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RESEARCH ARTICLE

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Inverse Polyamidoamine (i-PAMAM) Dendrimer Antimicrobials

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This Publication is dedicated to Prof. *Robert Deschenaux*, a pioneer of dendrimers, on the occasion of his 65th birthday

Here we redesigned the branches of polyamidoamine (PAMAM) dendrimers by moving the amide carbonyl group on the other side of the amide nitrogen atom, transforming the β-alaninyl-amidoethylamine branch, which easily undergoes retro-*Michael* reactions and renders PAMAMs intrinsically unstable, into a more stable glycyl-amidopropylamine branch. The resulting inverse PAMAM (i-PAMAM) dendrimers have the same carbon framework as PAMAMs and only differ by the position of the carbonyl group. In contrast to PAMAMs which are prepared in solution and are difficult to purify, we synthesize i-PAMAMs using high-temperature solid-phase peptide synthesis by iterative coupling and deprotection of the commercially available *N,N*-bis(N′-Fmoc-3-aminopropyl)glycine and purify them preparative reverse phase HPLC. Our i-PAMAM dendrimers show no detectable degradation over time. We demonstrate this new class of dendrimers with the synthesis of antimicrobial dendrimers with potent yet non-membrane disruptive activities against both *Gram*-negative and *Gram*-positive bacteria.

Keywords: antimicrobials, dendrimers, PAMAM dendrimers, peptides, polycations, solid-phase synthesis.

Introduction

Besides it's esthetic appeal, the multi-branched, tree-like architecture of dendrimers is unavoidably attractive to synthetic chemists because it allows to assemble relatively large molecules in a few iterative coupling steps, as has been exemplified over the years using various building blocks and coupling chemistries.[1–4] Most interestingly, dendrimers display emerging properties not available in their constituent building blocks such as microenvironment effects in the dendrimer core as well as multi-valency, cooperativity, and local concentration effects in the dendrimer branches. These emergent properties have been exploited in diverse applications ranging from technology to medicine.[5–9]

A large part of dendrimer chemistry has been realized by functionalizing the terminal amino groups of polyamidoamine (PAMAM) dendrimers, which consist of β-alanyl-amidoethylamine branches connecting tertiary amine branching points,[10,11] as exemplified in the recently reported antimicrobial lipidated PAMAM **1** (*Figure 1*).[12] PAMAMs are synthesized by double *Michael* addition of ethyl acrylate to a primary amine followed by aminolysis of the two ester groups with ethylene diamine, repeating the cycle until a given size has been reached (*Figure 1*). Unfortunately, the blessing of this simple synthetic procedure is also its curse because the *Michael* addition is slowly reversible, such that PAMAM dendrimers can undergo retro-*Michael* reactions of the β-alanine unit in their branches resulting in partial disassembly.[13,14] The labile β-alanine unit was maintained in a PAMAM analog prepared on solid support by iterative *Michael* addition using ethyl acrylate and 1,3-diaminopropane building blocks,[15] as well as in an inverse PAMAM

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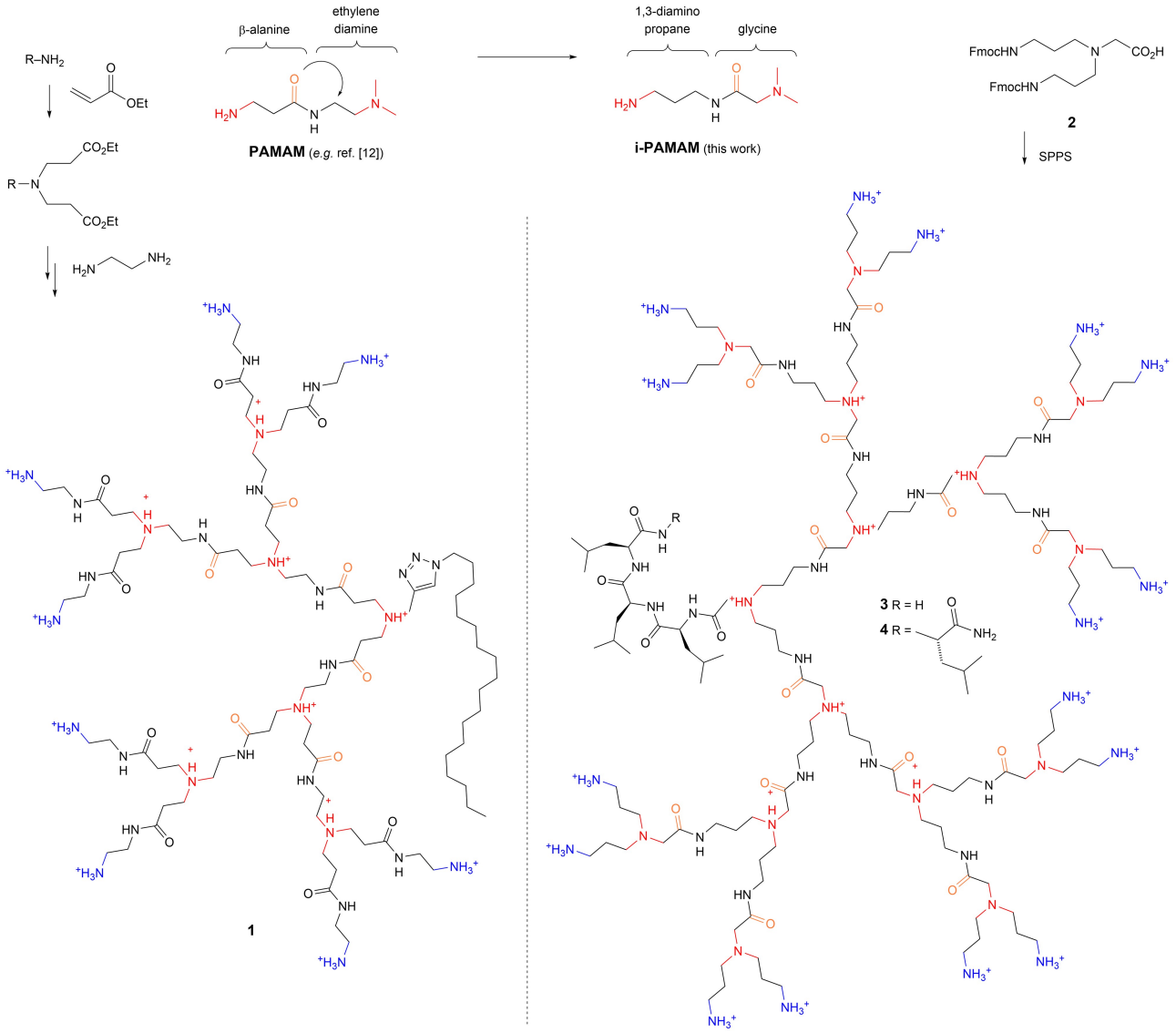


Figure 1. Design, synthesis concept and examples of antibacterial PAMAM (**1**) and i-PAMAM (**3**, **4**) dendrimers. The dendrimers are shown in their protonation state as predicted for neutral pH. See *Figures S4* and *S5* for predicted pK_a values.

obtained using solid-phase peptide synthesis by iterative coupling of *N,N*-bis(N′-Boc-3-aminopropyl)β-alanine.[16,17]

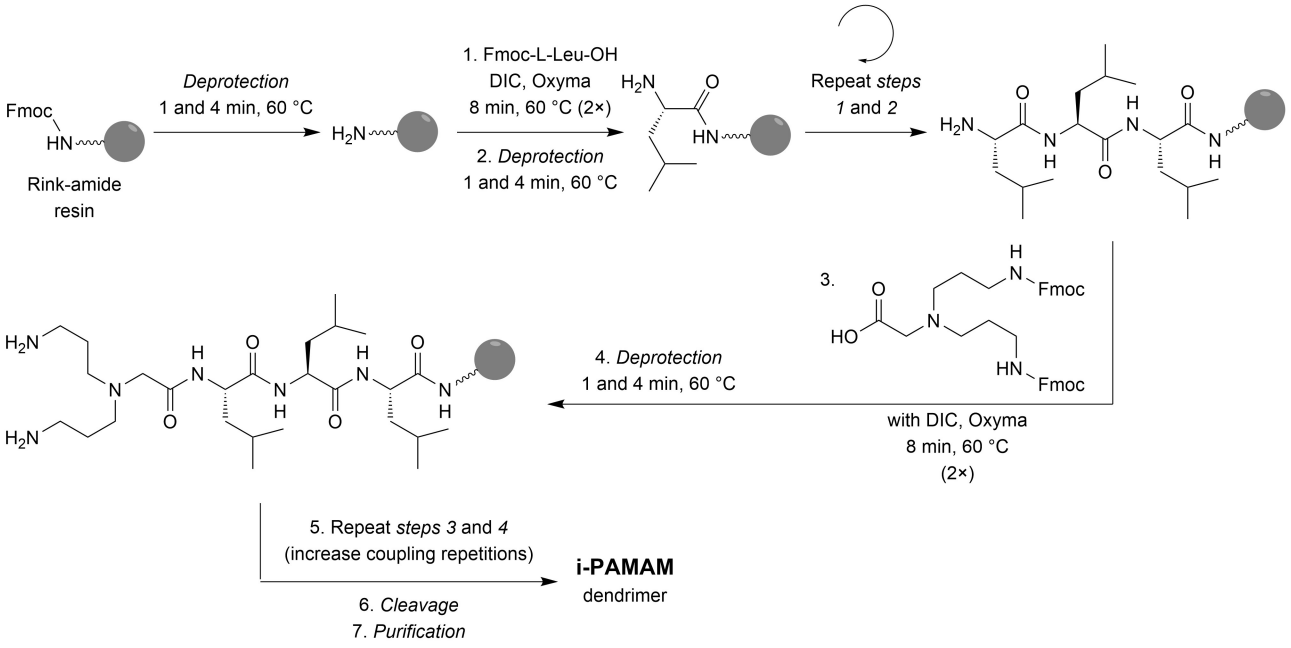
Here we built on our own experience of obtaining peptide dendrimers[18–21] as pure and stable products from solid-phase peptide synthesis (SPPS).[22–24] We envisioned that a stable PAMAM analog should result from simply moving the carbonyl group of β-alanine across the amide bond. Indeed, this simple change transforms the β-alanine unit into 1,3-diaminopropane and the ethylene diamine unit into glycine, thereby eliminating the possibility of a retro-*Michael* reaction (*Figure 1*). This transformation furthermore inverts the direction of the amide bond, such that the dendrimer should be accessible by SPPS using *N,N*-bis(N′-Fmoc-3-aminopropyl)glycine **2**. This building block is commercially available and has been used to prepare branched peptides,[25] but to the best of our knowledge has never been tested for dendrimer synthesis. Herein we demonstrate this approach at the example of i-PAMAM dendrimers **3** and **4** obtained by extending a tri- respectively tetra-leucine peptide with four successive generations of the i-PAMAM dendron **2** (*Figure 1*). The i-PAMAM dendrimers **3** and **4** exhibit strong antibacterial effects on *Gram*-negative bacteria. In contrast to other antimicrobial peptide dendrimers[26–30] and related compounds, however, **3**

and **4** do not disrupt the bacterial membrane and presumably act on intracellular targets.

Results and Discussion

Design, Synthesis and Properties of i-PAMAMs

In view of our previous experience with antimicrobial peptide dendrimers with a hydrophobic core[27] and the recent report of the similar antimicrobial PAMAM **1**,[12] we hypothesized that extending a hydrophobic tri- or tetra-leucine peptide by iterative coupling of the inverted PAMAM building block **2** might provide a dendrimer with sufficient hydrophobicity to be purifiable by reverse-phase HPLC and possibly exhibiting interesting antimicrobial properties. We carried out our synthesis by Fmoc SPPS on Rink-amide resin using *OxymaPure*[31] and diisopropyl carbodiimide as coupling reagents in DMF at high temperature (60 °C) with eight minutes coupling time (*Scheme 1*). Couplings were repeated twice during the formation of the core, the first generation and the second generation, four and six times in the third and fourth generations, respectively. Acidic cleavage and deprotection followed by purification using preparative reversed-phase HPLC yielded i-PAMAMs **3** and **4** in 10% and 8% yield, which are comparable to SPPS yields for peptide dendrimers synthesized under similar reaction conditions, and excellent purity as assessed by LCMS and HRMS data (*Table 1* and *Supporting Information*).



Scheme 1. Synthetic scheme for the SPPS.

Table 1. Synthesis and physico-chemical properties of i-PAMAM.

Compound	Sequence[a] i-PAMAM:	SPPS Yield[b]	Retention Time[c]	MS Analysis[d] calc./obs.	Hydrodynamic Radius (R_h)[e]
3	$(Bag)_8(Bag)_4(Bag)_2Bag$LeuLeuLeu	43 mg (10%)	1.80 min	2923.34/2923.3228	2.51 nm
4	$(Bag)_8(Bag)_4(Bag)_2Bag$LeuLeuLeuLeu	35 mg (8%)	1.99 min	3036.42/3036.4061	2.41 nm

[a] Three letter code for amino acids, Bag = bis(3-aminopropyl)glycine and Leu = L–Leucine, branching residues in italics. [b] Yield given for RP-HPLC purified product, calculated from the theoretical 100% yield from the quantity and the resin loading given by the supplier. [c] Measured on analytical RP-HPLC with a 7.50 min gradient, from solvent system A/D 100:0 to 0:100. Elution solutions were A) *MilliQ* deionized water containing 0.05% TFA; D) *MilliQ* deionized water/acetonitrile (10:90, *v/v*) containing 0.05% TFA. $\lambda = 214$ nm. [d] Electrospray ionization mass spectrometry (positive mode), the calculated monoisotopic masses and the observed masses for [M] are reported. [e] Hydrodynamic radius (R_h) in nm, calculated from the diffusion coefficient D [$m^2 \cdot s^{-1}$] obtained by DOSY NMR and after solving the *Stokes–Einstein* equation: $R_h = k_B \cdot T/(6 \cdot \pi \cdot \eta \cdot D)$ with *Boltzmann* constant $k_B = 1.380 \cdot 10^{-23}$ [$J \cdot K^{-1}$], temperature $T = 298$ [K] and viscosity of D_2O $\eta = 1.089$ [mPa·s].

The two i-PAMAM dendrimers exhibited very hydrophilic properties compared to our previously reported dendrimers,[29,30] as indicated by their very short retention time in the column of the RP-HPLC, even for compound **4** bearing four leucines in its core. ^{1}H-NMR spectra were consistent with the expected structures, with only minor impurities, which appeared to be low molecular weight by diffusion ordered spectroscopy (DOSY, see *Supporting Information*). The DOSY analysis, carried out for the trifluoroacetate salts in D_2O, provided an estimate of 2.40 to 2.50 nm for the hydrodynamic radii, which is comparable to previous measurements with related peptide dendrimers.[26] Acid-base titration of the trifluoroacetate salts of **3** and **4** with NaOH showed a broad buffering zone from pH ~ 3 to pH ~ 6 followed by a sharp rise to pH ~ 8 and a second buffering zone up to pH ~ 11 (*Figure S2*). This data was consistent with neutralization at low pH of excess trifluoroacetic acid and eight tertiary amines in the G4 unit, predicted to have acidic pK_a, followed by neutralization at high pH of the tertiary amines in the G1, G2 and G3 levels and the terminal primary amines, all predicted to have basic pK_a (*Figures S3–S5*). Interestingly, both dendrimers were entirely stable against degradation in human serum after incubation for 24 hours at 37 °C, showing that the i-PAMAM dendrimer is resistant to proteolysis and additionally shields the dendrimer core from proteases (*Table 1* and *Supporting Information*).

Antimicrobial Activities

The antimicrobial properties of the two dendrimers **3** and **4** were first evaluated on the *Gram*-negative bacterium *P. aeruginosa* PAO1. While there was no activity when tested in full medium (*Mueller–Hinton* broth *MHB*), we observed strong antibacterial effects in diluted medium (12.5 % *MHB*, *Table 1*), conditions under which proline rich AMPs such as oncocin show their activity.[32–35] Dilute media better reproduce the situation in tissues where nutrients are scarce.[36] The two dendrimers showed a similar activity under slightly basic conditions (pH 8.5), which we have found to increase the activity of polycationic dendrimers and of the cyclic peptide polymyxin B.[37] The dendrimers showed very low hemolysis of human red blood cells (hRBC) as measured by minimum hemolytic concentration (*MHC* = > 1000 μg/mL, *Table 1*), indicating a good selectivity for bacteria over eukaryotic cells.

We observed similarly strong activities against a broader panel of bacteria, including *P. aeruginosa* strains selected to be resistant to dendrimers,[38] further *Gram*-negative (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli*) and *Gram*-positive (*Staphylococcus aureus* COL 'MRSA') bacteria listed as 'priority pathogens' from the World Health Organization (*Table 2*).[39] However, these strong activities only appeared at pH 8.5 in dilute medium. Activities were much weaker at pH 7.4 and completely absent in full medium.

Time-kill assays, which assess the number of remaining colony forming units (cfu) over time upon exposure to the compounds, confirmed the bactericidal effect of **3** and **4** (*Figure 2,a* and *2,b*). However, the rate of killing was comparable to that of polymyxin B and somewhat slower than for membrane disruptive compounds such as our peptide dendrimers, which typically completely kill bacteria within 30 minutes.[24, 29, 37] Indeed, both dendrimers were entirely inactive in a vesicle leakage assay measuring the release of 5(6)-carboxyfluorescein from synthetic vesicles consisting of egg white phosphatidyl glycerol (EYPG) which mimics bacterial membranes (*Figure 2,b*).[40] Both dendrimers were also entirely inactive on vesicle consisting of egg white phosphatidyl choline (EYPC) that mimics eukaryotic membranes, in line with the fact that they were non-hemolytic. The absence of membrane activity suggests that our dendrimers do not kill the bacteria via a membrane-disruptive mode of action, therefore they probably have an intracellular target, similar to observations made with many polycationic antimicrobial peptides.[41–46]

Conclusions

In summary, we synthesized two i-PAMAM dendrimers consisting of a hydrophobic oligo-leucine peptide combined with four successive generations of the *N,N*-bis(aminopropyl)glycine dendron. We used a conventional high-temperature SPPS protocol with commercially available building blocks, and obtained good yields and purities of isolated products without optimization. These i-PAMAMs behave as standard peptides and can be characterized by their LC/MS profiles and High-Resolution Mass Spectra. Initial assessment showed that our test i-PAMAMs **3** and **4** are stable against serum degradation, are non-hemolytic, and display interesting non-membrane disruptive antibacterial activities, reproducing the properties of non-membrane targeting antimicrobial peptides such as oncocins. Due to their ease of synthesis, stability and biocompatibility, we believe that i-PAMAMs have

Table 2. Antimicrobial activities (*MIC*)[a], hemolytic activity (*MHC*)[b] and serum stability of i-PAMAMs.

Compound	Medium (*MHB*)	pH	*P. aeruginosa* PAO1	*P. aeruginosa* PA14	*P. aeruginosa* PA14 4.13	*P. aeruginosa* PA14 4.18	*K. pneumoniae* NCTC418	*A. baumannii* ATCC19606	*E. coli* W3110	*S. aureus* COL (MRSA)	hRBC *MHC*[b] [μg/mL]	Serum stability after 24 h [%][c]
3	full	7.4	>32				>32	>32	>32	>32	>1000	96
3	full	8.5	32				>32	32	16	8–16	>1000	96
3	12.5%	7.4	2	1	2	1	16	8	4–8	16–32	>1000	96
3	12.5%	8.5	2	1	2	1	4	2	1	1	>1000	96
4	full	7.4	>32				>32	>32	>32	>32	>1000	99
4	full	8.5	n.d.				n.d.	n.d.	n.d.	n.d.	>1000	99
4	12.5%	7.4	2	0.5	1	1	32	8	8	16–32	>1000	99
4	12.5%	8.5	2	1	2	1	8	2–4	0.5–1	2	>1000	99
PMB[d]	full	7.4	0.5				0.25	0.25	0.25	>32		
PMB[d]	full	8.5	<0.125				<0.125	<0.125	<0.125	2		
PMB[d]	12.5%	7.4	0.5	0.5	0.5	0.5	0.5	0.25	0.5	16		
PMB[d]	12.5%	8.5	0.5	0.25	0.25	0.25	0.25–0.5	0.25	0.25	2		
VCM[e]	full	7.4	>16				>16	>16	>16	0.5		
VCM[e]	full	8.5	>16				>16	>16	>16	2		
VCM[e]	12.5%	7.4	>16	>16	>16	>16	>16	>16	8	2		
VCM[e]	12.5%	8.5	>16	>16	>16	>16	16	>16	16	0.5		

[a] Minimum inhibitory concentration (*MIC*), in μg mL^{-1}, was determined on bacteria in diluted *Mueller–Hinton* broth (12.5% MHB), at pH 7.4 and 8.5, after incubation for 16–20 h at 37 °C. Values represent at least two different triplicate *MIC* determinations. [b] Minimum Hemolytic Concentration (*MHC*) measured on human red blood cells (hRBC) in 10 mM phosphate buffer, 150 mM NaCl, pH 7.4, 25 °C, 4 h. [c] Serum stability assay of the respective compound (200 μM), incubated with human serum (12.5% in TRIS buffer, 0.1 M, pH 7.4), at 37 °C. Normalized undegraded dendrimer values determined by RP-HPLC analysis using 4-hydroxybenzoic acid as internal standard. Data given as a percentage of undegraded compound after 24 h of incubation. [d] Polymyxin B. [e] Vancomycin.

a great potential as dendrimer scaffolds for biological applications. Future experiments will address the optimization of antimicrobial properties of i-PAMAMs by varying the dendrimer core, branches and N-termini, as well as the exploration of other types of biological activities accessible to biocompatible dendrimers.

Experimental Section

Peptide Synthesis

Material and Methods. DMF (*N,N*-dimethylformamide) was purchased from *Thommen-Furler AG*, Ethyl 2-cyano-2-(hydroxyimino)acetate (*Oxyma pure*) was purchased from *SENN AG*, *N,N′*-diisopropyl carbodiimide (DIC) and *N,N*-bis(N′-Fmoc-3-aminopropyl)glycine were purchased from *Iris Biotech GMBH*, piperazine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1-butanol, *N,N*-diisopropylamine (DIPEA), triisopropylsilane (TIS), phenylsilane was purchased from *TCI* (*Tokyo Chemical Company*) and trifluoroacetic acid (TFA) purchased by *Fluorochem Ltd. Chemicals* were used as supplied and solvents were of analytical grade. The L-leucine amino acid was purchased from *Shanghai Space Peptides Pharmaceuticals Co., Ltd* and used as the following derivatives: Fmoc-Leu-OH and Fmoc-protected Rink Amide *TentaGel S RAM* resin was purchased from *Rapp Polymere GmbH*.

LC and MS. Analytical RP-HPLC was performed with an *Ultimate 3000 Rapid Separation* LC–MS System (*DAD-3000RS* diode array detector) using an *Acclaim RSLC 120 C18* column (2.2 μm, 120 Å, 3×50 mm, flow 1.2 mL/min) from *Dionex*. Data recording and processing was done with Dionex Chromeleon Management System Version 6.80 (analytical RP-HPLC). All RP-HPLC were using HPLC-grade acetonitrile and *Milli-Q* deionized water. The elution solutions were *A*) *MilliQ* deionized water containing 0.05% TFA; *D*) *MilliQ* deionized water/acetonitrile (10:90, *v/v*) containing 0.05% TFA. Preparative RP-HPLC was performed with a *Waters* automatic Prep LC Controller system containing the four following modules: *Waters 515* HPLC pump, *Waters 2489* UV/vis detector, *SQ Detector 2* (single quadrupole mass detector and *Waters 2767* Sample Manager, Injector and Collector. A *Dr. Maisch GmbH Reprospher* column (*C18-DE*, 100×30 mm, particle size

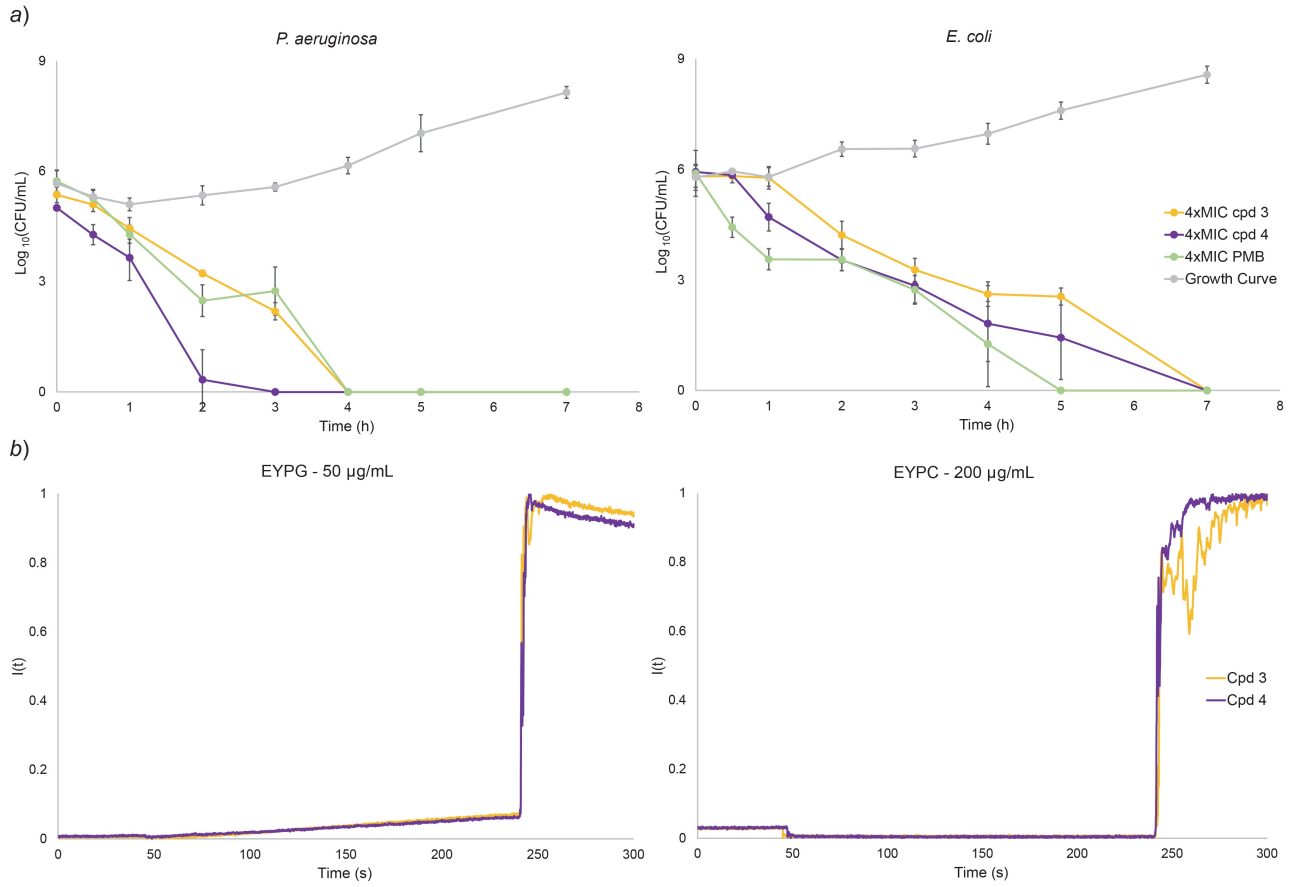


Figure 2. Killing profile and activities of i-PAMAM on bacteria and 'membrane-like' vesicles. *a*) Bacteria killing assay against *P. aeruginosa* PAO1 and *E. coli* W3110. The assay was performed with a compound's concentration equal to 4× its *MIC* value for the respective bacteria strain; **3** and **4** (8 µg/mL for *P. aeruginosa* PAO1 and 4 µg/mL for *E. coli* W3110), **PMB** (2 µg/mL for both strains) as a positive control and the growth curve as a negative control. Each point represents the average value of triplicates, measured twice. $Log_{10}(CFU/mL) = 0$ means no bacteria could be detected anymore. *b*) Lipid vesicles containing 5(6)-carboxyfluorescein made of egg yolk phosphatidyl choline (EYPC), mimicking eukaryotic membranes and egg yolk phosphatidyl glycerol (EYPG) that mimics bacterial membranes, were both suspended a TRIS buffer (10 mM TRIS, 107 mM NaCl, pH 7.4). After 45 s, compound **3** or **4** was added at the indicated concentration. *Triton X-100* (1.2%) was added after 240 s for complete fluorescein release. The data were normalized to the minimal and maximal intensities.

5 µm, pore size 100 Å, flow rate 40 mL/min) was used. Compounds were collected according to their mass using SQ Detector 2. Data recording and processing was performed with MassLynx software version 4.2. The elution solutions were: *A*) *MilliQ* deionized water containing 0.05% TFA; *D*) Acetonitrile containing 0.05% TFA. Method used was a gradient from 2% to 50% of solvent *D* in 14 min with a total runtime of 20 min. MS Spectra, recorded on a *Thermo Scientific LTQ OrbitrapXL*, were provided by the MS analytical service of the Department of Chemistry, Biochemistry and Pharmaceutical Sciences at the University of Bern (group PD Dr. *Stefan Schürch*).

NMR Spectra (^1H, ^{13}C). NMR Spectra were recorded at 22 °C unless otherwise stated. Chemical shifts (δ) are reported in ppm relative to the signal of tetramethylsilane (TMS) and residual solvent signals in ^{1}H- and ^{13}C-NMR spectra were used as internal reference. Atom to peak assignment was performed through standard 2D-NMR techniques such as COSY, HSQC, HMBC, and NOESY. ^{1}H- and ^{13}C-NMR spectra were measured either on a *Bruker Avance III 300* spectrometer (at 300 MHz and 75 MHz, resp.) or on a *Bruker Avance II 400* spectrometer (at 400 MHz and 101 MHz, resp.).

DOSY NMR. Standard diffusion NMR experiments were performed using a *Brucker Avance 400* MHz with diluted solutions of dendrimers in D_2O (*ca.* 7–

10 mg mL^{-1}, pH ~ 3, at 298 K). The gradient with a maximum strength of 50·10^{-4} T·cm^{-1} was calibrated using the HOD proton signal in D_2O (99.997 %). The diffusion time Δ was set at 125 ms and the gradient duration δ at 7 ms. Data analysis was performed by using MestReNova V14.2.0 (software from *Mestrelab Research*, https://mestrelab.com/) and the diffusion coefficient D [m^2·s^{-1}] was derived from peak integrals or intensities. The hydrodynamic radii were calculated from the diffusion coefficient *D* using the *Stokes–Einstein* equation $R_h = k_B \cdot T/(6 \cdot \pi \cdot \eta \cdot D)$ with *Boltzmann* constant $k_B = 1.380 \cdot 10^{-23}$ J·K^{-1}, temperature *T* in K and viscosity $\eta = 1.089$ mPa·s for D_2O.

Synthesis of i-PAMAM (SPPS). Inverted PAMAM were synthesized manually following the standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis procedures. The syntheses were performed at 60 °C under nitrogen bubbling. All dendrimer syntheses were carried out by using Rink Amide *TentaGel S RAM* (100–200 mesh), unloaded (0.22 mmol/g), 0.066 mmol scale (300 mg of resin). The resin was swollen in DMF for 30 min at 60 °C and deprotected twice, 1 and 4 min, respectively, using a deprotection cocktail containing piperazine (5 %), DBU (2 %) and butanol (10 %) in DMF, at 60 °C. 5 equiv. of Fmoc-protected amino acid/*N,N*-bis(N′-Fmoc-3-aminopropyl)glycine with a concentration of 0.2 M together with 5 equiv. of *Oxyma* and 6 equiv. of DIC, with a respective concentration of 0.4 M and 0.45 M, were used as coupling reagents in 4.5 mL of DMF. The reaction was stirred for 8 min at 60 °C, washed two times with 6 mL DMF, and a second coupling step was performed under the same conditions. The resin was then washed three times with 6 mL DMF. For the second generation of the dendrimer, four couplings were performed (4×8 min, 60 °C, 2× washing in between) and six coupling steps were used for the third generation. After each coupling steps, the Fmoc protecting group was removed with two times 6 mL of a deprotection cocktail containing piperazine (5 %), DBU (2 %) and butanol (10 %) in DMF, respectively for 1 and 4 min at 60 °C. The resin was then washed four times with DMF (6 mL). The cleavage was carried out by treating the resin with 7 mL of a TFA/TIS/H_2O (94 : 5 : 1, *v/v/v*) solution for 3 h at room temperature. The dendrimer solution was then precipitated with 25 mL of *tert*-butyl methyl ester (TBME), centrifuged for 10 min at 4′000 rpm (twice), evaporated and dried with argon. The dried crude was dissolved in a water/acetonitrile mixture, filtered (pore size 0.45 µm) and purified by two consecutive preparative RP-HPLC with a gradient of 2–20 % solvent *B* in 20 min. The pure fraction was analyzed by LC–MS with a 5 min gradient. Pure products were obtained as white solids after lyophilization. The yields were calculated for the TFA salts.

i-PAMAM dendrimer 3 was obtained as a foamy white solid after preparative RP-HPLC (43.1 mg, 10 %). Analytical RP-HPLC: $t_R = 1.80$ min (*A/D* 100:0 to 0:100 in 7.50 min, $\lambda = 214$ nm). ESI-MS (pos.): $C_{138}H_{291}N_{49}O_{18}$ calc./obs. 2923.34/2923.3228 Da [M].

i-PAMAM dendrimer 4 was obtained as a foamy white solid after preparative RP-HPLC (34.7 mg, 8 %). Analytical RP-HPLC: $t_R = 1.99$ min (*A/D* 100:0 to 0:100 in 7.50 min, $\lambda = 214$ nm). ESI-MS (pos.): $C_{140}H_{302}N_{50}O_{19}$ calc./obs. 3036.42/3036.4061 Da [M].

Antimicrobial Activity (MIC)

i-PAMAM dendrimers cytotoxicity was assayed against *P. aeruginosa* PAO1, *P. aeruginosa* PA14, *P. aeruginosa* PA14 4.13, *P. aeruginosa* PA14 4.18, *P. aeruginosa* PA14 2P4, *K. pneumoniae* NCTC418, *E. coli* W3110, *A. baumannii* ATCC 19606 and *S. aureus* COL (clinical isolate of MRSA).

To determine the Minimal Inhibitory Concentration (*MIC*), Broth Microdilution method was used.[47] A colony of bacteria from glycerol stock was grown in Lysogeny broth (LB) overnight at 37 °C and agitated at 180 rpm. The compounds were prepared as stock solutions of 2 mg/mL in sterilized *MilliQ* deionized water, added to the first well of a 96-well sterile, round bottom microtiter plates in polypropylene (*Costar*®, untreated) and diluted serially by ½. The concentration of the bacteria was quantified by measuring absorbance at 600 nm and diluted to an OD_{600} of 0.022 in 12.5 % MHB (pH 7.4 or 8.5). The sample solutions (150 µL), prepared at the desired concentration in 12.5 % MHB (pH 7.4 or 8.5), were mixed with 4 µL diluted bacterial suspension with a final inoculation of about 5×10^5 CFU. For each test, two columns of the plate were kept for sterility control (12.5 % MHB only, pH 7.4 or 8.5), growth control (12.5 % MHB with bacterial inoculum, no compound, pH 7.4 or 8.5). The positive control, Polymyxin B (starting with a concentration of 16 µg/mL) in 12.5 % MHB (pH 7.4 or 8.5) with bacterial inoculums, was introduced in the two first lines of the plate. The plates were incubated at 37 °C for *ca.* 18 h under static conditions. 15 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT)[48] (1 mg/mL in sterilized *MilliQ* deionized water) were added to each well and the plates were incubated for 30 min at room temperature. The minimal inhibitory concentration (*MIC*) was defined as the lowest concentration of the compound that inhibits the visible growth of the tested bacteria (yellow) with the unaided eye.

Time-Killing Assay

A single colony of *P. aeruginosa* PAO1 and *E. coli* was picked and grown overnight with shaking (180 rpm) in LB (*Sigma Aldrich*, Buchs, Switzerland) medium 5 mL overnight at 37 °C. The overnight bacterial culture was diluted to OD_{600} of 0.002 (2×10^6 CFU/mL) in fresh diluted MHB (*Sigma Aldrich*, Buchs, Switzerland, 12.5% at pH 8.5) medium. Stock solutions of dendrimers in sterilized *MilliQ* water were prepared in 1 mg/mL and were diluted to two times more than required concentration in 12.5% MHB at pH 8.5. 100 μL prepared bacteria solution in MHB and 100 μL samples in MHB were mixed in 96-well microtiter plate (TPP, untreated, *Corning Incorporated*, Kennebunk, USA). Untreated bacteria at 1×10^6 CFU/mL were used as a growth control. 96-well microtiter plates were incubated in 37 °C with shaking (180 rpm). Surviving bacteria were quantified at 0, 0.5, 1, 2, 3, 4, 5 and 7 h by plating 10-fold dilutions of sample in sterilized normal saline on LB agar plates. LB agar plates were incubated at 37 °C for 10 h and the number of individual colonies was counted at each time-point. The assay was performed twice in triplicate.

Hemolysis Activity

To determine the minimal hemolytic concentration (*MHC*), stock solutions of 8 mg/mL of the dendrimer in sterilized *MilliQ* water deionized were prepared and 50 μL were diluted serially by $^1/_2$ in 50 μL PBS (pH 7.4) in 96-well plate (*Costar* or *Nunc*, polystyrene, untreated). Human red blood cells (hRBC) were obtained by centrifugation of 1.5 mL of whole blood, from the blood bank of Bern, at 3000 rpm for 15 min at 4 °C. Plasma was discarded and the pellet was re-suspended in a 15 mL *Falcon* tube in 5 mL of PBS. The washing was repeated three times and the remaining pellet was re-suspended in 10 mL of PBS at a final hRBC concentration of 5%. The hRBC suspension (50 μL) was added to each well and the plate was incubated at room temperature for 4 h. Minimal hemolytic concentration (*MHC*) end points were determined by visual determination of the wells after the incubation period. Controls on each plate included a blank medium control (50 μL PBS + 50 μL of hRBC suspension) and a hemolytic activity control *t*-Octylphenoxypolyethoxyethanol (*Triton-X100*; 1% in *MilliQ*-deionized water) 50 μL + 50 μL hRBC suspension.

Lipid Vesicle Leakage Assay

5(6)-Carboxyfluorescein (CF) was purchased from *Sigma Aldrich*. Egg Yolk Phosphatidylcholine (EYPC), Egg Yolk Phosphatidylglycerol (EYPG) and a Mini-Extruder were purchased from *Avanti Polar Lipids*. Egg PC or Egg PG thin lipid layers were prepared by evaporating a solution of 100 mg EYPC or EYPG in 4 mL MeOH/$CHCl_3$ (1:1) on a rotary evaporator at room temperature and then dried in vacuo overnight. The resulting film was then hydrated with 2 mL CF buffer (50 mM CF, 10 mM TRIS, 10 mM NaCl, pH 7.4) for 30 min, subjected to freeze-thaw cycles (7×) and extrusion (15×) through a polycarbonate membrane (pore size 100 nm). Extra vesicular components were removed by gel filtration (*Sephadex G-50*) with 10 mM TRIS, 107 mM NaCl, pH 7.4 buffer. Final conditions: *ca.* 2.5 mM PC or PG; inside: 50 mM CF, 10 mM TRIS, 10 mM NaCl, pH 7.4 buffer; outside: 10 mM TRIS, 107 mM NaCl, pH 7.4. PC or PG stock solutions (37.5 μL) were diluted to 3000 μL with a buffer (10 mM TRIS, 107 mM NaCl, pH 7.4) in a fluorescence cuvette (at 25 °C) and gently stirred (final lipid concentration *ca.* 31 μM). CF efflux was monitored at λ_{em} 517 nm (λ_{ex} 492 nm) as a function of time after addition of the desired volume of dendrimer (1 or 20 mg/mL stock in *MilliQ* water) at $t = 45$ s. The following final concentrations of dendrimer were monitored: 10 and 200 μg/mL for EYPC, 10 and 50 μg/mL for EYPG. Finally, 30 μL of 1.2% *Triton X-100* was added to the cuvette (0.012% final concentration) at t = 240 s to reach the maximum intensity. Fluorescence intensities were then normalized to the maximal emission intensity using $I(t) = (I_t - I_{max})/(I_{max} - I_{min})$ where I_{min} at lowest intensity value and $I_{max} = I_t$ at saturation of lysis.

Serum Stability Assay

Human serum was diluted in 0.1 M filtered TRIS buffer pH 7.4 (25%, 1:3, *v/v*). i-PAMAM dendrimers were diluted in 0.1 M filtered TRIS buffer pH 7.4 to a concentration of 400 μM and 0.1 mg/mL of 4-hydroxybenzoic acid was added as internal standard. Aliquots of dendrimer solution (50 μL) were added to aliquots of serum (50 μL) in sterile *Eppendorf* tubes, to reach a dendrimer concentration of 200 μM during the assay.

Samples were incubated at 37 °C under gentle stirring (350 rpm). Different samples (triplicates) were quenched at different time points (0/1/6/10/24 h) by precipitating serum proteins through the addition of (0.1 M) $ZnSO_4$·7 H_2O/MeCN (1:1) (0.1 M, 100 μL) and cooling in ice bath for 10 min. Protein precipitates were pelleted under centrifugation (5 min at 4'000 rpm) and the supernatants were analyzed by LC/MS. Experiment controls included a precipitation control for each dendrimer to test their resistance to the protein precipitation conditions and serum blanks to check reproducibility over different serum batches. Peaks corresponding to the internal standard and the undegraded dendrimers were integrated, with the ratio dendrimer/standard at $t = 0$ h as 100%.

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Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Author Contribution Statement

E. B. co-designed the project, carried out all experiments, and wrote the paper. *J.-L. R.* designed and supervised the project and wrote the paper.

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