A Minimalist Design Approach to Antimicrobial Agents Based on a Thionin Template

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Abstract: Numerous studies have been devoted to the stabilization of secondary structure elements to improve receptor−ligand recognition. We report a novel application of this principle to create new antimicrobial agents using the highly folded thionin from *Pyrularia pubera* as a template. Non-native disulfide bonds have been used to induce two short linear segments of the protein into an amphipathic helix. The resulting 13- and 9-residue peptides are significantly more active than their linear counterparts and have an activity similar to that of native thionin.

Stabilization of secondary structure has been widely used in medicinal chemistry for the de novo design of peptide molecules with desired structural properties and activity.[1,2] Major attention has been focused on helices as they play a crucial role in protein folding and protein−protein interactions. In particular, conformational restriction of helical peptide ligands was successfully used to improve the affinity for protein receptors.[3] Several approaches for the stabilization of helical structures using natural[4,5] and non-natural amino acid substitutions and metal complexation[6,7] have been reported.[8−10] Covalent side chain cross-linking has also been used to lock helical structures through amide,[11,12] hydrazone,[13] all-hydrocarbon,[3,14] or disulfide bonds.[15,16]

In this paper we describe how potent antimicrobial drug candidates with simple peptide structures can be evolved from a complex, highly folded plant peptide by a structural minimization and conformational constraint. Growing interest on antimicrobial peptides (AMPs) as potential sources of anti-infective drug leads[17,18] is driven by the fact that emergence of resistance for AMPs is demonstrably lower than for classical antibiotics.[19] This is because AMP mechanisms, mostly involving membrane perturbation,[20] can only be effectively subverted by the coordinated action of not a small number of mutations, an unlikely event. The amphipathic α-helix, characteristic of many membrane active AMPs,[21−23] is one of the most extensively studied helical motifs, and a large number of studies were devoted to elucidate its role and the roles of other structural features on AMP activity.[19,24] However, prediction of activity for de novo designed sequences is not trivial, since requirements for activity (charge, helicity, amphipathic character, etc.) are strongly and complexly interrelated; hence, natural peptides are still considered ideal templates to develop effective, noncytotoxic, and affordable drug candidates.

We recently reported[25] a structurally guided dissection of the antimicrobial thionin from *Pyrularia pubera* (PpTH)[26] and showed that its antiparallel double helix core (residues 7−32) is the key feature for antimicrobial activity. Interestingly, while the two short helical segments (residues 7−19 and 24−32) that make up this core are highly cationic and amphipathic in the native structure, the attempt to mimic them as simple linear sequences produced inactive peptides devoid of structural organization and unable to bind model membranes.[25] We hypothesized that if those linear sequences could be induced into native-like helical conformation by appropriate structural restriction, membrane binding and thus antimicrobial potency might be regained.

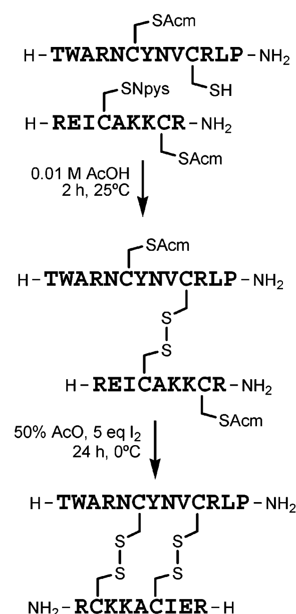

Figure 1. Synthetic route to heterodimeric peptide TH(7−19)-(24−32R). Npys-mediated formation of the first disulfide is followed by regioselective iodine-promoted deprotection and oxidation under mild conditions.

Table 1. Synthesized Peptides

peptide[a]	sequence	[M + H]$^{+}$ [b] calcd	[M + H]$^{+}$ [b] exptl
TH (7−19)(24−32R[c])	TWARNC[12]YNVC[16]RLP + REIC[27]AKKC[31]	2697.25	2697.85[a]
TH(7−19)Abu	TWARN-Abu-YNV-Abu-RLP	1558.82	1559.09
TH(7−19)cC	TWARNcYNVCRLP	1592.76	1592.79
TH(7−19)cH	TWARNcYNV-Hcy-RLP	1606.73	1606.97
TH(24−32R[c])Abu	REI-Abu-AKK-Abu-R	1069.71	1069.34
TH(24−32R[c])cC	REIcAKKCR	1103.58	1103.76
TH(24−32R[c])cH	REIcAKK-Hcy-R	1117.60	1117.65

[a] All peptides were prepared by standard Fmoc solid-phase synthesis methods.[33] Disulfides were formed by air oxidation except TH(7−19)-(24−32R) (see text for details). [b] Average [M + H]$^{+}$ values for TH (7−19)(24−32R); monoisotopic for all other peptides. For single intramolecular disulfide peptides, the mass of the oxidized product is given. [c] R indicates the replacement of the Asp residue at position 32 by Arg, a modification previously shown to expand the antimicrobial spectrum to include Gram-negative bacteria.[25]

A prerequisite for the above direction was to show that the double helical motif of thionin could be replicated as a functional entity. We therefore designed a chimeric construct, the heterodimeric TH(7−19)(24−32R) peptide, in which the disulfide bonds linking the two helical fragments in the native structure were preserved. This TH(7−19)(24−32R) peptide was obtained (Figure 1) by stepwise, directed disulfide formation. In the first step, the TH(7−19)Cys12(Acm) peptide, with a free Cys residue, reacted with the Cys-activated TH(24−32R)Cys27-(Npys)Cys31(Acm) to give the Cys16,27 disulfide.[27,28] The

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Table 2. Inhibition of Bacterial and Fungal Pathogen Growth by Native Thionin and Derived Peptides

no.	peptide	EC_{50} (μM)[d] Gram-positive *C. michiganensis*	Gram-negative *R. meliloti*	Gram-negative *X. campestris*	fungi *B. cinerea*	fungi *P. cucumerina*
1	native thionin	0.30 ± 0.04	>20 ± 0	3.65 ± 0.58	0.32 ± 0.19	0.36 ± 0.01
2	TH32R[a,b]	0.37 ± 0.00	0.8 ± 0	0.3 ± 0.00	0.80 ± 0.00	0.36 ± 0.00
3	TH(7−32R[a])[b]	0.80 ± 0.04	4 ± 0	4.60 ± 0.37	0.80 ± 0.19	7.5 ± 0.00
4	TH(7−19)(24−32R[a])	0.03 ± 0.00	2.07 ± 1.2	1.03 ± 0.04	0.08 ± 0.0	0.16 ± 0.03
5	TH(7−19)[b,c]	>50 ± 0.00	>50 ± 0	>50 ± 0.00	5.50 ± 0.00	29 ± 5.72
6	TH(7−19)Abu	3.27 ± 1.25	>50 ± 0	>50 ± 0.00	1.60 ± 0.00	3.97 ± 0.12
7	TH(7−19)cC	0.51 ± 0.12	>50 ± 0	29.33 ± 5.73	0.20 ± 0.00	2.77 ± 0.21
8	TH(7−19)cH	0.74 ± 0.14	>50 ± 0	18.33 ± 0.47	0.19 ± 0.01	3.50 ± 0.00
9	TH (24−32R[a])[b]	21 ± 2.55	>50 ± 0	>50 ± 0.00	3.50 ± 0.35	20 ± 0.00
10	TH (24−32R[a])Abu	5.13 ± 0.11	>50 ± 0	>50 ± 0.00	4.10 ± 0.25	15.67 ± 1.78
11	TH (24−32R[a])cC	0.90 ± 0.00	>50 ± 0	>50 ± 0.00	0.55 ± 0.24	0.80 ± 0.00
12	TH (24−32R)cH	0.90 ± 0.00	>50 ± 0	>50 ± 0.00	0.40 ± 0.07	0.78 ± 0.00

[a] These peptides include the Arg-for-Asp replacement at residue 32 (see Table 1, footnote c). [b] Synthesis and antimicrobial activity as previously reported.[25] [c] The two native Cys residues 12 and 16 mutated to Ser. [d] Effective concentration for 50% of growth inhibition (mean of three experiments ± SD).

second disulfide was regioselectively generated by iodine-mediated Cys(Acm) cleavage and oxidation. Careful optimization of reaction conditions[25,27,29] at this step was necessary to minimize unexpected oxidative cleavage at the C-terminus of Trp (see Supporting Information); the improved protocol used 15 μM peptide in 50% AcOH, with 2.5 equiv of I_2 per Cys(Acm) group at 0 °C in complete darkness. As hypothesized, TH(7−19)(24−32R) was an active peptide with antimicrobial potency superior to full length PpTH (Table 2, entries 1 and 4) against representative plant pathogens. This proved that thionin could be used as a template from which active, structurally simplified analogues could be evolved.

Once the proof of principle was satisfactorily established, we moved on to the design of even simpler peptides by conformational restriction of the 7−19 or the 24−32 segments into amphipathic helices (see Table 1 for a complete list of all designed and synthesized peptides). Since the Cys residues in the 7−19 and 24−32 helical regions of native PpTH are in a i, $i + 4$ disposition, we created internal disulfide linkages to favor the helical conformation. MD simulations of the TH(7−19) and TH(24−32) thionin fragments were run for 1 ns using the MOLARIS software to assess the compatibility of the amphipathic helix with the i, $i + 4$ internal disulfide. Results showed that a D-Cys^{i}-L-Cys^{i+4} but not a L-Cys^{i}-L-Cys^{i+4} bridge was compatible with a helical structure (Figures 2 and 3), as shown earlier for i, $i + 3$ disulfides.[30]

Accordingly, an L-to-D Cys mutation was included in all new thionin analogues. To further evaluate the contribution of the disulfide linkage to the conformational restriction and activity of the cyclic peptides, analogues TH(7−19)Abu and TH(24−32R)Abu (Table 1) were prepared with both Cys residues mutated to aminobutyric acid (Abu), an isoster of disulfide-bridged cysteines[31,32] with no constraining effects. Finally, to explore the effect of the Cys side chain length on the disulfide-stabilized helices, two peptides with Cys-to-Hcy replacement, TH(7−19)cH and TH(24−32R)cH (Table 1), were also made (see Supporting Information for synthesis details).

An antimicrobial assay of the peptides against a panel of representative plant pathogens (three bacteria and two fungi, Table 2) satisfactorily confirmed our hypothesis; i.e., all disulfide-containing peptides evolved from PpTH(7−19) and PpTH(24−32) fragments had significant antimicrobial activity. Thus, the conformationally constrained cyclic disulfide TH(7−19)cC (Table 2, entry 7) was equipotent to PpTH against *C. michiganensis*, a typical Gram-positive species, and against the fungi *B. cinerea*, despite the drastic (>70%) reduction in size vs the parent PpTH. The inactivity of this peptide against Gram-negative *R. meliloti* and *X. campestris* (Table 2) was not unexpected and parallels that of native PpTH. The similarly restricted analogue, TH(24−32R)cC (entry 11, Table 2), with a >80% size reduction over the parent structure, exhibited comparable potency and spectrum. Likewise, the presence of an additional CH_2 in the disulfide ring preserved the enhanced activity of the two Hcy-containing analogues (entries 8 and 12, Table 2). It also induced a comparable tendency to become

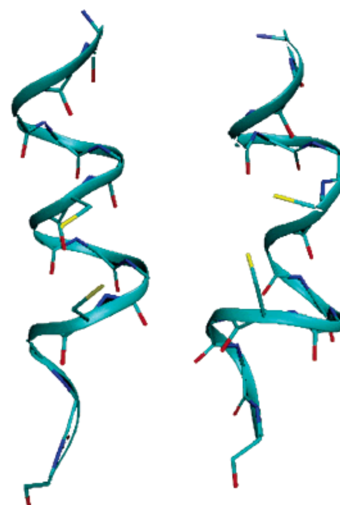

Figure 2. Backbone structure of peptides TH(7−19)cC (left) and TH(7−19)CC (right), both containing an internal disulfide bond, after 1 ns of molecular dynamics simulation. c and C denote D- and L-Cys, respectively. Results show that the helical structure for TH(7−19)cC is almost preserved while that of TH(7−19)CC is highly distorted.

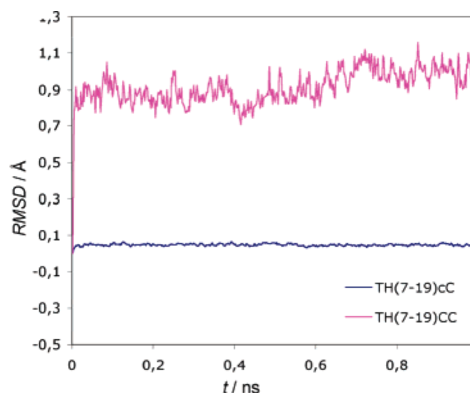

Figure 3. Plot of backbone rmsd vs time for TH(7−19) based helices. Results show that TH(7−19)CC quickly deviates from the original helical structure forced by the disulfide, while for TH(7−19)cC the D-Cys-L-Cys disulfide is compatible with the preservation of the initial structure.

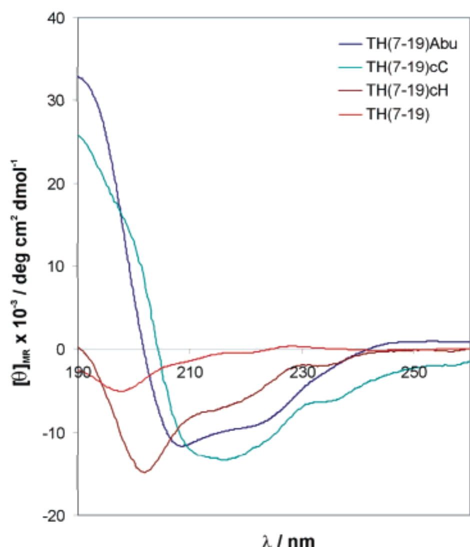

Figure 4. CD spectra of TH(7−19), TH(7−19)Abu, TH(7−19)cC, and TH(7−19)cH at 30 μM in 25 mM phosphate, pH 6.0, in the presence of DMPG liposomes (peptide/lipid is 1:100). Disulfide- and Abu-containing peptides display characteristic helical signatures, while the linear TH(7−19) (Cys residues mutated to Ser) is a completely random coil.

structured in the presence of artificial membranes, though with different CD profiles than the all-Cys analogues (see below and Supporting Information).

CD analysis was performed to assess the conformational effects of the structural modifications introduced. For instance, TH(7−19)cC (Figure 4) illustrates the tendency to adopt substantial levels of helical structure upon binding to negatively charged model membranes, suggesting that the internal cyclization restricts the conformational repertoire to a narrow set of membrane-active (i.e., amphipathic), native-like populations.

For the cyclic analogue TH(24−32)cC, correlation between activity and a helical CD signature (see Supporting Information) was less obvious probably because with nine residues it is not large enough to promote sizable levels of structure. In any event, the decisive influence of the cyclic restriction on activity is substantiated by the average 10-fold drop in antimicrobial potency of both Abu-containing analogues (Table 2, entries 6 and 10) relative to their disulfide counterparts, despite the similar levels of helicity achieved upon binding to DMPG liposomes (Figure 4). A plausible interpretation of this different behavior could be that, while both peptides are able to adopt helical structures, the limited conformational freedom of the disulfide-restricted peptides results in energetically favorable access to bioactive membrane-binding structures and ensuing killing effects.

In conclusion, internal i, $i + 4$ disulfide linking is a powerful tool to stabilize membrane-active helical conformations and, in our particular case, a successful route to the smallest, highly active antimicrobial peptides thus far derived from a complex, highly folded natural peptide. This approach should be of general applicability, and its relevance to other AMP structures is currently under study.

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Supporting Information Available: Experimental details including synthesis of disulfide bond containing peptides and their characterization; table of purity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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