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Date:
2017-01-05

Citation:
Li, W., O'Brien-Simpson, N. M., Yao, S., Tailhades, J., Reynolds, E. C., Dawson, R. M., Otvos, L., Hossain, M. A., Separovic, F. & Wade, J. D. (2017). C-Terminal Modification and Multimerization Increase the Efficacy of a Proline-Rich Antimicrobial Peptide. Chemistry A European Journal, 23 (2), pp.390-396. https://doi.org/10.1002/chem.201604172.

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To be cited as: 10.1002/chem.201604172

Link to VoR: http://dx.doi.org/10.1002/chem.201604172

C-terminal modification and multimerization increase the efficacy of a proline-rich antimicrobial peptide

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Abstract: Two series of branched tetramers of the proline-rich antimicrobial peptide, Chex1-Arg20, were prepared to improve antibacterial selectivity and potency against a panel of Gram negative nosocomial pathogens including *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*. First, tetramerization was achieved *via* dithiomaleimide (DTM) conjugation of two C-terminal-cysteine bearing dimers that also incorporated *C*-terminal peptide chemical modification. DTM-linked tetrameric peptides containing a *C*-terminal hydrazide moiety on each monomer exhibited highly potent activities in the minimum inhibition concentration (MIC) range of 0.49-2.33 µM. A second series of tetrameric analogues with C-terminal hydrazide modification was prepared using alternative peptide linkers including *trans*-1,4-dibromo-2-butene, α,α′-dibromo-*p*-xylene, or 6-bismaleimidohexane to determine the effect of length on activity. Each displayed potent and broadened activity against Gram-negative nosocomial pathogens, particularly the butene-linked tetrameric hydrazide. Remarkably, greatest MIC activity is against *P. aeruginosa* (0.77 µM/8 µg/mL) where the monomer is inactive. None of these peptides showed any cytotoxicity to mammalian cells up to 25 times the MIC. A diffusion NMR study of the tetrameric hydrazides showed that the more active antibacterial analogues were those with more compact structure having smaller hydrodynamic radii. The results show that C-terminal PrAMP hydrazidation together with its rational tetramerization is an effective means for increasing both diversity and potency of PrAMP action.

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Introduction

In the past decade, the significant increase in bacterial multi-drug resistance (MDR) has been associated with major clinical pathogenic infections and led to widespread calls for increased research funding and pharma intervention to develop new antimicrobial agents[1]. Antimicrobial peptides (AMPs), more correctly known as host-defense peptides, are considered to be potent alternatives to conventional antibiotics. These display broad-spectrum activities and possess distinct modes of action to combat pathogens[2]. Among many AMPs, proline-rich antimicrobial peptides (PrAMPs) show broad-spectrum activity against Gram-negative bacteria[3]. They also display a multi-modal mechanism of action which includes membrane rupture[4], inhibition of the bacterial shock heat protein DnaK[5], blockade of bacterial ribosomal protein expression[6], and immunostimulatory activity[7].

We have previously shown that the designed PrAMP, Chex1-Arg20, undergoes an alteration of mechanism of interaction with Gram-negative *Escherichia coli* membranes upon multimerization from its linear monomeric form to either a discontinuous dimer or tetramer[8]. These observations were broadly confirmed by the use of different model membranes to investigate the mode of interaction by Chex1-Arg20 and its multimers[9]. We also recently demonstrated that *C*-terminal chemical modifications of the Chex1-Arg20 monomer can significantly improve both the peptide's potency and spectrum of bacterial action. Greatest beneficial effect was observed on addition of a *C*-terminal hydrazide moiety leading to a broadening of the activity from *E. coli* and *Klebsiella pneumoniae* to include the other Gram-negative species, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*[10].

Our previously developed tetramer was formed by a disulfide bond between two Cys-functionalized dimers. While effective, the relative instability of the disulfide linker under cellular conditions prompted us to confirm or otherwise the unique range of activity of the homotetramer using alternative tethering chemistries and also the effect of inclusion of the aforementioned beneficial *C*-terminal peptide chemical modification. Numerous chemical linkers have been developed with one of the most popular being thiol-based conjugation, which uses mild reaction conditions and wide variety of reactions ranging from thiol-yne to thiol-maleimide as well as the thiol-ene[11]. More recently, dibromomaleimide (DBM), unlike the conventional maleimide, has shown versatile conjugation properties which allow double thiol attachments to substitute for the bromo-group[12]. Protein modifications and conjugations with DBM for dithiolmaleimide (DTM) formations have been

developed and demonstrated by several groups[13]. Importantly, these linkers have been demonstrated to possess inherent fluorescent properties[13c, 14] that enable their use in, for example, high resolution microscopy. Such inbuilt fluorescence would be an advantage in tetrameric PrAMPs, as we have previously shown that addition of a bulky AlexFluor moiety to our first tetramer resulted in significant loss of antimicrobial activity[8, 15]. Consequently, we undertook the use of dibromomaleimide conjugation of Chex1-Arg20 dimers possessing various *C*-terminal chemical modifications to form non-disulfide-linked tetramers with this inbuilt-fluorescent capability. A second series of tetrameric analogues was also prepared using monomeric peptide linked *via trans*-1,4-dibromo-2-butene, α,α′-dibromo-*p*-xylene or 6-bismaleimidohexane as tether to determine by diffusion NMR the effect of tether length on antimicrobial activity.

Results and Discussion

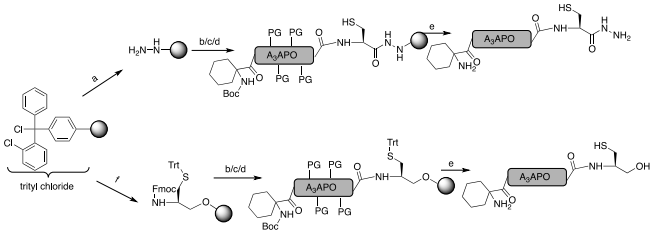


Figure 1. General conditions for the synthesis of C-terminal modified dimer (A3-APO) peptides: (a) hydrazine monohydrate (2 equiv), triethylamine (3 equiv), DMF; (b) Fmoc-AA-OH (4 equiv), HATU (3.8 equiv), DIPEA (4 equiv), 1 h; (c) 20% piperidine in DMF, 10 min; (d) 1-aminocyclohexanecarboxylic acid (1.5 equiv), HATU (1.4 equiv), DIPEA (4 equiv), 1 h; (e) TFA/anisole/TIPS/DODT (95:3:1:1), 2 h; (f) N-Fmoc-cysteine-β-amino-alcohol, tetrahydrofuran, pyridine, 60°C, 6 h. The A3-APO block represents –RPDKPRPYLPRPRPPRPVR\ /Dab–/ –RPDKPRPYLPRPRPPRPVR while PG represents the protecting groups.

Homotetramers with *C*-terminal modification of PrAMPs. Table 1 summarizes the synthetic Chex1-Arg20 peptide analogues that were prepared during this study. Our previous results have shown that certain *C*-terminal modifications, in particular hydrazide, alcohol or amide, of Chex1-Arg20 resulted in expanded diversity of activity against a pool of Gram-negative bacteria[10]. Dimeric Chex1-Arg20 peptides with each of these *C*-terminal modifications were prepared *via* Fmoc/tBu solid phase peptide synthesis (SPPS) with different functionalized 2-chlorotrityl chloride resins which were prepared in presence of hydrazine or *N*-Fmoc-*S*-trityl-L-cysteine-β-amino-alcohol (Figure 1). Each *C*-terminal modified dimer bearing a single *C*-terminal region Cys residue was then subjected to reaction in solution with a dibromomaleimide moiety under mild conditions (guanidine hydrochloride buffer, pH 8.0) to produce three dithiolmaleimide-linked tetramers (Table 1 and Figure 2). Tetrameric PrAMP-hydrazide was also prepared using either *trans*-1,4-dibromo-2-butene, 1,6-bimaleimidohexane, or α,α'-dibromo-*p*-xylene as tether (Table 1 and Figure 2) to investigate the effect of length on antibacterial activity. Each tetramer was obtained in modest overall yield of *ca.* ~15% relative to crude Chex1-Arg20 dimer (Table 1) and then subjected to comprehensive chemical characterization including analytical RP-HPLC and MALDI-TOF MS to confirm their purity (SI_Figure S2).

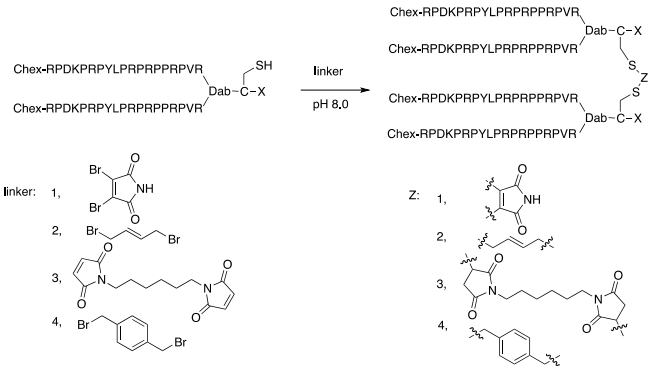
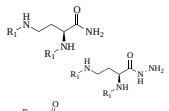
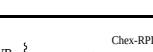
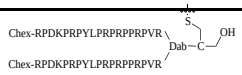


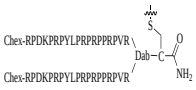
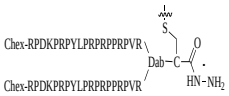
Figure 2. The preparation of dithiolmaleimide linked tetrameric PrAMPs with *C*-terminal modifications, as well as the tetrameric PrAMP-hydrazide with different linkers. For linker 1, the X represents amide group ( ), hydroxyl group ( ), or hydrazide group ( ) and Z represents Z1. For linker 2-4, the X represents hydrazide group ( ) and Z represents Z2-Z4.

Antibacterial activity. Each *C*-terminal modified PrAMP DTM-linked tetramer was assayed against nosocomial Gram-negative bacteria, *E. coli* ATCC 29222, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606 and *P. aeruginosa* ATCC 47085. The results are given in Table 2. The tetramers tetramer-DTM-NH2, tetramer-DTM-ol, and tetramer-DTM-$NHNH_2$ maintained activity against *E. coli* (MIC of 0.93-3.67 μM) and *K. pneumoniae* (MIC 0.49-2.33 μM). Moreover, compared to the monomer, the tetramers had improved potency against *A. baumannii* (MIC 2.33-13.29 μM) and *P. aeruginosa* (MIC 1.14-2.05 μM), which are strongly associated with hospital associated/acquired infections[21]. Among these tetramers, tetramer-DTM-$NHNH_2$ showed particularly impressive activity against all of the Gram-negative nosocomial bacteria with MIC = 0.49-2.33 μM, which was significantly improved compared with the original disulfide-linked tetramer and both the monomer and dimer (A3-APO) hydrazides (Table 2). These results highlight the significant advantage conferred to bacterial killing by increased multimerization together with *C*-terminal peptide hydrazide modification. We are unaware of any other report of the utility of *C*-terminal peptide hydrazidation as a basis of improving antibacterial activity. However, the chemical basis for the improvement afforded by the presence of the *C*-terminal hydrazide of Chex1-Arg20 in both bacterial selectivity and potency remains unknown. Hydrazide-reactive peptides have been used to label bacterial cell lysates[22], and may possibly react more strongly with proteins. This requires future examination.

Table 1. Structure of tetramer analogues used in this report. The monomer, monomer-hydrazide and A3-APO (dimer) were reported previously[8, 10] and compared here.

Head 1[a]	Tetramer analogue	Structure*	Yield (overall)	MW
1	monomer[8]	Chex-RPDKPRPYLPRPRPPRPVR—NH_2	40%	2475.0
2	monomer-$NHNH_2$[10]	Chex-RPDKPRPYLPRPRPPRPVR—NH–NH_2	52%	2490.0
3	A3-APO (dimer)[8]		30%	5030.0
4	dimer-$NHNH_2$		25%	5048.0
5	tetramer-DTM-ol		12.3%	10340.3
6	tetramer (disulfide)[8]		15.6%	10270.5
7	tetramer-DTM-NH_2		15.9%	10365.3
8	tetramer-DTM-$NHNH_2$		16.6%	10357.4
9	tetramer-butene-$NHNH_2$		16.1%	10354.4
10	tetramer-bismal-$NHNH_2$		13.3%	10578.6
11	tetramer-xylene-$NHNH_2$		15.0%	10403.4

* R1 represent Chex-RPDKPRPYLPRPRPPRPVR–, R2 

R3  , and R4  .

Although the DTM tether has been demonstrated to be reducible by glutathione leading to release of the dansylthiol as reported for a DTM moiety-linked protein[23], the rate of cleavage is substantially slower than of the disulfide bond that was used in the previous generation of tetrameric Chex1-Arg20. The alternative functional linkers examined here are each expected to have similar strong or complete stability against the reductive conditions within cells. The resulting tetrameric hydrazide PrAMPs (Figure 2) were also tested against the same nosocomial Gram-negative bacteria. The MIC values also showed significantly improved antibacterial activities for all the peptides with the tetramer-$NHNH_2$ bearing the shortest linker (butene) showing an MIC of 0.77 µM-1.89 µM against *E. coli, K. pneumoniae, A. baumannii and P. aeruginosa* (Table 2). The two other tetramer-$NHNH_2$ peptides (linked *via* bismaleimide or xylene) had similar activities (MIC 1.51 µM - 6.24 µM) against *E. coli, K. pneumoniae and P. aeruginosa,* but not against *A. baumannii* (MIC 10.35 µM - 12.70 µM). Especially noteworthy are the pronounced actions of the tetrameric peptides against *P. aeruginosa*. This bacterium is consistently more difficult to treat than many other, if not all, Gram negative bacteria and the observed MIC values are in general 4-8-fold higher than for other nosocomial pathogens[24]. In particular, the tetramer-butene-$NHNH_2$ possesses an MIC of just 0.77 µM.

Table 2. Antibacterial activity, MIC (µM), of PrAMPs with different linkages against Gram-negative nosocomial pathogens. The MIC values expressed in µg/mL are in brackets.

No	*E. coli*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*
1	2.6±0.77 [6.3±1.9]	0.80±0.02 [2.0±0.1]	>100 [>250]	56.3±4.58 [140±11.3]
2	4.0±1.5 [10.0±3.7]	1.7±0.1 [4.2±0.3]	52.5±9.5 [130±20]	28.9±2.7 [72±6.7]
3	2.6±1.14 [13.0±5.7]	1.4±0.12 [7.2±0.6]	8.0±1.09 [40.2±5.5]	3.1±0.05 [15.7±0.3]
4	3.0±1.00 [15.1±5.1]	3.6±0.02 [18.3±0.1]	5.3±0.73 [26.8±3.7]	3.0±0.18 [15.2±0.9]
5	3.7±0.25 [38.0±2.6]	2.2±0.01 [24.2±0.1]	11.6±1.44 [120.1 ±14.9]	1.9±0.07 [19.2±0.7]
6	5.8±1.30 [59.2±13.4]	3.5±0.33 [35.5±3.4]	15.8±1.51 [162.3±15.5]	3.6±1.46 [37.1±15.0]
7	3.0±0.99 [30.7±10.3]	2.3±0.01 [24.2±0.1]	13.3±0.10 [137.8±1.0]	2.0±0.08 [21.3±0.8]
8	0.9±0.02 [9.6±0.2]	0.5±0.01 [5.1 ±0.1]	2.3±0.48 [24.1±5.0]	1.1±0.03 [11.8±0.3]
9	0.9±0.04 [9.7±0.4]	0.8±0.02 [8.4±0.2]	1.9±0.63 [19.6±6.5]	0.8±0.02 [8.0±0.2]
10	3.9±0.04 [41.6±0.4]	3.2±0.03 [33.6±0.3]	10.3±0.22 [109.5±2.3]	1.6±0.11 [17.1±1.2]
11	6.2±0.53 [64.9±5.5]	4.5±0.56 [46.7±5.8]	12.7±0.96 [132.1±10.0]	1.5±0.33 [15.7±3.4]

Photoluminescence analysis. The photoluminescent properties of the DTM-linked homotetramers were determined and showed a $\lambda_{ex,max}$ of 392-395 nm and $\lambda_{em,max}$ of 554-555 nm (Figure 3). The excitation and emission wavelength of DTM fluorescence matched with the Qdot® 565 probe (Thermo Fisher Scientific) for potential imaging and flow cytometry applications. These properties augur well for the use of these tetrameric peptides in their use in, for example, mechanism studies involving high resolution microscopy and are an alternative to the bulky AlexFluor 647 or 430 that can cause loss of antibacterial activity[8, 15].

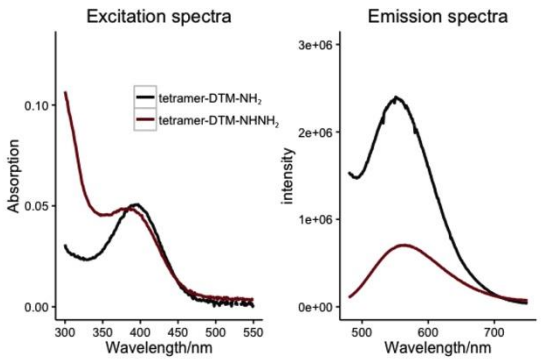


Figure 3. Excitation and emission spectra of DTM-linked tetrameric PrAMP in MilliQ water.

Cytotoxicity *In vitro* cytotoxicity was also determined *via* the Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay[8] using the mammalian cell lines HEK-293 (ATCC CRL 1573) and H-4-II-E (ATCC CRL-1548) (SI_Table S1). All of the tetramer analogues showed no toxicity on either mammalian cell line at the highest tested concentration (25 □M), which is much higher than the antibacterial activity MIC (Table 2) on nosocomial Gram-negative bacteria. It needs to be mentioned however that lack of *in vitro* toxicity of AMPs to mammalian cells seldom reflects *in vivo* adverse effects, either transient or permanent, lethargy and internal bleeding being the most frequently observed ones.

Diffusion NMR. To further delineate the potential relationship between the size/shape of these peptides with different chemical tethers in isotropic solution and their activity against pathogens, translational diffusion coefficients of PrAMPs were measured using pulsed field gradient (PFG) NMR (SI, Figure S4) and the results are summarized in Figure 4 and Table 3. Based on the well-known Stokes-Einstein equation, $D_t = k_BT/(6\square\square R_h)$, the molecules with larger effective hydrodynamic radius R_h would display a slower diffusion coefficient *D* in solution.

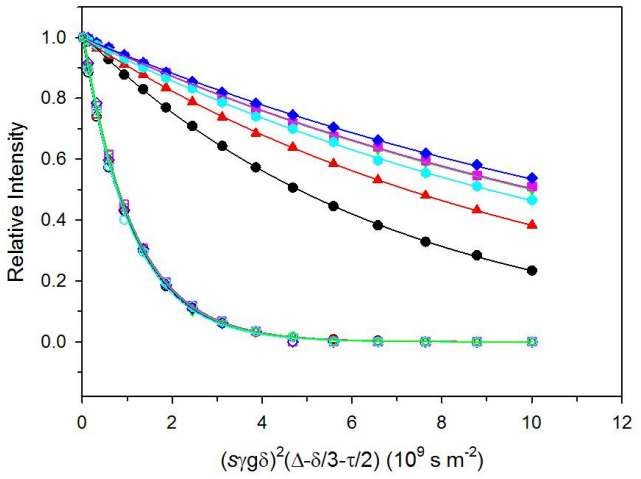


Figure 4. Translational diffusion coefficients of PrAMPs and dioxane, as internal reference for hydrodynamic radius, measured in ^2H_2O at 298 K. The translational diffusion-induced signal attenuation in the presence of pulsed-field gradients for PrAMPs (● monomer, ▲ dimer, ▼ tetramer-butene-$NHNH_2$, ■ tetramer-bismal-$NHNH_2$, ◆ tetramer-xylene-$NHNH_2$, ⬢ tetramer-DTM-$NHNH_2$) and dioxane (open symbols) are shown as relative intensities versus the strength of the diffusion encoding, $\gamma^2s^2g^2\delta^2(\Delta-\delta/3-\tau/2)$. Lines represent the results of non-linear regression to Eq. 1. Resultant diffusion coefficients of the non-linear regression are listed in Table 3.

Compared with monomer-$NHNH_2$, the dimer-$NHNH_2$ exhibited a 35% reduction (Table 3) in translational diffusion coefficient, which is in excellent agreement with a reduction of 33% as predicted by the Stokes-Einstein equation for spheres. In contrast, the tetramer-hydrazides displayed a further reduction in their diffusion coefficients ranging from 20% to 35% (Table 3) when compared with dimeric hydrazide. Such range in reduction suggests no aggregation or oligomeric formation of the tetrameric PrAMPs.

Amongst the four different linked tetramer-hydrazides, tetramer-bismal-$NHNH_2$ and tetramer-xylene-$NHNH_2$ displayed an increased hydrodynamic radius by ~ 35% and ~ 54%, respectively, compared to dimer-$NHNH_2$ (Table 3). Slightly less increase in effective hydrodynamic radii was observed for tetramer-DTM-$NHNH_2$ (29%) and tetramer-butene-$NHNH_2$ (34%). This indicates that the tetramer-DTM-$NHNH_2$ and tetramer-butene-$NHNH_2$ are more compact than the tetramer-bismal-$NHNH_2$ and tetramer-xylene-$NHNH_2$ in solution, which may correlate with the observed increased activity against Gram-negative bacteria. The peptide chains may possibly act synergistically against the membrane in a similar manner to PGLa/magainin against *E. coli* lipid extract membrane bilayers[25], where PGLa inserts across the bilayer and magainin adopts a surface orientation, and together more severely disrupt the membrane. Possibly some peptide chains could insert into the membrane with others from the tetramer lying on the surface and act in a similar manner to PGLa/magainin.

As an indication of the secondary structures of these tetramer-hydrazide PrAMPs, circular dichroism (CD) spectroscopy was used. The CD spectra indicated no significant difference in secondary structure among these peptides which were mainly unstructured in 20 mM, phosphate buffer, pH 7.4, (SI, Figure S3) as reported for other similar PrAMPs[25]. Two-dimensional ^1H NMR spectra of monomeric and dimeric forms of PrAMP (SI, Figure S5) also indicated a primarily unstructured conformation in solution, most likely due to the high positive charge on the peptides.

Table 3. NMR diffusion coefficients of PrAMPs with *C*-terminal hydrazide*.

No.	C (□M)	D^p (×10-10 m2 s-1)	D^d (×10-10 m2 s-1)	R_h (Å)
2	49	1.462 ± 0.006	9.216 ± 0.127	13.36 ± 0.19
4	61	0.956 ± 0.004	9.052 ± 0.087	20.07 ± 0.21
8	17	0.767 ± 0.004	9.391 ± 0.177	25.96 ± 0.51
9	31	0.694 ± 0.002	8.814 ± 0.111	26.92 ± 0.35
10	26	0.688 ± 0.004	8.807 ± 0.038	27.14 ± 0.20
11	43	0.626 ± 0.003	9.130 ± 0.104	30.92 ± 0.38

* C is the concentration of the PrAMPs used for the diffusion NMR measurements, D^p refers to the diffusion coefficient of PrAMPs in deuterated water, D^d to the diffusion coefficient of dioxane in deuterated water, and R_h is the effective hydrodynamic radius of the PrAMPs.

Conclusions

In summary, a series of DTM-linked tetrameric PrAMPs based on the designed PrAMP, Chex1-Arg20, bearing *C*-terminal modifications were obtained under mild conditions. Among these tetramers, the tetramer-DTM-$NHNH_2$ displayed the most potent

and broadened antibacterial activity against a panel of Gram-negative nosocomial bacteria. As well, tetrameric hydrazides with different lengths including *trans*-1,4-dibromo-2-butene, 1,6-bimaleimidohexane and α,α′-dibromo-*p*-xylene were obtained under mild conditions *via* an S_N2 mechanism or addition reaction. All of these tetrameric hydrazides exhibited potent antibacterial activity as well as broader bacterial selectivity against the Gram-negative nosocomial pathogens. Particularly noteworthy was the significant effect on the notoriously difficult-to-kill *P. aeruginosa*. Diffusion NMR spectroscopy of tetrameric hydrazides indicated their similar translational diffusion coefficients with a smaller hydrodynamic radius for tetramer-DTM-$NHNH_2$ and tetramer-butene-$NHNH_2$, which were correspondingly more effective against the Gram-negative bacteria. However, NMR and CD spectroscopy of the tetrameric hydrazide peptides showed a mainly disordered structure possibly due to their high charge. The tetramers displayed no cytotoxicity against two mammalian cell lines at a concentration far above their MIC to bacteria. Our findings highlight the advantages of modern synthetic chemistry methods to develop novel *C*-terminal-modified multimerized PrAMPs with both more potent and broad spectrum antibacterial activity, as well as inherent fluorescent properties, that have significant potential for use in both therapeutic applications, either alone or in combination with other antibacterial agents, and cellular mechanism studies.

Experimental Section

Materials. 9-Fluorenylmethoxylcarbonyl (Fmoc)-L-amino acids, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylamonium hexafluorophosphate (HCTU), and 2-chlorotrityl chloride resin were purchased from GL Biochem (Shanghai, China). TentaGel-MB-RAM-resin was from Rapp Polymere (Tubingen, Germany). N,N-Diisopropylethylamine (DIPEA), dimethylformamide (DMF), and trifluoroacetic acid (TFA) were obtained from Auspep (Melbourne, Australia). 1,6-Bimaleimidohexane was obtained from TCL (Gillman, Australia). Isobutyl chloroformate (IBCF), $NaBH_4$, ethyl acetate, 2,3-dibromomaleimide, α,α′-dibromo-*p*-xylene, *trans*-1,4-dibromo-2-butene, piperidine, triisopropylsilane (TIPS), anisole, 3,6-dioxa-1,8-octanedithiol (DODT), hydrazine monohydrate, and acetonitrile (CH_3CN) were all obtained from Sigma (Sydney, Australia).

Peptide preparation. The peptides were synthesized by Fmoc/tBu solid-phase methods[16]. Dimeric peptide synthesis was carried out on a CEM Liberty microwave-assisted synthesizer using TentaGel-MB-RAM-resin or 2-chlorotrityl chloride resin as previously described[8] (Figure 1). The *C*-terminal modified peptides were prepared on 2-chlorotrityl chloride resin functionalized with hydrazide or N-Fmoc-amino acid alcohol prior to SPPS. Standard Fmoc-chemistry was used throughout with a 4-fold molar excess of the Fmoc-protected amino acids in the presence of 4-fold HCTU and 8-fold DIPEA. The peptides were cleaved from the solid support resin with TFA in the presence of anisole, TIPS and DODT as scavenger (ratio 95:2:2:1) for 2 h at room temperature. After filtration to remove the resin, the filtrate was concentrated under a stream of nitrogen, and the peptide products were precipitated in ice-cold diethyl ether and washed three times. The peptides were then purified by reversed-phase high performance liquid chromatography (RP-HPLC) in water and acetonitrile containing 0.1% TFA. The final products were characterized by both RP-HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Preparation of different length linked tetramer-hydrazides. To a solution of dimeric PrAMP-hydrazide (0.5 mmol) in guanidine hydrochloride buffer (pH 8.0, 150 µL) was added dropwise a solution of linker including (DTM, trans-1,4-dibromo-2-butene, 1,6-bismaleimidohexane, or α,α'-dibromo-*p*-xylene) that was dissolved in acetonitrile (0.25 mmol, 100 µL). The mixture was reacted for 2-6 h at room temperature and the resulting tethered tetrameric peptides were purified by RP-HPLC in aqueous acetonitrile containing 0.1% TFA in overall moderate yield (Figure 2). The final products were characterized by RP-HPLC and MALDI-TOF MS (SI_Figure S2).

Antibacterial assay. Antibacterial assays were undertaken to determine the minimal inhibitory concentrations (MIC) as described previously[10]. Four Gram-negative nosocomial bacteria, namely *E. coli* ATCC 29222, *K. pneumoniae* ATCC13883, *A. baumannii* ATCC 19606, and *P. aeruginosa* ATCC 47085 were chosen to test the antibacterial activities of the tetrameric PrAMPs, with 2.5×10^5 cells/ml in MHB at 37°C immediately prior to the determination of MIC, with gentamycin (Table S2) as the positive control, and the non-treated bacteria as the negative control.

Photoluminescence. Spectra were obtained using a HORIBA Jobin Yvon Fluorolog fluorescence spectrophotometer. To minimise reabsorption effects, the optical absorptions of the sample solutions were kept around 0.10 at the excitation wavelength. The sample solutions of tetramer-DTM-NH_2 or tetramer-DTM-$NHNH_2$ were prepared in MilliQ water in BRAND® disposable cuvettes and spectra were recorded immediately after the UV-vis absorbance measurements.

Diffusion NMR spectroscopy. Translational diffusion coefficients of PrAMPs were measured at 298 K on a Bruker Avance II 800 MHz spectrometer using a TXI cryoprobe equipped with a single field gradient (*Gz*). Diffusion measurements were carried out using a standard BPP-STE sequence without modification (*stebpgp1s*, Bruker pulse sequence library). The field gradient strength of *Gz* was calibrated by measuring the self-diffusion coefficient[17] of residual H_2O in a 100% ^2H_2O sample at 298.13 K using a diffusion coefficient of 1.9×10^{-9} m^2 s^{-1} for the residual H_2O [18]. Spectra were processed in TOPSPIN (Version 3.2, Bruker). Diffusion coefficient, *D*, was determined by fitting diffusion weighted intensities of well-resolved and intense peaks using the T_1/T_2 relaxation module in TOPSPIN (Version 3.2, Bruker) and *SigmaPlot* (version 12.5, *Systat Software*) to the following equation (1):

$$I = I_0 \exp\{-\gamma^2 s^2 g^2 \delta^2 (\Delta - \delta/3 - \tau/2) D\} \qquad (1)$$

where γ is the gyromagnetic ratio of protons and *s*, g, δ, and Δ are the shape factor, amplitude, duration and separation, respectively, of the single pair of gradient pulses, and τ is the time interval within the bipolar pulse pair. Sinusoidal shaped gradient pulses were used in the present study. The effective hydrodynamic radius of PrAMPs were subsequently estimated from experimentally measured translational diffusion coefficients using the following relationship equation (2)[19]:

$$R_h = R_h^{REF} (D^{REF}/D) \qquad (2)$$

where R_h^{ref} and D^{ref} are the hydrodynamic radius and translational diffusion coefficient, respectively, of the reference molecule. In the present study, dioxane with R_h^{ref} of 2.12 Å was used as the reference molecule[20].

Cell proliferation test. The proliferation of HEK-293 (ATCC® CRL-1573™) and H-4-II-E (ATCC® CRL-1548™) cells were tested with tetrameric PrAMPs with CellTiter 96 AQ_{ueous} Non-Radioactive Cell

Proliferation Assay (Promega) as described before[10], which was measured at 490 nm.

Acknowledgements

Ms Mina Barzegar Amiri Olia (School of Chemistry, University of Melbourne) assisted with photoluminescence measurements. We gratefully acknowledge support of the studies undertaken in the authors' laboratory by ARC Discovery Project grants (DP150103522) to JDW and MAH, and (DP140102127) to FS, and NHMRC Project grants (APP1029878) to NMOBS and (APP1008106) to ECR and NMOBS. JDW is an NHMRC (Australia) Principal Research Fellow. WL is the recipient of an MIRS PhD award and Dr Albert Shimmins postgraduate writing-up award. Research at the FNI was also supported by the Victorian Government's Operational Infrastructure Support Program.

Keywords: proline-rich antimicrobial peptides • conjugation • tetramer • expended activity • compacted structure

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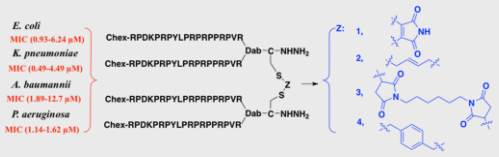

C-terminal PrAMP hydrazidation together with its rational tetramerization have been shown to be an effective means for increasing both diversity and potency of PrAMP action against nosocomial Gram-negative bacteria.

Wenyi Li, Neil M. O'Brien-Simpson, Shenggen Yao, Julien Tailhades, Eric C. Reynolds, Raymond M. Dawson, Laszlo Otvos, M. Akhter Hossain, Frances Separovic and John D. Wade**

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C-terminal modification and multimerization increase the efficacy of a proline-rich antimicrobial peptide