\ \mu g\,mL^{-1}$) on both Gram positive and Gram negative bacteria. From studies with antibacterial α- and β-peptides,[11] we reasoned that increasing the hydrophobic content of **3** could increase its potency, albeit at the cost of mammalian versus bacterial cell selectivity. However, substituting *i*Bu for *i*Pr side chains in **3** (to give **4**) led only to a modest improvement (MIC $= 128\ \mu g\,mL^{-1}$ on the three bacterial strains). In contrast, the same modification on **2** gave the most potent antibacterial oligourea (**5**) reported to date with MIC and MBC values as low as 8 and 32 $\mu g\,mL^{-1}$ on *S. aureus*, respectively.

We next examined the susceptibility of host cell membranes to oligoureas and γ-peptides by monitoring lysis of sheep red blood cells. Oligourea **2** ($HD_{100} = 256\ \mu g\,mL^{-1}$) compared favorably with melittin, which is strongly toxic towards red blood cells ($HD_{100} = 32\ \mu g\,mL^{-1}$). γ^4-Peptides **3** and **4** display the highest HD_{100} values.

With the aim to investigate further the effects of insertion of γ^4-amino acid residues on 2.5-helix propensity and antimicrobial activity, we prepared mixed oligourea/γ^4-peptides **6**–**8** containing two to four γ^4-amino acid residues. Information about the conformational preferences of oligomers **6**–**8** was gained by monitoring chemical shift differences ($\Delta\delta$) in the ^{1}H NMR spectra between diastereotopic $^{\alpha}$CH protons of "diaminoethylene" residues (Figures S4–S6 and Table S1 in the Supporting Information)[9] and by circular dichroism (CD) spectroscopy (Figure S7 in the Supporting Information),[9] as described previously.[3] Comparison with oligomer **2** revealed several trends. The insertion of an

Table 1: Antibacterial and haemolytic activities of compounds **2**–**8** compared to melittin and omiganan.[a]

Compound	Bacteria						HD_{100}[b]
	E. coli (ATCC 25922)		*P. aeruginosa* (ATCC 27853)		*S. aureus* (ATCC 25923)		
	MIC	MBC	MIC	MBC	MIC	MBC	
omiganan	64	>256	128	>256	32	>256	nt[c]
melittin	64	64	128	256	32	64	32
2	32	32	64	128	32[d]	64[d]	256
3	>256	>256	256	>256	>256	>256	>512
4	128	>256	128	>256	128	>256	>512
5	16	32	16	64	8	32	64
6	64	128	32	128	16	64	512
7	32	128	64	128	32	128	nt
8	128	>256	128	256	128	>256	nt

[a] MIC is the minimum inhibitory concentration and MBC the minimum bactericidal concentration. MIC, MBC, and haemolysis in $\mu g\,mL^{-1}$. [b] HD_{100} values are concentrations required to lyse 100% of the cells after 48 h. [c] nt = not tested. [d] Identical MIC and MBC values were obtained on a clinical isolated methicillin-resistant *S. aureus* strain (IBS 45-501).

additional γ^4-amino residue at positions P5 and P8 has a local destabilizing effect on the preceding residues (i.e. residues at P4 and P7), but it does not seem to alter the overall helical conformation. In contrast, the insertion of two γ^4-amino acid residue pairs such as in **8** has a more pronounced effect on the overall structure, with significantly reduced $\Delta\delta$ values. CD spectroscopy experiments show that the positive molar ellipticity at 203 nm per urea moiety decreases significantly as a result of γ^4-amino acid insertion, which may be interpreted as 2.5-helix destabilization.[9,12] Similarly, antibacterial activity was found to decrease with the number of γ-amino acid residues in the backbone. However, octamer **6**, with a central γ^4-Val residue, compares favorably with **2** in term of both bactericidal activity and cytotoxicity against mammalian cells. This increased selectivity makes mixed amide/urea oligomer **6** an interesting lead compound for future studies.

Oligomers **6**–**8** were also evaluated against three ATCC isolates of *Candida*, namely *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. albicans* ATCC 90028.[13] Again, oligourea **6** was the most active compound in the series, with MICs of 16–64 $\mu g\,mL^{-1}$ and minimal fungicidal concentrations (MFCs) against all three *Candida* isolates either equal to or twice as large as the MICs (Table S2 in the Supporting Information).[9]

Evidence for a direct interaction between oligoureas and bacterial membranes was gained from immunoelectron microscopy experiments using postembedding immunogold labeling experiments.[9] The ultrastructural localization of the biotinylated analogue of **2** (biot-**2**) on Gram negative bacteria (*E. coli*) reveals the presence of small and well-defined clusters in the vicinity of the inner and outer bacterial membranes (Figure 2). These results may suggest that oligoureas either alone or as aggregates are able to cross bacterial membranes.

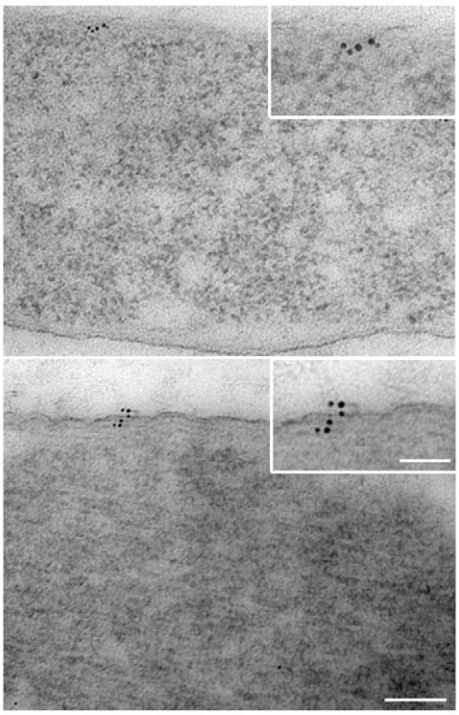

Figure 2. Immunoelectron microscopy (biotin, 6 nm) of biot-**2** on *E. coli* after high-pressure freezing and freeze-substitution. Scale bar 50 nm; inset 10 nm.

To determine whether the functional difference between oligourea and oligoamide backbones results from differential membrane disruption activities, we have undertaken physicochemical investigations using negatively charged phospholipid membranes as model systems. Both oligourea **2** and γ^4-peptide **3** carry indole side chains at positions 3 and 6 in their sequence, thus endowing the molecules with intrinsic fluorescence. We first studied the interaction of **2** and **3** with large unilamellar vesicles (LUV) of egg yolk phosphatidylcholine (EYPC) and egg yolk phosphatidylglycerol (EYPG) in a 70:30 molar ratio by measuring indole emission fluorescence and steady-state fluorescence anisotropy upon addition of an increasing concentration of phospholipids. At a lipid-to-oligomer molar ratio $R_i = 20$, shifts to lower wavelength (blue shift) of the indole emission peak (λ_{max}) of 23 nm and 19 nm were observed for **2** and **3**, respectively (Figure S8 in the Supporting Information),[9] thus indicating significant penetration of the indole moieties into the hydrophobic lipid bilayer. The restricted mobility of the indole moiety upon interaction with the membrane was also inferred from an increase in fluorescence anisotropy. Titrations of **2** and **3** with EYPC/EYPG LUVs (Figure S9 in the Supporting Information) were used to quantify the binding of both oligomers to vesicles.[9] The apparent dissociation constants ($1.4\times10^{-7}\,mol\,L^{-1}$ for **2** and $9\times10^{-7}\,mol\,L^{-1}$ for **3**) calculated by curve fitting using the binding model given in Equation (13) in the Supporting Information indicate that γ-peptide **3** has a sixfold lower affinity for the membrane than oligourea **2**.

To assess the extent to which peptides **2** and **3** disrupt phospholipid membranes, we measured their abilities to induce efflux of carboxyfluorescein from EYPC/EYPG (70:30 molar ratio) LUVs. The time course of the leakage of encapsulated carboxyfluorescein was detected by the corresponding increase in fluorescence intensity at 518 nm (Figure 3). Whereas γ-peptide **3** was not able to induce significant efflux of carboxyfluorescein at 4.16 μM even after prolonged time, very fast and strong carboxyfluorescein release was observed when LUVs were treated with **2** at the same concentration. This series of experiments further confirms the different membrane-disruption properties exhibited by oligoamide and oligourea backbones.

A number of recent studies have shown that conformational preorganization is not necessarily a prerequisite to design oligomers with antimicrobial activity.[14,15] Thus, it is unlikely that the difference in helix stability between oligoamides and oligoureas alone accounts for the observed dichotomy in membrane interaction and antibacterial activities. Instead, helix geometry (side-chain projection) and backbone polarity are two factors that vary substantially between the two oligomeric systems and that need to be considered. Moreover, it is tempting to speculate that structural features of helical oligoureas revealed by X-ray diffraction studies,[4] that is, conformational adaptability (helix plasticity) and directional aggregation, may have

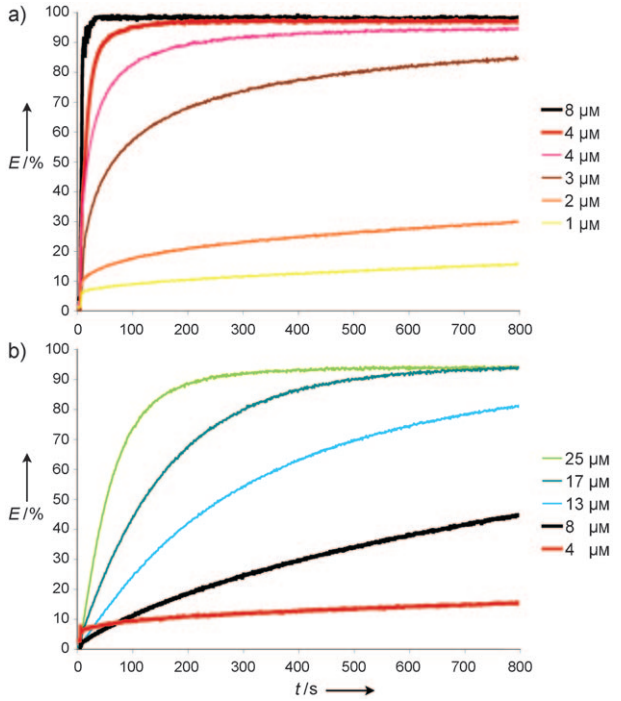

Figure 3. Time course of carboxyfluorescein efflux from LUV EYPC/EYPG (70:30 molar ratio) upon addition of increasing concentrations of a) **2** and b) **3**.

some relevance to the unique antimicrobial activities of oligoureas and related hybrids.

In conclusion, we have documented a case of dichotomy in the foldamer γ-peptide lineage whereby two apparently isostructural helical backbones (i.e. oligoamide and oligourea) bearing identical side chains appear to strongly differ in their antibacterial and biomolecular recognition properties. Our results also point to heterogeneous helical urea/amide backbones, which may become advantageous in the development of more potent yet less cytotoxic antimicrobial helical foldamers for in vivo applications.[16]

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Keywords: amphiphiles · antimicrobial agents · helical structures · peptidomimetics · structure–activity relationships

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