Antimicrobial Peptides Hot Paper

DOI: 10.1002/anie.201409270

Combining Topology and Sequence Design for the Discovery of Potent Antimicrobial Peptide Dendrimers against Multidrug-Resistant *Pseudomonas aeruginosa***

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Abstract: *Multidrug-resistant opportunistic bacteria, such as Pseudomonas aeruginosa, represent a major public health threat. Antimicrobial peptides (AMPs) and related peptidomimetic systems offer an attractive opportunity to control these pathogens. AMP dendrimers (AMPDs) with high activity against multidrug-resistant clinical isolates of P. aeruginosa and Acinetobacter baumannii were now identified by a systematic survey of the peptide sequences within the branches of a distinct type of third-generation peptide dendrimers. Combined topology and peptide sequence design as illustrated here represents a new and general strategy to discover new antimicrobial agents to fight multidrug-resistant bacterial pathogens.*

The emergence of multidrug-resistant bacteria is a public health threat of increasing importance.[1] There is an urgent need for new antibiotics, in particular against Gram-negative bacteria, such as multidrug-resistant *P. aeruginosa*. This opportunistic pathogen causes chronic infections in cystic-fibrosis patients as well as acute infections of burn wounds, the urinary tract, and the respiratory system in intubated patients.[2]

Antimicrobial peptides (AMPs), which are part of the defense system of virtually all multicellular organisms,[3] offer an interesting option to address resistant strains. AMPs typically contain up to 50 amino acids with many basic residues (lysine or arginine) and at least 30% hydrophobic side chains,[4] and mostly act by folding into amphipathic conformations inducing membrane disruption, an activity type also described for various peptidomimetic systems.[5] Whereas amino acid sequence variations have been extensively explored for linear and cyclic AMPs,[6] alternative topologies of the peptide chain have not been studied in depth for AMP design. In particular, the dendrimer topology has only been used as a tool to achieve multivalency of pre-existing AMP sequences or single amino acids, typically by attachment to a dendritic poly(lysine) tree.[7] Herein, we combined topology and sequence design by distributing amino acids within the branches of a distinct type of third-generation peptide dendrimer that consists of multiple short dipeptides connected by branching lysine residues. Antimicrobial peptide dendrimers (AMPDs) were identified that display potent activities against *P. aeruginosa* and *A. baumannii,* two of the most problematic antibiotic-resistant nosocomial pathogens (e.g., G3KL; Figure 1). Mechanistic investigations show that the activity is triggered by a dendritic effect, but also requires a precise sequence of hydrophobic and cationic residues within the dendrimer branches as well as conformational flexibility at the water–membrane interface to enable membrane disruption. The dendritic topology furthermore greatly facilitates the synthesis of these AMPDs and endows them with excellent serum stability despite the exclusive use of natural L-amino acids.

We recently showed that peptide dendrimers of up to 40 residues that contain multiple short mono-, di-, or tripeptide branches can be efficiently prepared by solid-phase peptide synthesis (SPPS) and display a range of biomimetic functions.[8] Their dendritic topology induces a flexible yet relatively compact molten globule conformation[9] resulting in good resistance to proteolysis.[10] Considering that glycosylated versions showed useful lectin inhibition and anti-biofilm activities against *P. aeruginosa*,[11] we asked the question whether a systematic survey of amino acid sequences within this multi-branched topology might lead to peptide dendrimers with direct AMP-like antimicrobial effects.

Initial combinatorial experiments identified bH1, with the sequence $L_8(BL)_4(BF)_2BK$ (B = branching 2,3-diaminopropanoic acid, Dap), as a simple, relatively small AMPD with only one residue per branch whose positive charges were provided by the eight amino termini.[12] This dendrimer showed significant activity against *P. aeruginosa* presumably by membrane disruption;[12,13] however, sequence variations were ineffective in improving its activity. Considering that an increase in size and the number of positive charges is often key to promoting membrane interactions in polycationic compounds, we set out to investigate third-generation dendrimers with dipeptide branches offering 30 variable positions

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[**] This work was supported financially by the University of Bern, the Swiss National Science Foundation, the European Marie Curie Initial Training Network BioChemLig, and Swiss TransMed.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201409270.

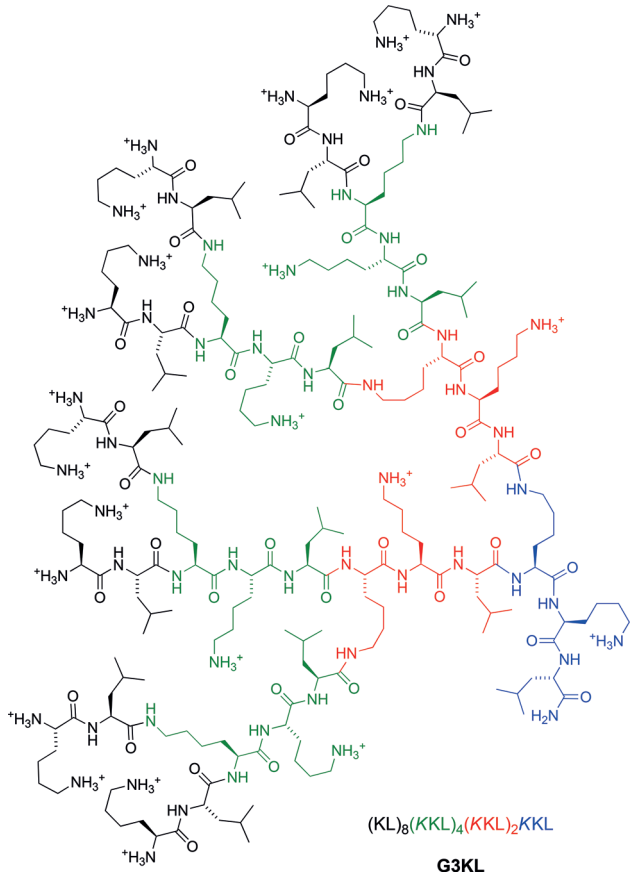

Figure 1. Structure of AMPD G3KL.

suitable for installing a larger number of positive charges and hydrophobic groups. Inspired by the prevalence of leucine and lysine in many AMPs, including tridecapeptide KYKKALKKLAKLL (LinKYL13) used here as positive control,[14] six dendrimers (G3KL, G3LK, G3KK1, G3KK2, G3LL1, G3LL2) featuring various sequences of these two key residues within their branches were prepared by Fmoc-SPPS (Fmoc = 9-fluorenylmethoxycarbonyl) on Rink-amide resin and purified by preparative HPLC (Table 1).

Profiling against *P. aeruginosa*, *E. coli*, and *B. subtilis* in a broth dilution assay[15] showed that G3KL with the sequence $(KL)_8(KKL)_4(KKL)_2KKL$ (*K* = branching lysine, Figure 1) combined a good broad-spectrum activity with >200-fold selectivity against hemolysis. The other five dendrimers with different distributions of lysines and leucines in the branches gave significantly less potent and more hemolytic dendrimers. Dendrimer G3KL with the repeated KL sequence was therefore further investigated. The activity was comparable to that of bG3KL, which was built with the shorter Dap linker; however, with bG3KL, increased hemolysis was observed. The enantiomer DG3kl showed comparable activities to G3KL, suggesting membrane disruption rather than specific receptor-mediated effects as a possible mechanism of action.

Experiments with dipeptide KL and the G1 and G2 analogues revealed a strong dendritic effect in the antimicrobial activity of the third-generation dendrimer G3KL. Thus, whereas G3KL showed good activity on all three bacteria, its second-generation analogue G2KL was only active on *P. aeruginosa*, and the first-generation analogue G1KL and dipeptide KL were inactive. Furthermore, the linear tetradecapeptide LinKL7 was more hemolytic than antimicrobial, showing that the dendritic topology has a favorable effect on selectivity. Exchanging lysines in G3KL with more basic arginines gave G3RL with slightly weaker antimicrobial effects but 10-fold stronger hemolysis, a trend further exacerbated when using the shorter Dap linker in bG3RL. The antimicrobial effect was mostly preserved when replacing the hydrophobic leucines by phenylalanines or tryptophans in G3KF and G3KW; however, the corresponding exhaustive Leu→Tyr exchange gave the inactive G3KY. G3KA, which features an altered sequence with partial replacement of Leu and Lys by Ala and a KL→LK exchange, in G2 provided a useful non-hemolytic and inactive control dendrimer with the same number of formal positive charges as G3KL. Interestingly, the related G3LA with only 13 positive charges also had significant activity against *P. aeruginosa*, showing that charge alone cannot explain activity.

The AMPDs G3KL, DG3kl, and G3RL were selected for further investigation. Antimicrobial activity against PAO1 was retained in the presence of human serum[16] for G3KL (MIC = 2 $\mu g\,mL^{-1}$; see the Supporting Information, Table S2) despite partial degradation (60% after 24 h; Figure S1) and for DG3kl (MIC = 0.5 $\mu g\,mL^{-1}$, no degradation after 24 h), which was slightly more active than polymyxin (2–4 $\mu g\,mL^{-1}$) under these conditions. On the other hand, G3RL and our previously reported dendrimer bH1 were inactive in serum where they underwent complete degradation ($t_{1/2} \approx 5$ h), whereas the reference AMP LinKYL13 showed weaker activity (MIC = 32 $\mu g\,mL^{-1}$) and rapid degradation ($t_{1/2} = 2$ h).

The activities of the AMPDs were next tested against four clinical isolates of *P. aeruginosa* (ZEM9A, ZEM1A, PEJ2.6, PEJ9.1, provided by CHUV, Lausanne, Switzerland), which are resistant to at least two different classes of antibiotics, including β-lactams (cephalosporins, carbapenems), aminoglycosides (amikacin, gentamicin, tobramycin), or quinolones (norfloxacin, ciprofloxacin), and the Gram-negative reference strains *A. baumannii* ATCC19606, *E. coli* W3110, and *E. aerogenes* 13048. For all strains except ZEM9A, at least one of the three AMPDs showed an MIC ≤ 16 $\mu g\,mL^{-1}$, whereas our previous dendrimer bH1 was mostly inactive (Table 2).

The dendrimers showed comparable activity against lipopolysaccharide (LPS) mutant strains of *P. aeruginosa* lacking either the A-band LPS (PAO1*rmd*) composed of D-rhamnose trisaccharides, the A- and B-band LPS (PAO1*rmlC*), or the A-, B-band LPS and part of the outer-core LPS (glucose residues; AK1012 (*algC*)).[17] The data showed that the LPS layer, which serves as an efficient barrier against hydrophobic compounds, did not impede the activity of G3KL, DG3kl, or G3RL.

Conformational changes upon interaction with cell membranes and folding into amphiphilic secondary structures such as α-helices in the membrane environment have been described as essential features for AMP activity.[3a,b,4,18] In the case of peptide dendrimers with the same topology and size as G3KL, MD simulations and CD and FTIR spectros-

Table 1: Synthesis and bioactivity of antimicrobial peptide dendrimers (AMPDs).[a]

Compound	Sequence[b]	Yield, mg (%)[c]	mass calc./obs.[c]	Pos.[d]	AAs[e]	*P. aeruginosa* PAO1	*E. coli* DH5α	*B. subtilis* BR151	MHC[f]
Polymyxin	Cyclic peptide	–	1301.6	5	11	2	0.3	20	>2000
Tobramycin	Aminoglycoside	–	467.5	5	–	0.5	1	0.6	>2000
bH1	$(L)_8(BL)_4(BF)_2BK$	22 (7)	2401.1/2400.0	9	22	18	3.9	2.9	>2000
LinKYL13	KYKKALKKLAKLL	56 (9)	1544.0/1545.2	7	13	2	<1	4	420
G3KL	$(KL)_8(KKL)_4(KKL)_2KKL$	52 (8)	4534.2/4535.0	23	37	2	4	3	840
G3LK	$(LK)_8(KLK)_4(KLK)_2KLK$	8 (1)	4534.2/4534.0	23	37	9	56	3	210
G3KK1	$(KK)_8(KKK)_4(KLL)_2KLL$	15 (2)	4669.3/4669.0	31	37	>95	>95	6	60
G3KK2	$(KK)_8(KLL)_4(KKK)_2KLL$	5 (1)	4609.0/4612.0	28	37	>66	>66	12	120
G3LL1	$(LL)_8(KLL)_4(KKK)_2KKK$	15 (1)	4399.1/4399.0	14	37	>67	60	35	4
G3LL2	$(LL)_8(KKK)_4(KLL)_2KKK$	7 (1)	4460.1/4460.7	18	37	12	>62	3	230
bG3KL	$(KL)_8(BKL)_4(BKL)_2BKL$	68 (3)	4239.0/4240.0	23	37	2	1	2	400
DG3kl	$(kl)_8(kkl)_4(kkl)_2kkl$	17 (2)	4534.2/4536.7	23	37	4	1	2	680
KL	KL	15 (30)	258.4/259.6	2	2	>74	>74	>74	>1500
G1KL	$(KL)_2KKL$	52 (44)	869.2/869.8	5	7	>91	>91	>91	>1800
G2KL	$(KL)_4(KKL)_2(KKL$	45 (15)	2090.9/2091.0	11	17	7	>85	>85	>1700
LinKL7	KLKLKLKLKLKLKL	10 (1)	1706.3/1707.0	8	14	>28	13	14	4
G3RL	$(RL)_8(KRL)_4(KRL)_2KRL$	13 (2)	4954.4/4953.5	23	37	8	3	11	90
bG3RL	$(RL)_8(BRL)_4(BRL)_2BRL$	7 (<1)	4659.8/4660.0	23	37	7	3	4	25
G3KF	$(KF)_8(KKF)_4(KKF)_2KKF$	17 (2)	5044.4/5048.8	23	37	6	7	2	270
G3KW	$(KW)_8(KKW)_4(KKW)_2KKW$	3 (<1)	5630.0/5630.3	23	37	12	7	6	nd
G3KY	$(KY)_8(KKY)_4(KKY)_2KKY$	21 (2)	5284.4/5290.1	23	37	68	68	20	nd
G3KA	$(KA)_8(KLK)_4(KKL)_2KAL$	37 (6)	4140.5/4141.0	22	37	83	83	29	830
G3LA	$(LA)_8(KLK)_4(KLA)_2KKL$	28 (4)	4083.4/4084.0	13	37	14	78	27	580

[a] The MIC (minimal inhibitory concentration) in $\mu g\,mL^{-1}$ was determined by a 2/3 serial dilution in Luria–Bertani (LB) broth in a 96 well plate after incubation for 18–22 hours at 37 °C. The MIC values were determined in independent triplicates with at least two experiments giving the same value. The values of weighed lyophilized TFA salts were corrected for peptide content determined by amino acid analysis. [b] One letter code for amino acids (upper case: L-, lower case: D-). Branching diamino acids in italics. *B* = branching 2,3-diaminopropanoic acid. All peptides are carboxamides ($CONH_2$) at the C terminus and have free amino termini. [c] Yields given for the RP-HPLC purified product. Mass calc. for M^+, observed by positive-ion mode ESI as $[M+H]^+$ or positive-ion mode MALDI as $[M+H]^+$ or as higher protonation states, see the Supporting Information for details. [d] Position: Number of formal cationic charges (*N*-termini, Lys and Arg side chains). [e] AAs = total number of amino acids. [f] The MHC (minimal hemolytic concentration) in $\mu g\,mL^{-1}$ was determined on human red blood cells (hRBCs). MHC values were determined in duplicate and averaged.

Table 2: MIC values of AMPDs for multidrug-resistant isolates and LPS mutant strains of *P. aeruginosa*.[a]

Strain	G3KL	DG3kl	G3RL	bH1	Polymyxin
ZEM1.A	4	4	8	>64	0.25
ZEM9.A	32	32	32	>64	8
PEJ2.6	8	4	8	>64	1
PEJ9.1	32	32	8	>64	2
A. baumannii	8	16	8	16	1
E. coli	4	2	4	8	0.5
E. aerogenes	64	32	16	64	0.5
PAO1	4	4	8	>64	1
PAO1*rmd* (A-)	4	4	8	>64	1
PAO1*rmlC* (A-B-)	4	4	4	64	0.5
AK1012 (*algC*)	2	2	4	16	1

[a] MIC values were determined by serial two-fold dilutions in Mueller–Hinton (MH) broth in 96 well plates after incubation for 18–22 hours at 37 °C. Experiments were performed in triplicates with at least two independent experiments giving the same value. For the resistance profiles, see Table S1. bH1 was active against PAO1 in LB broth (Table 1), but inactive in MH broth (this table).

copy studies have shown that they are flexible molecules without a well-defined conformation.[9,19] For the present AMPDs, CD signals indicative of random coils were observed in aqueous neutral phosphate-buffered saline (PBS, pH 7.4) with a minimum at 200 nm for the L-enantiomers (G3KL, G3KA, G3KK1) and a maximum at 200 nm for the D-enantiomer DG3kl. Addition of TFE (trifluoroethanol, 50 %)[20] or sodium dodecylsulfate (SDS; 20 mM) induced a substantial change in the CD profile towards an α-helix-like signal suggesting a conformational rearrangement in the membrane environment; however, the changes were similar for the active and inactive dendrimers (Figure 2A and Figure S2).

Conformational changes were furthermore visible for the active AMPD G3KL by variations in the hydrodynamic radius R_h in different media as determined by diffusion NMR spectroscopy. G3KL contracted upon neutralization from acidic pH values (pH 2, $R_h = 2.10 \pm 0.01$ nm) to neutral aqueous pH values (pH 7.4, $R_h = 1.08 \pm 0.01$ nm), and opened up again to a more extended conformation in a membrane-like environment (pH 7.4, 50 % TFE, $R_h = 2.58 \pm 0.02$ nm). The remarkably compact conformation of G3KL in neutral aqueous medium probably reflects a hydrophobic collapse of its fifteen hydrophobic leucine side chains, which is partially compensated for by electrostatic repulsion between multiple protonated ammonium groups at low pH values and by a weakened hydrophobic effect in the membrane-like TFE environment. In contrast, these variations were much smaller in the inactive dendrimer G3KA where the hydrophobic Leu residues in the G3 branch had been exchanged for Ala (pH 2,

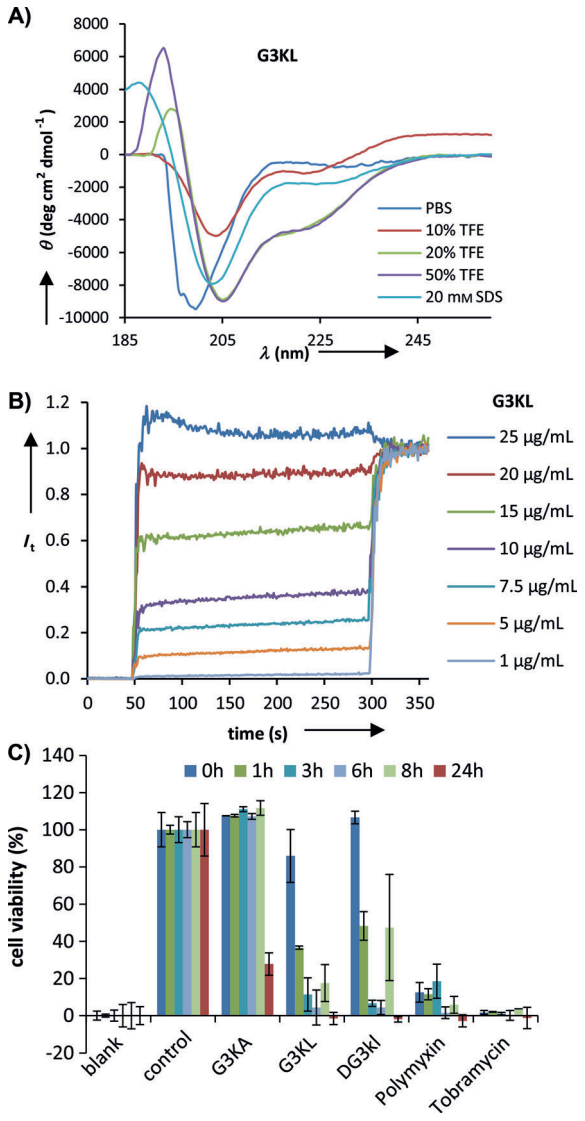

Figure 2. A) CD spectra of G3KL (100 μg mL^{-1}) in different media. B) 5(6)-Carboxyfluorescein leakage from phosphatidylglycerol lipid vesicles. Addition of G3KL to a lipid vesicle solution in buffer (10 mM TRIS, 107 mM NaCl, pH 7.4) at 50 s and addition of 1.2% Triton X-100 at 300 s. Fluorescence intensities were normalized to the fractional emission intensity $I(t)$ using $I(t) = (I_t - I_0)/(I_\infty - I_0)$ where $I_0 = I_t$ at peptide dendrimer addition, $I_\infty = I_t$ at saturation of lysis. C) Cell viability of active and inactive peptide dendrimers measured in two independent duplicates, one experiment shown. *P. aeruginosa* were incubated with the antimicrobial compound (25 μg mL^{-1}) at 37 °C for 0, 1, 3, 6, 8, 24 h. After addition of WST-8 and incubation, the absorbance was measured at 450 nm. See the Supporting Information for details and additional data.

$R_h = 2.41 \pm 0.02$ nm; pH 7.4, $R_h = 2.10 \pm 0.03$ nm; pH 7.4, 50% TFE, $R_h = 2.46 \pm 0.1$ nm), which suggests that these residues not only enhance the hydrophobicity of G3KL but also enable medium-dependent conformational changes, which might contribute to the antimicrobial effect.

The preferential interaction of AMPDs with the bacterial membrane was further studied by fluorescein leakage experiments with large unilamellar vesicles (LUVs) composed either of phosphatidyl glycerol (PG), an anionic lipid mimicking a bacterial membrane, or of phosphatidyl choline (PC), a neutral head group lipid representative of the mammalian cell membrane.[21] Addition of the AMPDs G3KL or DG3kl or the known membrane disruptor polymyxin[1d] to fluorescein-loaded LUVs consisting of the anionic lipid PG resulted in a concentration-dependent increase of fluorescence (Figure 2B and Figure S3). In contrast, the inactive AMPDs G3KA and G3KK1 showed no immediate increase even at 100 μg mL^{-1} and only negligible leakage at longer incubation times. Tobramycin, an antibiotic that interferes with the biosynthesis of proteins in the bacteria, also did not show release of CF (CF = 5(6)-carboxyfluorescein). Exposing fluorescein-loaded neutral PC-LUVs to G3KL, dG3kl, G3KA, G3KK1, tobramycin, or polymyxin did not induce CF release even at high concentrations. Overall, the experiments showed that active AMPDs interacted with the negatively charged lipids in LUVs to release CF whereas inactive peptide dendrimers did not, supporting the hypothesis that AMPDs act by a membrane-disruption mechanism.

Although fast killing is generally expected from membrane-disrupting compounds,[3b] we found that AMPDs were generally rather slow-acting. The kinetics of bacterial cell death were determined by measuring the amount of live bacteria as a function of time using phenazine ethosulfate and the formazan dye precursor WST-8.[22] The AMPDs G3KL and DG3kl required three hours for complete killing, and G3KA was inactive, whereas the control antibiotics polymyxin and tobramycin were both fast-acting independent of their mechanism of action (Figure 2C).

Considering that AMPDs should have a mode of action similar to AMPs, it is expected that treatment with AMPDs should not lead to a fast resistance development.[23] Resistance development against G3KL and DG3kl was investigated only for *B. subtilis* using the standard broth dilution assay with daily dilution from the $^1/_2$ MIC well upon a period of 15 days without significant changes in MIC values, suggesting that resistance development to AMPDs might indeed be slow.

In summary, a systematic survey of the peptide sequences distributed in the branches of G3 peptide dendrimers led to the discovery of G3KL and analogues as a new type of AMPDs with strong antimicrobial activities against *P. aeruginosa* and *A. baumannii* strains, including multidrug-resistant clinical isolates, as well as low hemolysis and good stability and strong activity in human serum. These favorable properties may be assigned to the combination of a specific dendrimer topology giving access to a relatively large structure (37 residues) with a precise amino acid sequence enabling the disruption of bacterial membranes. The rapid synthesis (only 11 SPPS cycles) and the use of standard amino acids only present significant advantages for the potential development of AMPDs as a new class of antimicrobial agents. The combination of peptide topology and sequence design as illustrated here represents a new and general strategy for the discovery of new antimicrobials to fight multidrug-resistant bacterial pathogens. Further activity optimization and biological evaluation studies of the AMPDs are currently underway.

Received: September 19, 2014
Published online: October 24, 2014

Keywords: antibiotics · antimicrobial peptides · dendrimers · *Pseudomonas aeruginosa* · peptides

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