Bioorganic & Medicinal Chemistry 19 (2011) 2918–2926

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Comparing micellar, hemolytic, and antibacterial properties of di- and tricarboxyl dendritic amphiphiles

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A R T I C L E I N F O

Article history:
Received 23 May 2010
Revised 10 March 2011
Accepted 17 March 2011
Available online 12 April 2011

Keywords:
Dendritic amphiphiles
Staphylococcus aureus
MRSA
Critical micelle concentrations
Hemolytic activities

A B S T R A C T

Homologous dicarboxyl dendritic amphiphiles—$RCONHC(CH_3)(CH_2CH_2COOH)_2$, **4**($n$); and $ROCONHC(CH_3)(CH_2CH_2COOH)_2$, **5**($n$), where $R = n\text{-}C_nH_{2n+1}$ and n = 13–22 carbon atoms—were synthesized. Critical micelle concentrations (CMCs) in aqueous triethanolamine solutions and at pH 7.4 were measured along with hemolytic activity (effective concentrations, EC_{10}) in phosphate-buffered saline (PBS). Log CMC showed a linear dependence on chain length (n); the longest chain in each series had the lowest CMC—in triethanolamine: **4**(21), 180 μM and **5**(22), 74 μM and at pH 7.4: **4**(21), 78 μM and **5**(22), 33 μM. These two series, **4**(n) and **5**(n), and three series of homologous tricarboxyl dendritic amphiphiles—$RCONHC(CH_2CH_2COOH)_3$, **1**(n); $ROCONHC(CH_2CH_2COOH)_3$, **2**(n); $RNHCONHC(CH_2CH_2COOH)_3$, **3**(n), where $R = n\text{-}C_nH_{2n+1}$ and n = 13–22 carbon atoms—were tested for growth inhibition of *Staphylococcus aureus* strain ATCC 6358 and methicillin-resistant *S. aureus* (MRSA) strain ATCC 43330 by microdilution in 0.1-strength brain heart infusion broth (BHIB). Amphiphiles **4**(19), **4**(21), **5**(18), and **5**(20) showed the strongest antibacterial activity (2.2–3.4 μg/mL) against *S. aureus* (vancomycin, MIC = 0.25 μg/mL). These four plus **1**(21), **2**(20), **2**(22), and **3**(20) exhibited the strongest antibacterial activity (1.7–6.8 μg/mL) against MRSA (vancomycin, MIC = 0.25 μg/mL). The MICs of these amphiphiles against six clinical MRSA were similar to those against the ATCC strain. In PBS, EC_{10}s of the most active homologues ranged from 7 to 18 μg/mL and 18 to 220 μg/mL for di- and tricarboxyl dendritic amphiphiles, respectively. To assess the potential safety of using dendritic amphiphiles as drugs, measurements of micellar and hemolytic properties were conducted in the same medium (full-strength BHIB) that was used for antibacterial activity. The CMCs (9–36 μg/mL, ~18–72 μM) of ten amphiphiles were measured by microdilution (log 2 progression) with dye-covered beads. The EC_{10}s were similar to those in PBS. The MICs of most amphiphiles (14–72 μg/mL) and vancomycin (1.1–2.2 μg/mL) against both *S. aureus* and MRSA increased significantly compared to the MICs measured in 0.1-strength BHIB. The one exception, **5**(18), had an MIC against *S. aureus* of 1.1 μg/mL compared to vancomycin (2.2 μg/mL). With CMC (9–18 μg/mL) and EC_{10} (16 μg/mL) values higher than the MIC, **5**(18) was discovered as a lead for further development.

1. Introduction

Long-chain, tricarboxyl dendritic amphiphiles show a broad spectrum of antimicrobial activity.[1,2] Three homologous series, **1**(n)–**3**(n) (Fig. 1), represent starting points for antibacterial development. These amphiphiles, designed to enable aqueous solubility of very long-chain fatty alkyl groups (C_{13}–C_{22}), provide a range of hydrophobicities for probing the responses of microbes to the physicochemical properties of antimicrobial agents. For example, amphiphiles **1**(n)–**3**(n) exhibit antimicrobial activities at concentrations below their critical micelle concentrations (CMCs),[2,3] and antibacterial assays of these homologous amphiphiles show both

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doi:10.1016/j.bmc.2011.03.036

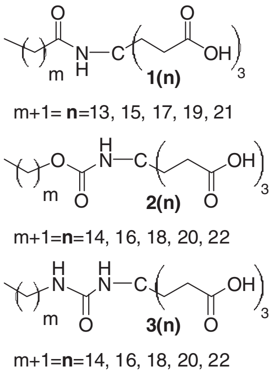


Figure 1. Antimicrobial tricarboxyl dendritic amphiphiles.

species and chain-length specificities.[1,2] Given the 'epidemic of antibiotic-resistant infections' [4] (e.g., the increasing incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections[5,6]), we continue to pursue these amphiphiles as potential topical and systemic antimicrobial agents.

A question surfaced from previous work;[1] were the tricarboxyl dendritic amphiphiles as trianions too hydrophilic to be effective antibacterial agents? If so, they would be less likely to partition into a cell (or cell envelope) and, consequently, they would be less active. To answer this question, we synthesized two homologous series of dicarboxyl dendritic amphiphiles, **4**(*n*) and **5**(*n*) (Fig. 2), that are, as dianions, less hydrophilic analogues of trianions, **1**(*n*) and **2**(*n*), respectively. Another question arose as to whether minimal inhibitory concentrations (MICs = MIC_{99}s) of dendritic amphiphiles would show an inoculum effect in assays against *S. aureus* and MRSA. This question was motivated by four results: (1) Glycerol monolaurate exhibited an inoculum effect against *S. aureus*.[7] (2) Various antibiotics showed inoculum effects against *S. aureus*.[8–10] (3) Some homologues of **1**(*n*)–**3**(*n*) showed, at high inoculum density, only modest inhibition against *S. aureus* and MRSA.[1] (4) Series **3**(*n*) showed a significant 'inoculum effect' in assays against *Mycobacterium smegmatis*.[3] Assessing the inoculum effect could be crucial to identifying novel antimicrobial agents.[8]

In this study, we report excellent antibacterial activity for several homologues of **1**(*n*)–**5**(*n*) and a strong inoculum effect on the MICs of dendritic amphiphiles and vancomycin. To assess the potential safety of these amphiphiles, we have measured the CMCs and hemolytic activity. Comparing these measurements provides insights into designing safe, amphiphilic antibacterial agents.

2. Results and discussion

2.1. Synthetic chemistry

Synthesis of the dicarboxyl dendritic amphiphiles, **4**(*n*) and **5**(*n*), required making the diesteramine[11] (**7**) and isocyanatodiester (**8**) dendrons, respectively (Scheme 1). Adding 2 equiv of *tert*-butyl acrylate to nitroethane catalyzed by DBU[12] gave **6** in good yield after chromatography. Hydrogenating **6** catalyzed by Raney Ni produced **7** in high yield.[11] Combining **7** with DMAP and di-*tert*-butyl dicarbonate (Boc_2O) in dichloromethane for 15 min[13] gave **8** in good yield after chromatography. Compounds **6** and **7** were fully characterized; **8** gave the expected NMR and IR spectra as well as a satisfactory elemental analysis.

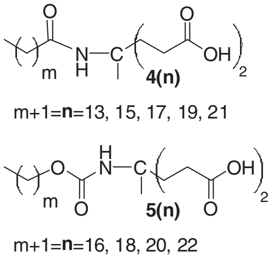


Figure 2. Antimicrobial dicarboxyl dendritic amphiphiles.

The two series of dicarboxyl dendritic amphiphiles were prepared similarly to their tricarboxyl counterparts.[1,14] The condensation of a fatty acid chloride with **7** gave an amide, **9**(*n*), in moderate yields after chromatography or recrystallization. Formolysis or trifluoroacetolysis of **9**(*n*) produced **4**(*n*) in moderate yields after recrystallization; trifluoroacetolysis, the faster method, was preferred. The addition of fatty alcohols to **8** catalyzed by triethylamine, which was employed as the solvent, gave good yields of **10**(*n*) after chromatography. Trifluoroacetolysis of **10**(*n*) gave **5**(*n*) in high yields.

2.2. Critical micelle concentrations (CMCs)

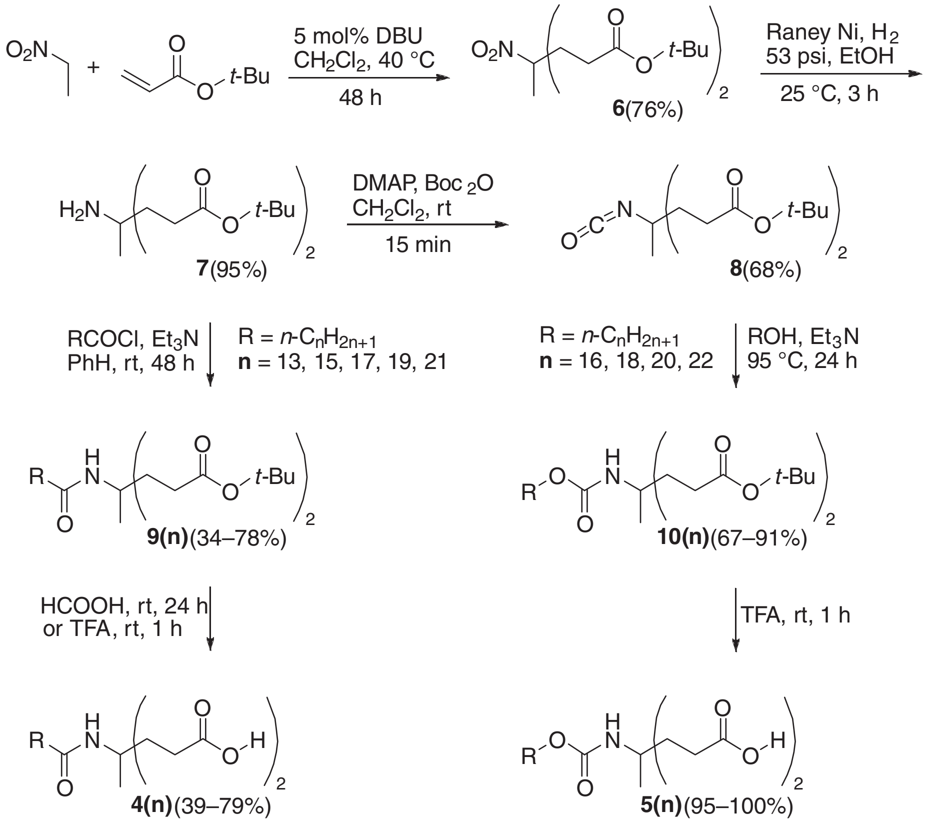
CMCs of **4**(13)–**4**(21) and **5**(16)–**5**(22) in triethanolamine/water [0.9% (w/v), pH ~9] were measured by using pyrene fluorescence as an indicator of micelle formation. CMCs of **4**(13)–**4**(21) were also measured by using a pendent-drop analyzer to measure surface tension. The log CMCs for the two series of dicarboxyl amphiphiles displayed nearly identical dependencies on chain length (Fig. 3, solid symbols). As expected,[15,16] the CMCs of **4**(*n*) and **5**(*n*) were lower than the CMCs[2,3] of the three series of tricarboxyl amphiphiles (Fig. 3, open symbols). The differences increased with chain length. Notably, the slopes of the lines for the dicarboxyl series are steeper than those for the tricarboxyl series. Comparing the CMCs of **4**(*n*) to those of **1**(*n*) showed a larger difference than comparing the CMCs of **5**(*n*) to those of **2**(*n*).

The CMCs decreased for all amphiphiles when the pH of the solution was lowered to 7.4 with phosphoric acid. In our previous work,[2] the CMCs for **1**(21) and **3**(16) at pH 7.4 were twofold and fourfold lower, respectively, than the CMCs in triethanolamine/water. CMC measurements at pH 7.4, the pH of the antibacterial assays, included all the strongly active homologues (see below) of the three tricarboxyl series and all homologues of the dicarboxyl series. The drop in pH consistently decreased the CMCs in the tricarboxyl series (Fig. 4) by an average of 3.3-fold. A similar average drop (3.4-fold) in CMCs occurred in the dicarboxyl series, but the range was wider (Fig. 5).

2.3. Antibacterial assays

2.3.1. Effect of inoculum density on antibacterial activity

Inoculum density affected the susceptibilities of *S. aureus* strain ATCC 6358 and MRSA strain ATCC 43330 to all dendritic amphiphiles and vancomycin. *S. aureus* strain ATCC 6358 and MRSA strain ATCC 43330 were grown to log phase and then diluted to different cell densities. MICs were measured in 0.1-strength brain heart infusion broth (BHIB). As illustrated in Figure 6 for MRSA, susceptibilities to **4**(19) and vancomycin decreased (higher MICs) as the inoculum density increased. The results with vancomycin confirmed the earlier work.[8] These results typified the susceptibilities of both *S. aureus* and MRSA to all compounds (data not shown). The consistent values of MIC observed at inoculum densities of $\leqslant 10^5$ colony forming units (CFU) suggested that MIC had reached a minimum (Fig. 6), which is defined as MIC_0, the intrinsic MIC.[3,9] For all the values of MIC reported herein, inocula were at $\sim 10^5$ CFU/mL.



Scheme 1. Synthesis of dicarboxyl dendritic amphiphiles, **4**(*n*) and **5**(*n*).

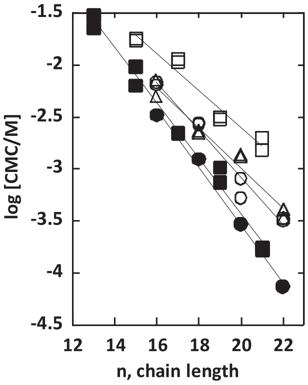


Figure 3. CMCs of dendritic amphiphiles in aqueous triethanolamine. M = molar. Amphiphiles: **1**(*n*) (open squares), **2**(*n*) (open circles), **3**(*n*) (open triangles), **4**(*n*) (solid squares), and **5**(*n*) (solid circles). Solid lines are linear-regression analyses with standard errors, where log CMC = for **1**(*n*): $(-0.18 \pm 0.01) \times n + 1.0 \pm 0.3$; for **2**(*n*): $(-0.23 \pm 0.01) \times n + 1.5 \pm 0.3$; for **3**(*n*): $(-0.19 \pm 0.01) \times n + 0.8 \pm 0.3$; for **4**(*n*): $(-0.27 \pm 0.01) \times n + 1.9 \pm 0.2$; and for **5**(*n*): $(-0.28 \pm 0.02) \times n + 2.0 \pm 0.3$.

2.3.2. Effect of structure on antibacterial activity

Chain length and head group affected the antibacterial activity against *S. aureus*; chain length affected activity against MRSA. The antibacterial activity of each homologue of **1**(*n*)–**5**(*n*) was measured (data not shown) against both *S. aureus* strain ATCC 6358 and MRSA strain ATCC 43330 at an inoculum density of ~10^5 CFU/mL. In general, the activity improved with increasing chain length. The two homologues with the longest chains in four series, **1**(*n*)–**4**(*n*), had the lowest MICs (Table 1). Compounds **5**(18) and **5**(20), which are respectively isosteres of **4**(19) and **4**(21), had the lowest MICs in the **5**(*n*) series; the MIC for **5**(22) was 50-fold higher than those.

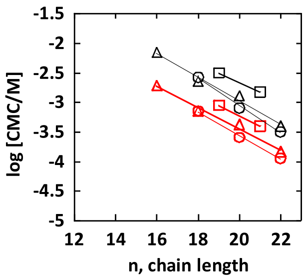


Figure 4. CMCs of tricarboxyl dendritic amphiphiles in aqueous triethanolamine at pH ~9 (black) and pH 7.4 (red). M = molar. Amphiphiles: **1**(*n*) (open squares), **2**(*n*) (open circles), and **3**(*n*) (open triangles). Solid lines are linear-regression analyses with standard errors, where log CMC at pH ~9 = for **1**(*n*): $(-0.16) \times n + 0.57$; for **2**(*n*): $(-0.23 \pm 0.02) \times n + 1.6 \pm 0.4$; for **3**(*n*): $(-0.20 \pm 0.02) \times n + 1.0 \pm 0.3$ and where log CMC at pH 7.4 = for **1**(*n*): $(-0.17) \times n + 0.28$; for **2**(*n*): $(-0.20 \pm 0.01) \times n + 0.4 \pm 0.3$; for **3**(*n*): $(-0.23 \pm 0.03) \times n + 1.1 \pm 0.6$.

Dicarboxyl dendritic amphiphiles had approximately tenfold and twofold lower MICs than tricarboxyl dendritic amphiphiles against *S. aureus* and MRSA, respectively (Table 1). These ten amphiphiles were then tested against recent clinical isolates to ensure that the patterns observed with the ATCC strains were typical. The activities of dicarboxyl dendritic amphiphiles, **4**(19), **4**(21), **5**(18), and **5**(20) were quite high (low MICs) for all strains (Table 1); 5(18) had a few MICs that were less than fivefold higher than vancomycin.

2.4. Hemolysis

To assess the ability of the amphiphiles to disrupt mammalian membranes, hemolysis (effective concentration for 10% hemolysis,

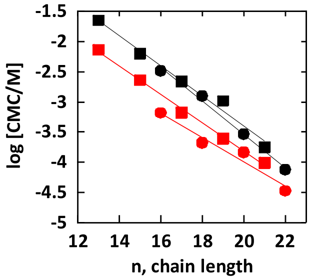


Figure 5. CMCs of dicarboxyl dendritic amphiphiles in aqueous triethanolamine at pH ~9 (black) and pH 7.4 (red). M = molar. Amphiphiles: **4**(n) (solid squares) and **5**(n) (solid circles). Solid lines are linear-regression analyses with standard errors, where log CMC at pH ~9 = for **4**(n): $(-0.25 \pm 0.02) \times n + 1.6 \pm 0.3$; for **5**($n$): $(-0.28 \pm 0.02) \times n + 2.0 \pm 0.3$, and where log CMC at pH 7.4 = for **4**(n): $(-0.236 \pm 0.008) \times n + 0.9 \pm 0.1$; for **5**($n$): $(-0.20 \pm 0.03) \times n + 0.0 \pm 0.6$.

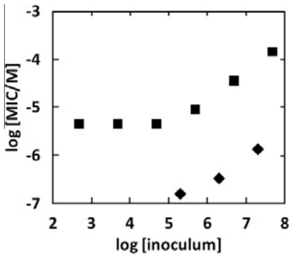


Figure 6. Effect of initial cell density on the activities **4**(19) (squares) and vancomycin (diamonds) against MRSA strain ATCC 43330. M = molar. CFU = colony forming units.

EC_{10}) was measured in phosphate-buffered saline (PBS) following a recent procedure.[17] Triton® X-100 served as the reference for complete hemolysis. In general, tricarboxyl dendritic amphiphiles had higher EC_{10}s (220–18 µg/mL) than dicarboxyl dendritic amphiphiles had (18–7 µg/mL). Amphiphiles **5**(18) and **5**(20) had the lowest EC_{10}s (7 and 14 µg/mL); **2**(20) and **3**(20) had the highest (220 and 54 µg/mL).

2.5. Comparing micellar, hemolytic, and antibacterial properties in the same medium

The micellar, hemolytic, and antibacterial data reported above were measured in three different media. The CMCs, EC_{10}s, and MICs were measured in pH-adjusted aqueous triethanolamine, PBS, and 0.1-strength BHIB, respectively. Although the CMCs and MICs provided comparisons to our previous work,[1,2] they did not allow a fair assessment of interrelationship of CMCs, EC_{10}s, and MICs. The challenge to find a medium to measure all three properties was solved by sacrificing the precision of the usual methods for measuring CMCs. The availability of dye-covered glass beads enabled using 96-well microtiter plates and measuring CMCs to the nearest twofold dilution similar to measuring EC_{10}s and MICs. As hemolysis could not be measured in 0.1-strength BHIB due to lysis of red-blood cells, measurements of all three properties were made in full-strength BHIB (Table 2). Changing the ionic strength of the medium and the concentration of nutrients significantly changed the values for most compounds in all three measurements.

Table 2
Comparing MICs of dendritic amphiphiles and vancomycin against *S. aureus* and MRSA with CMCs and hemolysis (EC_{10}s) for dendritic amphiphiles in full-strength BHIB by microdilution (twofold)[a]

Amphiphile	MIC[a] (µg/mL)		CMC (µg/mL)	EC_{10} (µg/mL)
	S. aureus ATCC 6358	MRSA ATCC 43330		
1(19)	144	36	18	31–63
1(21)	72	18	18	31–63
2(20)	51	27	18–36	31–63
2(22)	108	36	18–36	63
3(20)	36	14	9	31
3(22)	101	54	NM[b]	31
4(19)	18	27	9–18	7.8
4(21)	14	14	9–18	7.8
5(18)	1.1	18	9–18	16
5(20)	72	72	9–18	16
Vancomycin	2.2	1.1	NM[b]	NM[b]

[a] All measurements were made in duplicate.
[b] Not measured.

The CMCs (log CMC ~−4.7 to −4.4) in full-strength BHIB (Table 2) were lower than those measured at pH 7.4 in aqueous triethanolamine (log CMC ~−4.0 to −3.7, Fig. 4; ~−4.4 to −4.0, Fig. 5). However, CMCs measured in 0.1-strength BHIB (data not shown) were in the same range as those shown in Figures 4 and 5. To be conservative, we chose the first well that had an absorbance above the baseline as the CMC in full-strength BHIB. These CMCs were similar for all amphiphiles because the resolution of the measurements was limited by a twofold dilution in concentrations, a two-carbon difference in chain length in each series, and a very small change in absorbance (~0.04). A plateau for the maximum absorbance was not reached at the concentrations tested; thus, an inflection point could not be estimated. As expected, in all media, CMCs of tricarboxyl dendritic amphiphiles were slightly higher than those of dicarboxyl dendritic amphiphiles.

The EC_{10}s of the dendritic amphiphiles in full-strength BHIB were quite similar to those in PBS (Section 2.4); the values in broth

Table 1
The MICs of dendritic amphiphiles and vancomycin against *S. aureus*, MRSA, and recent clinical isolates of MRSA

Amphiphile	MIC (µg/mL)							
	S. aureus ATCC 6358	MRSA ATCC 43330	MRSA 523000	MRSA 522870	MRSA 34864	MRSA 36361	MRSA 53016	MRSA 34380
1(19)	54	45	28	57	23	57	28	28
1(21)	54	4.5	14	14	7	14	7	7
2(20)	27	4.5	7	7	7	14	7	5.3
2(22)	23	4.5	7	11	3.6	14	3.6	5.3
3(20)	54	4.5	7	14	11	28	11	7
3(22)	54	6.8	14	14	11	43	7	11
4(19)	2.2	2.2	3.6	5.3	3.6	3.6	3.6	3.6
4(21)	2.2	1.7	3.6	3.6	3.6	3.6	3.6	3.6
5(18)	2.2	2.2	1.7	NM[a]	2.2	2.2	2.2	2.2
5(20)	3.4	2.2	3.4	NM	4.5	2.2	3.4	4.5
Vancomycin	0.25	0.25	0.5	0.25	0.38	0.75	0.38	0.5

[a] Not measured.

were more consistent. The EC_{10}s were virtually identical for amphiphiles in a given series because the resolution of the measurements was limited by a twofold dilution in concentrations and by a two-carbon difference in chain length in each series. In both media, the tricarboxyl dendritic amphiphiles were less hemolytic than the dicarboxyl dendritic amphiphiles. For the dicarboxyl dendritic amphiphiles, **4**(19) and **4**(21) were more hemolytic than **5**(18) and **5**(20).

The MICs of the dendritic amphiphiles and vancomycin in full-strength BHIB (Table 2) were generally larger than those measured in 0.1-strength BHIB (Table 1). MICs of **3**(20) and **5**(18) against *S. aureus* plus the MIC of **1**(19) against MRSA were the exceptions. Of note, the MIC of **5**(18) against *S. aureus* was lower than that for vancomycin. The dendritic amphiphiles tested showed less solubility in full-strength BHIB than in 0.1-strength as evidenced by the appearance of a precipitate in the wells with the highest concentrations tested (precipitation did not occur in the measurements of CMC and EC_{10} as the time for those experiments were much shorter than the MIC measurements). At lower concentrations in measurements of MIC, the wells were clear until turbidity, due to bacterial growth, appeared. Spotting the solutions on agar and observing no growth verified that the precipitate at higher concentration did not contain any viable bacterial cells. As seen in 0.1-strength BHIB (Table 1), the MICs of the tricarboxyl dendritic amphiphiles were lower against MRSA than against *S. aureus*; the MICs of the dicarboxyl dendritic amphiphiles were similar against the two strains.

For comparison, the MICs of five fatty acids (C_{10}, C_{12}, C_{14}, C_{16}, and C_{18}) dissolved in aqueous triethanolamine showed very little activity against *S. aureus* and MRSA. The longer fatty acids, C_{20} and C_{22}, were insoluble under the conditions of the experiments. The MICs of C_{12} and C_{14} against *S. aureus* were 250 μg/mL; the MICs for the other three were >250 μg/mL. The MICs of C_{14} and C_{16} against MRSA were 250 μg/mL; the MICs for the other three were >250 μg/mL. The most active dendritic amphiphiles were substantially more active than the most active fatty acids.

2.6. Summary and conclusions

In answer to the questions posed in the introduction, (1) tricarboxyl dendritic amphiphiles are not too hydrophilic to be effective antibacterial agents, and (2) dendritic amphiphiles show inoculum effects with *S. aureus* and MRSA similar to that of vancomycin. Further, we find that increasing the strength of the broth decreases antibacterial activity for both dendritic amphiphiles and vancomycin. This finding agrees with others,[18] who observe that bacterial susceptibility to antimicrobial agents decreases as growth rate increases.

For activity against *S. aureus* in 0.1-strength BHIB (Table 1), dicarboxyl dendritic amphiphiles are ca. tenfold more antibacterial than tricarboxyl dendritic amphiphiles. In full-strength BHIB, the differential is smaller. One notable exception is **5**(18), which strongly inhibits growth of *S. aureus* in both media and has an MIC in full-strength BHIB slightly better than that of vancomycin. In contrast, both di- and tricarboxyl dendritic amphiphiles show similar activity against MRSA with MICs ranging from 2 to 7 μg/mL in 0.1-strength BHIB and 14–72 μg/mL in full-strength BHIB.

For either bacterial strain, the active microspecies of tricarboxyl (trianion, dianion, monoanion, or neutral) or dicarboxyl (dianion, monoanion, or neutral) dendritic amphiphiles has not been identified. For a specific microspecies and homologue, the $c\log P$s of di- and tricarboxyl dendritic amphiphiles are similar. As microspecies are in equilibrium, identifying the active form against a specific strain is challenging. For activity against *S. aureus*, dicarboxyl dendritic amphiphiles, especially **5**(18), are more active than the tricarboxyl dendritic amphiphiles. One cannot discern whether the greater activity is due to differences between dianions and trianions partitioning into cell walls or to a specific interaction of the dicarboxyl(ate) versus the tricarboxyl(ate) with the bacterium or a combination of these two effects. For activity against MRSA, the activities of di- and tricarboxyl dendritic amphiphiles are similar. As the CMCs and EC_{10}s are similar to the MICs, a nonspecific amphiphilic interaction likely leads to inhibition of MRSA.

The dye-covered-glass-bead method for measuring CMCs works well in full-strength BHIB and only uses small quantities of amphiphile. The method facilitates assessing micellar contributions to antibacterial and hemolytic activities. For dendritic amphiphiles, similarities of CMCs and EC_{10}s in full-strength BHIB support a conclusion that the two phenomena are closely related. Comparing MICs to CMCs or EC_{10}s (Table 2) assesses the safety of using an amphiphile.[17,19] As the MICs against *S. aureus* and MRSA are similar to the CMCs and EC_{10}s, the safety margin for using these dendritic amphiphiles is too small. The one exception, **5**(18), has an excellent activity against *S. aureus* and a safety margin >10.

The difference in antibacterial activities (Tables 1 and 2) against *S. aureus* between the di- and tricarboxyl dendritic amphiphiles raises questions about the mechanism of action of these agents. Although they are significantly more active than fatty acids, they may act similarly to them. Fatty acids appear to target the bacterial cell membrane.[20] For example, glycerol monolaurate and dodecanoic acid inhibit the production of *S. aureus* β-lactamase, toxic shock syndrome toxin-1, and other exoproteins by interfering with signal transduction.[7,21] They could be acting as surfactants (detergents)[22,23] by disrupting cell membranes to inhibit bacterial growth. As bacterial cell walls differ from red-blood-cell membranes, studies[24,25] are needed to demonstrate whether **5**(18) disrupts the cell wall of *S. aureus*.

In conclusion, dendritic amphiphiles can serve as a platform for developing novel, amphiphilic antibacterial agents. A recent review endorses developing membrane-active agents against dormant bacteria as a strategy for designing new antibiotics to treat persistent infections.[26] In that vein, the work reported here will be followed by studies of the mechanism of action of **5**(18) and further structure–activity work to improve the safety margin.

3. Methods

3.1. General methods for synthesis

Unless specified, solvents and reagents were used as received. Benzene was dried with 4 Å molecular sieves. Analytical thin layer chromatography was performed on plastic-coated Silica Gel 60 Å and detected by dipping in a solution of 5% ethanolic phosphomolybdic acid reagent (20 wt % solution in ethanol) and then heated with a heat gun for ~1 min. The R_f for a given compound ranged from 0.3 to 0.5; the compounds were eluted in the different solvent mixtures (see below for details). Preparative flash column chromatography was carried out on silica gel (60 Å). Solutions were concentrated by rotary evaporation. Melting point ranges were determined in open capillary tubes at 1 °C/min and uncorrected. NMR spectra of all compounds were recorded at 400 or 500 MHz for ^1H and 100.6 or 125.8 MHz for ^{13}C, respectively, and reported in ppm relative to TMS in $CDCl_3$, and DMSO-d_6 (2.54 δ for ^1H and 40.45 δ for ^{13}C) in DMSO-d_6. IR spectra were recorded on neat samples with an FTIR equipped with a diamond ATR system, and reported in cm^{-1}. HRMS data were obtained on a dual-sector mass spectrometer in FAB mode with 2-nitrobenzylalcohol as the proton donor and on an Accurate-MASS Time-of-Flight (TOF) LC/MS operating under electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Elemental analyses were performed by a commercial vendor.

3.1.1. Di-*tert*-butyl 4-methyl-4-nitroheptanedioate, 6

Nitroethane (1.01 g, 13.3 mmol) and *tert*-butyl acrylate (3.50 g, 27.3 mmol) were dissolved in CH_2Cl_2 (13 mL) and heated at 40 °C. To this colorless solution, DBU (0.100 mL, 0.67 mmol) was added in 0.025-mL portion every 10 min. The brown solution was stirred for 24 h; after 6 h the solution turned yellow and then green the following morning. The solution was allowed to sit for 24 h without heating and stirring. The green solution was concentrated at 30 °C to form a green solid, which was dried under high vacuum for 24 h (4.30 g, 97%). Eluting with 97.5:2.5 (v/v) $CHCl_3$/EtOAc, the TLC showed two spots with R_f values 0.63 and 0.26. The green solid was purified in batches. For example, the green solid (2.13 g) was purified by flash column chromatography—6.5 cm ID column, 97.5:2.5 (v/v) $CHCl_3$/EtOAc, silica gel (40.30 g)—to produce a white solid (1.66 g, 76% yield): mp 52.6–53.0 °C (lit.[11] 46–47 °C); ^1H NMR (400 MHz, $CDCl_3$, δ): 1.44 (s, 18H), 1.53 (s, 3H), 2.05–2.38 (m, 8H) (lit.[11] 250 MHz); ^{13}C NMR (125.8 MHz, $CDCl_3$, δ): 22.0, 28.3, 30.4, 34.6, 81.3, 90.2, 171.6; IR: 2981, 1722, 1533, 1160 cm^{-1}. HRMS–ESI (m/z): $[M+Na]^+$ calcd for $C_{16}H_{29}O_6NNa$, 354.1887; found, 354.1889.

3.1.2. Di-*tert*-butyl 4-amino-4-methylheptanedioate, 7

An aqueous slurry of Raney Ni (~11 g, Raney® 2800 nickel, slurry in H_2O) was placed in a hydrogenation flask; excess H_2O was removed by pipette to give a final weight of 10.29 g (~78 mmol of Ni, presuming 50% H_2O and 89% Ni). To the wet catalyst, **6** (8.37 g, 25.3 mmol) in 95% EtOH (160 mL) was added. The reaction mixture was shaken in a sealed container under H_2 (53 psi) for 3 h at 25 °C. The mixture was filtered cautiously through Celite® under continuous flow of N_2 to remove the catalyst. The clear filtrate was concentrated at rt and dried under high vacuum for 48 h to give a white solid (7.25 g, 95%): mp 36.3–37.0 °C (lit.[11] as a white oil); ^1H NMR (400 MHz, $CDCl_3$, δ): 1.03 (s, 3H), 1.14 (s, 2H), 1.44 (s, 18H), 1.64 (m, 4H), 2.27 (m, 4H) (lit.[11] 250 MHz); ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 27.1, 28.0, 30.4, 37.4, 50.7, 80.3, 173.2; IR: 2977.93, 1725, 1249, 1160 cm^{-1}; HRMS–ESI (m/z): $[M+H]^+$ calcd for $C_{16}H_{32}O_4N$, 302.2326; found, 302.2334.

3.1.3. Di-*tert*-butyl 4-isocyanato-4-methylheptanedioate, 8

A solution of **7** (0.550 g, 1.80 mmol) and DMAP (0.216 g, 1.80 mmol) in CH_2Cl_2 (20 mL) was mixed with a solution of Boc_2O (0.390 g, 1.80 mmol) in CH_2Cl_2 (10 mL) and stirred for 15 min at rt. TLCs, eluting with 50:50 (v/v) hexane/EtOAc, were run to check for completion of reaction. Upon completion, TLC showed three spots with R_f values of 0.64, 0.53, and 0.03. The solution was washed with 10% HCl (5 mL) and deionized H_2O (5 mL). The organic solution was dried overnight with $MgSO_4$. The filtrate was concentrated at rt and placed under high vacuum overnight to give a colorless, viscous liquid (0.51 g, 85%). TLC showed two spots with R_f values 0.64 and 0.53. Several reactions on larger scale were run by this method and produced similar results.

In order to purify the sample further, additional TLCs were run to find a better separation. Eluting with 90:10 (v/v) hexane/EtOAc showed only two spots with R_f values of 0.24 and 0.00. Colorless, viscous liquid (27.67 g) was purified by flash column chromatography—4.4 cm ID, silica gel (160.43 g), 90:10 (v/v) hexane/EtOAc, fraction size (50 mL), collecting and concentrating fractions 10 through 18—to produce a colorless, viscous liquid (22.07 g, 68%) that showed a single spot on TLC. ^1H NMR (500 MHz, $CDCl_3$, δ): 1.31 (s, 3H), 1.45 (s, 18H), 1.75–1.95 (m, 4H), 2.33 (m, 4H); ^{13}C NMR (125.8 MHz, $CDCl_3$, δ): 27.6, 28.3, 30.8, 37.1, 60.1, 80.9, 122.8, 172.4; IR: 2979, 2256, 1728, 1160 cm^{-1}. Anal. Calcd for $C_{17}H_{29}O_5N$: C, 62.36; H, 8.93; N, 4.28. Found: C, 62.59; H, 8.95; N, 4.35.

3.1.4. Di-*tert*-butyl 4-methyl-4-(1-oxooctadecylamino)heptanedioate, 9(17)

Diesteramine **7** (6.08 g, 20.2 mmol) was dissolved in dry PhH (55 mL). To the stirring solution, Et_3N (2.2 mL, 16 mmol) and octadecanoyl chloride (4.15 g, 13.7 mmol) were added, resulting immediately in a cloudy solution, which was stirred for 48 h at rt. The reaction mixture was washed with satd $NaHCO_3$ (1 × 55 mL), water (1 × 55 mL), 10% aq HCl (1 × 55 mL), and satd NaCl (1 × 55 mL). The solution was dried with $MgSO_4$. After filtration the solution was concentrated and the light brown solid was recrystallized three times from MeCN to produce a white solid (2.69 g, 4.73 mmol, 34%): mp 57.1–57.8 °C; ^1H NMR (400 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 31H), 1.44 (s, 18H), 1.58 (br m, 2H), 1.88 (dt, 2H), 2.06 (m, 4H), 2.24 (t, 4H), 5.69 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 14.3, 22.8, 23.9, 26.0, 28.2, 29.45, 29.50, 29.53, 29.7, 29.81, 29.85, 30.5, 32.1, 33.5, 37.8, 55.3, 80.7, 172.9, 173.4; IR: 3332, 2917, 2849, 1723, 1645, 1542, 1151 cm^{-1}; HRMS (FAB+) calcd for $C_{34}H_{66}O_5N$ $[M+H]^+$ 568.4943, found 568.4949. Anal. Calcd for $C_{34}H_{65}O_5N$: C, 71.91; H, 11.54; N, 2.47. Found: C, 72.05; H, 11.54; N, 2.45.

3.1.5. General procedure for 9(*n*), *n* = 13, 15, 19, 21

Diesteramine **7** (8.00 mmol) was dissolved in dry PhH (40 mL). To the stirring solution, Et_3N (9 mmol) and alkanoyl chloride (8.80 mmol) were added, resulting immediately in a cloudy solution, which was stirred for 48 h at rt. The reaction mixture was washed with satd $NaHCO_3$ (1 × 40 mL), H_2O (1 × 40 mL), 10% aq HCl (1 × 40 mL), and satd NaCl (1 × 40 mL). The light brown solution was dried with $MgSO_4$. After filtration, the solution was concentrated and dried under a high vacuum for 24 h to produce a light brown oil or off-white solid. TLC revealed several minor spots and one major spot (R_f 0.5). The oil was purified in two batches by flash chromatography—4.4 cm ID, silica gel (60 Å, 91 g). The flow rate (~23 mL/min) was controlled by compressed air. After eluting the solvent mixture (100 mL), fractions (35 mL) were collected. The product appeared in fractions 3–12.

3.1.5.1. Di-*tert*-butyl 4-methyl-4-(1-oxotetradecylamino)heptanedioate, 9(13). Chromatography with 4:1 (v/v) hexanes/EtOAc. White solid (3.4491 g, 6.73 mmol, 77%): mp 42.9–43.2 °C; ^1H NMR (400 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 23H), 1.44 (s, 18H), 1.58 (br t, 2H), 1.88 (m, 2H), 2.08 (m, 4H), 2.24 (t, 4H), 5.70 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 14.3, 22.8, 23.9, 26.0, 28.2, 29.44, 29.49, 29.52, 29.66, 29.78, 29.80, 29.83, 30.5, 32.1, 33.5, 37.8, 55.3, 80.7, 172.9, 173.4; IR: 3304, 2921, 2851, 1726, 1644, 1544, 1148 cm^{-1}; HRMS (FAB+) calcd for $C_{30}H_{58}O_5N$ $[M+H]^+$ 512.4318, found 512.4332. Anal. Calcd for $C_{30}H_{57}O_5N$: C, 70.41; H, 11.23; N, 2.74. Found: C, 70.33; H, 11.31; N, 2.66.

3.1.5.2. Di-*tert*-butyl 4-methyl-4-(1-oxohexadecylamino)heptanedioate, 9(15). Chromatography with 3:1 (v/v) hexanes/EtOAc. White solid (77%): mp 46.2– 46.6 °C; ^1H NMR (400 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 27H), 1.44 (s, 18H), 1.58 (br q, 2H), 1.88 (m, 2H), 2.06 (m, 4H), 2.24 (t, 4H), 5.70 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 14.1, 22.7, 23.8, 25.8, 28.0, 29.28, 29.35, 29.37, 29.5, 29.62, 29.64, 29.67, 29.68, 30.3, 31.9, 33.3, 37.6, 55.2, 80.5, 172.7, 173.2; IR: 3304, 2920, 2851, 1725, 1644, 1543, 1148 cm^{-1}; HRMS (FAB+) calcd for $C_{32}H_{62}O_5N$ $[M+H]^+$ 540.4630, found 540.4644. Anal. Calcd for $C_{32}H_{61}O_5N$: C, 71.20; H, 11.39; N, 2.59. Found: C, 71.37; H, 11.23; N, 2.58.

3.1.5.3. Di-*tert*-butyl 4-methyl-4-(1-oxoicosylamino)heptanedioate, 9(19). Chromatography with 8.5:1.5 (v/v) hexanes/EtOAc. White solid (75%): mp 61.9–62.7 °C; ^1H NMR (400 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 35H), 1.44 (s, 18H), 1.58 (br m, 2H), 1.88 (dt, 2H), 2.08 (m, 4H), 2.24 (t, 4H), 5.70 (s, 1H); ^{13}C

NMR (100.6 MHz, $CDCl_3$, δ): 14.3, 22.8, 23.9, 25.9, 27.3, 28.2, 29.1, 29.4, 29.50, 29.52, 29.66, 29.79, 29.84, 30.4, 32.1, 33.5, 37.8, 55.3, 80.7, 172.9, 173.4; IR: 3328, 2916, 2848, 1723, 1645, 1542, 1154 cm^{-1}; HRMS (FAB+) calcd for $C_{36}H_{70}O_5N$ $[M+H]^+$ 596.5256, found 596.5309. Anal. Calcd for $C_{36}H_{69}O_5N$: C, 72.56; H, 11.67; N, 2.35. Found: C, 72.62; H, 11.72; N, 2.34.

3.1.5.4. Di-*tert*-butyl 4-methyl-4-(1-oxodocosylamino)heptanedioate, 9(21). Chromatography with 9.5:0.5 (v/v) $CHCl_3$/EtOAc. White solid (78%): mp 66.4–67.2 °C; ^1H NMR (400 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 39H), 1.44 (s, 18H), 1.58 (br m, 2H), 1.88 (dt, 2H), 2.08 (m, 4H), 2.24 (t, 4H), 5.70 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 14.3, 22.8, 23.9, 25.9, 27.3, 28.2, 29.1, 29.44, 29.50, 29.52, 29.66, 29.79, 29.84, 30.4, 32.1, 37.8, 55.3, 80.7, 172.9, 173.4; IR: 3335, 2918, 2849, 1724, 1646, 1543, 1155 cm^{-1}; HRMS (FAB+) calcd for $C_{38}H_{74}O_5N$ $[M+H]^+$ 624.5569, found 624.5612. Anal. Calcd for $C_{38}H_{73}O_5N$: C, 73.14; H, 11.79; N, 2.24. Found: C, 73.00; H, 11.79; N, 2.21.

3.1.6. 4-Methyl-4-(1-oxooctadecylamino)heptanedioic acid, 4(17)

Formic acid (20 mL) and unstabilized THF (2.25 mL) were added to **9**(17) (2.24 g, 3.94 mmol) resulting in a slightly cloudy solution, which was stirred for 24 h at rt to give a thick white solution. The mixture was concentrated, then a chase solvent (Me_2CO, 2 × 35 mL then CH_2Cl_2, 2 × 30 mL) was added and the mixture was re-concentrated to produce a white solid, which was recrystallized twice from glacial HOAc. The solid was dried under high vacuum for 48 h to give a white solid (1.42 g, 3.12 mmol, 79%): mp 133.6–134.0 °C; ^1H NMR (400 MHz, CD_3OD, δ): 0.89 (t, 3H), 1.21 (s, 3H), 1.28 (m, 28H), 1.58 (br q, 2H), 1.84 (m, 2H), 2.14 (t, 2H), 2.26 (m, 6H), 7.41 (s, 1H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ): 14.0, 22.1, 23.3, 25.5, 28.64, 28.66, 28.74, 28.82, 29.00. 29.04, 29.07, 31.3, 32.8, 35.9, 54.0, 172.0, 174.7; IR: 3404, 2916, 2848, 1723, 1689, 1614, 1521 cm^{-1}; HRMS (FAB+) calcd for $C_{26}H_{50}O_5N$ $[M+H]^+$ 456.3690, found 456.3694. Anal. Calcd for $C_{26}H_{49}O_5N$: C, 68.53; H, 10.84; N, 3.07. Found: C, 68.70; H, 10.86; N, 3.06.

3.1.7. General procedure for 4(*n*), *n* = 13, 15, 19, 21

After adding TFA (3.0 mL, 40 mmol) to **9**(*n*) (5.84 mmol), the solution was stirred for 1 h at rt. The light yellow liquid was concentrated, then a chase solvent (CH_2Cl_2, 10 mL) was added and the mixture was re-concentrated. This was performed repeatedly until an off-white solid appeared. The off-white solid was recrystallized twice from glacial HOAc. The solid was dried under vacuum for 48 h.

3.1.7.1. 4-Methyl-4-(1-oxotetradecylamino)heptanedioic acid, 4(13). White solid (52%): mp 127.5–128.2 °C; ^1H NMR (400 MHz, CD_3OD, δ): 0.90 (t, 3H), 1.22 (s, 3H), 1.29 (m, 20H), 1.58 (br q, 2H), 1.85 (m, 2H), 2.15 (t, 2H), 2.27 (m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ): 14.0, 22.1, 23.3, 25.5, 28.61, 28.65, 28.71, 28.78, 28.97, 29.02, 29.04, 29.06, 31.3, 32.8, 54.0, 172.0, 174.7; IR: 3407, 2915, 2848, 1725, 1687, 1618, 1521 cm^{-1}; HRMS (FAB+) calcd for $C_{22}H_{42}O_5N$ $[M+H]^+$ 400.3064, found 400.3072. Anal. Calcd for $C_{22}H_{41}O_5N$: C, 66.13; H, 10.34; N, 3.51. Found: C, 66.35; H, 10.45; N, 3.47.

3.1.7.2. 4-Methyl-4-(1-oxohexadecylamino)heptanedioic acid, 4(15). White solid (39%): mp 131.3–131.8 °C; ^1H NMR (400 MHz, CD_3OD, δ): 0.90 (t, 3H), 1.21 (s, 3H), 1.29 (m, 24H), 1.58 (br q, 2H), 1.85 (m, 2H), 2.15 (t, 2H), 2.27 (m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ); 14.0, 22.2, 23.3, 25.47, 25.51, 28.67, 28.78, 28.86, 29.04, 29.08, 29.11, 29.12, 31.4, 32.81, 32.85, 35.9, 54.0, 172.1, 174.7; IR: 3364, 2916, 2848, 1725, 1689, 1618, 1519 cm^{-1}; HRMS (FAB+) calcd for $C_{24}H_{46}O_5N$ $[M+H]^+$ 428.3377, found 428.3358. Anal. Calcd for $C_{24}H_{45}O_5N$: C, 67.41; H, 10.41; N, 3.28. Found: C, 67.44; H, 10.72; N, 3.27.

3.1.7.3. 4-Methyl-4-(1-oxoicosylamino)heptanedioic acid, 4(19). White solid (57%): mp 133.7–134.6 °C; ^1H NMR (400 MHz, CD_3OD, δ): 0.90 (t, 3H), 1.22 (s, 3H), 1.29 (m, 32H), 1.58 (br q, 2H), 1.85 (m, 2H), 2.15 (t, 2H), 2.27 (m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ): 14.0, 22.1, 23.3, 25.5, 28.64, 28.65, 28.74, 28.83, 29.01, 29.03, 29.06, 31.3, 32.8, 35.9, 54.0, 172.0, 174.7; IR: 3386, 2915, 2848, 1722, 1692, 1613, 1537 cm^{-1}; HRMS (FAB+) calcd for $C_{28}H_{54}O_5N$ $[M+H]^+$ 484.4003, found 484.3981. Anal. Calcd for $C_{28}H_{53}O_5N$: C, 69.52; H, 11.04; N, 2.90. Found: C, 69.79; H, 11.13; N, 2.90.

3.1.7.4. 4-Methyl-4-(1-oxodocosylamino)heptanedioic acid, 4(21). White solid (39%): mp 134.5–135.4 °C; ^1H NMR (400 MHz, CD_3OD, δ): 0.90 (t, 3H), 1.22 (s, 3H), 1.29 (m, 36H), 1.58 (br q, 2H), 1.86 (m, 2H), 2.15 (t, 2H), 2.27 (m, 6H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ): 14.0, 22.1, 23.3, 28.64, 28.65, 28.73, 28.83, 29.01, 29.03, 29.04, 29.06, 31.3, 32.8, 54.0, 172.0, 174.7; IR: 3385, 2916, 2849, 1723, 1693, 1614, 1537 cm^{-1}; HRMS (FAB+) calcd for $C_{30}H_{58}O_5N$ $[M+H]^+$ 512.4316, found 512.4301. Anal. Calcd for $C_{30}H_{57}O_5N$: C, 70.41; H, 11.23; N, 2.74. Found: C, 70.59; H, 11.31; N, 2.72.

3.1.8. General procedure for 10(*n*), *n* = 16, 18, 20, 22

In Et_3N (5 mL), **8** (5 mmol) and alkan-1-ol (5 mmol) were dissolved. The clear solution was refluxed in an oil bath at 95 °C for 24 h. The pale yellow solution was concentrated; Et_2O (150 mL) was added. The organic solution was washed with 2 M HCl (3 × 10 mL), satd $NaHCO_3$ (3 × 10 mL), and satd NaCl (30 mL). The solution was dried with $MgSO_4$ for overnight. The organic solution was concentrated to give colorless viscous liquid or white solid. TLC analysis was performed to optimize conditions for purification by flash chromatography.

3.1.8.1. Di-*tert*-butyl 4-(hexadecyloxycarbonylamino)-4-methylheptanedioate, 10(16). Eluting with 6:2.5:2.5 (v/v/v) hexane/Et_2O/MeOH, the TLC showed three spots with R_f values 0.52, 0.24, and 0.08. The liquid (3.05 g) was purified by flash column chromatography—4.2 cm ID, silica gel (125.03 g), 6:2.5:2.5 (v/v/v) hexane/Et_2O/MeOH, fraction size (20 mL), collecting and concentrating fractions 12 through 27—to give a clear viscous liquid (86%), which solidified when placed in a freezer (−20 °C). The melting point range, mp 20–23 °C, was determined by attaching a capillary of frozen liquid to a thermometer and then placed in the reservoir of a circulating water bath. ^1H NMR (500 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.26 (m, 29H), 1.44 (s, 18H), 1.57 (m, 2H), 1.85 (m, 2H), 2.03 (m, 2H), 2.24 (t, 4H), 3.97 (s, 2H), 4.71 (s, 1H); ^{13}C NMR (125.8 MHz, $CDCl_3$, δ): 14.4, 23.0, 24.1, 26.2, 28.4, 29.3, 29.63, 29.67, 29.87, 29.91, 29.97, 29.99, 30.01, 30.6, 32.2, 34.0, 54.6, 64.8, 80.7, 155.2, 173.2; IR: 3377, 2924, 2854, 1730, 1694, 1524, 1160 cm^{-1}; HRMS–ESI (*m*/*z*): $[M+Na]^+$ calcd for $C_{33}H_{63}O_6N$-Na, 592.4548; found 592.4540. Anal. Calcd for $C_{33}H_{63}O_6N$: C, 69.55; H, 11.14; N, 2.46. Found: C, 69.58; H, 11.10; N, 2.53.

3.1.8.2. Di-*tert*-butyl 4-methyl-4-(octadecyloxycarbonylamino)heptanedioate, 10(18). Eluting with 6:2.5:2.5 (v/v/v) hexane/Et_2O/MeOH, the TLC showed two spots with R_f values of 0.39 and 0.18. A viscous liquid was purified by—4.2 cm ID, 5″ height, silica gel (70.12 g), 6:2.5:2.5 (v/v) hexane/Et_2O/MeOH, fraction size (30 mL), collecting and concentrating fractions 13 through 26—to give clear viscous liquid (91%). Extended (4 d) drying under high vacuum gave a white solid: mp 33.4–34.0 °C; ^1H NMR (500 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 33H), 1.44 (s, 18H), 1.57 (m, 2H), 1.85 (m, 2H), 2.03 (m, 2H), 2.24 (t, 4H), 3.97 (s, 2H), 4.68 (s, 1H);

^{13}C NMR (125.8 MHz, $CDCl_3$, δ): 14.5, 23.0, 24.2, 26.3, 28.4, 29.4, 29.67, 29.71, 29.90, 29.95, 30.00, 30.03, 30.05, 30.6, 32.3, 34.0, 54.6, 64.8, 80.8, 155.3, 173.3; IR: 3344, 2919, 2849, 1726, 1691, 1532, 1255, 1160 cm^{-1}; HRMS–ESI (m/z): [M+H]$^+$ (m/z): calcd for $C_{35}H_{68}O_6N$, 598.5041; found 598.5043. Anal. Calcd for $C_{35}H_{67}O_6N$: C, 70.31; H, 11.29; N, 2.34. Found: C, 70.26; H, 11.28; N, 2.32.

3.1.8.3. Di-*tert*-butyl 4-(icosyloxycarbonylamino)-4-methylheptanedioate, 10(20). Eluting with 6:5:5 (v/v/v) hexane/Et_2O/MeOH, the TLC showed two spots with R_f values of 0.19 and 0.09. The white solid was purified by flash column chromatography—4.0 cm ID, 7″ height, silica gel (102.59 g), 6:5:5 (v/v/v) hexane/Et_2O/MeOH, fraction size (20 mL), collecting and concentrating fractions 6 through 24—to get white solid (67%): mp 37.6–38.0 °C; ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 37H), 1.44 (s, 18H), 1.57 (m, 2H), 1.85 (m, 2H), 2.03 (m, 2H), 2.24 (t, 4H), 3.97 (s, 2H), 4.68 (s, 1H); ^{13}C NMR (125.8 MHz, $CDCl_3$, δ): 14.5, 23.0, 24.2, 26.3, 28.4, 29.4, 29.68, 29.71, 29.91, 29.95, 30.00, 30.01, 30.04, 30.05, 30.6, 32.3, 34.0, 54.6, 64.8, 80.8, 155.3, 173.3; IR: 3340, 2918, 2849, 1727, 1692, 1532, 1254, 1160 cm^{-1}; HRMS–ESI (m/z): [M+Na]$^+$ calcd for $C_{37}H_{71}O_6NNa$, 648.5174; found 648.5154. Anal. Calcd for $C_{37}H_{71}O_6N$: C, 70.99; H, 11.43; N, 2.24. Found: C, 70.99; H, 11.20; N, 2.21.

3.1.8.4. Di-*tert*-butyl 4-(docosyloxycarbonylamino)-4-methylheptanedioate, 10(22). Eluting with 6:2.5:2.5 (v/v/v) hexane/Et_2O/MeOH, the TLC showed two spots with R_f values 0.22 and 0.12. The white solid was purified by flash column chromatography—4.2 cm ID, 8.5″ height, silica gel (127.58 g), 6:2.5:2.5 (v/v/v) hexane/Et_2O/MeOH, fraction size (10 mL), collecting and concentrating fractions 12 through 24—to give white solid (88%): mp 48.7–49.0 °C; ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.88 (t, 3H), 1.25 (m, 41H), 1.44 (s, 18H), 1.58 (m, 2H), 1.85 (m, 2H), 2.03 (m, 2H), 2.24 (t, 4H), 3.97 (s, 2H), 4.68 (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$, δ): 14.5, 23.0, 24.2, 26.3, 28.4, 29.4, 29.68, 29.71, 29.91, 29.95, 30.01, 30.05, 30.6, 32.3, 34.0, 54.6, 64.8, 80.8, 155.4, 173.2; IR: 3346, 2910, 2849, 1723, 1690, 1531, 1160 cm^{-1}; HRMS–ESI (m/z): [M+Na]$^+$ calcd for $C_{39}H_{75}O_6NNa$, 676.5487; found 676.5466. Anal. Calcd for $C_{39}H_{75}O_6N$: C, 71.62; H, 11.56; N, 2.14. Found: C, 71.56; H, 11.75; N, 2.16.

3.1.9. General procedure for 5(*n*), *n* = 16, 18, 20, 22

After adding TFA (1.2 mL, 16 mmol) to **10**(*n*) (1.10 mmol), the solution was stirred at rt for 1 h. The solution was concentrated at 70 °C to give a viscous liquid. This liquid was dissolved in CH_2Cl_2 (20 mL), and the solution was concentrated; this process was repeated nine times to chase all traces of TFA to finally give a white solid. This solid was kept under high vacuum for 1 week.

3.1.9.1. 4-(Hexadecyloxycarbonylamino)-4-methylheptanedioic acid, 5(16). White solid (100%): mp 100.0–100.5 °C; ^{1}H NMR (500 MHz, DMSO-d_6, δ): 0.89 (t, 3H), 1.10 (s, 3H), 1.27 (s, 26H), 1.54 (m, 2H), 1.71 (m, 2H), 1.97 (m, 2H), 2.16 (m, 4H), 3.91 (t, 2H), 6.78 (s, 1H), 12.06 (s, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ): 14.9, 23.0, 24.2, 26.3, 29.59, 29.66, 29.928, 29.934, 29.96, 30.00, 32.3, 34.0, 54.2, 64.0, 155.8, 175.6; IR: 3425, 3177, 2915, 2847, 1725, 1671, 1518, 1202 cm^{-1}; HRMS–ESI (m/z): [M+Na]$^+$ calcd for $C_{25}H_{32}O_6NNa$, 480.3296; found 480.3300. Anal. Calcd for $C_{25}H_{32}O_6N$: C, 65.61; H, 10.35; N, 3.06. Found: C, 65.48; H, 10.28; N, 2.98.

3.1.9.2. 4-Methyl-4-(octadecyloxycarbonylamino)heptanedioic acid, 5(18). White solid (95%): mp 102.6–102.8 °C; ^{1}H NMR (500 MHz, DMSO-d_6, δ): 0.89 (t, 3H), 1.10 (s, 3H), 1.27 (s, 30H), 1.54 (m, 2H), 1.72 (m, 2H), 1.98 (m, 2H), 2.16 (m, 4H), 3.91 (t, 2H), 6.78 (s, 1H), 12.06 (s, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ): 14.6, 22.8, 23.8, 26.0, 29.28, 29.36, 29.37, 29.63, 29.64, 29.66, 29.68, 32.0, 33.6, 53.9, 63.7, 155.4, 175.3; IR: 3424, 3181, ~2900, 2847, 1725, 1673, 1519, 1228 cm^{-1}; HRMS–ESI (m/z): [M+H]$^+$ calcd for $C_{27}H_{37}O_6N$, 486.3789; found 486.3783. Anal. Calcd for $C_{27}H_{36}O_6N$: C, 66.77; H, 10.58; N, 2.88. Found: C, 67.04; H, 10.57; N, 2.83.

3.1.9.3. 4-(Icosyloxycarbonylamino)-4-methylheptanedioic acid, 5(20). White solid (97%): mp 100.6–101.1 °C; ^{1}H NMR (500 MHz, DMSO-d_6, δ): 0.89 (t, 3H), 1.10 (s, 3H), 1.27 (s, 34H), 1.54 (m, 2H), 1.72 (m, 2H), 1.98 (m, 2H), 2.16 (m, 4H), 3.91 (t, 2H), 6.77 (s, 1H), 12.06 (s, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6, δ): 14.9, 23.1, 24.2, 26.4, 28.7, 29.58, 29.680, 29.688, 29.98, 30.00, 32.2, 33.9, 54.3, 64.1, 155.7, 175.6; IR: 3425, ~2900, 2847, 1725, 1673, 1520, 1228 cm^{-1}; HRMS–ESI (m/z): [M+H]$^+$ calcd for $C_{29}H_{41}O_6N$, 514.4102; found 514.4099. Anal. Calcd for $C_{29}H_{40}O_6N$: C, 67.80; H, 10.79; N, 2.73. Found: C, 67.99; H, 10.60; N, 2.71.

3.1.9.4. 4-(Docosyloxycarbonylamino)-4-methylheptanedioic acid, 5(22). White solid (100%): mp 107.8–108.2 °C; ^{1}H NMR (500 MHz, DMSO-d_6, δ): 0.89 (t, 3H), 1.10 (s, 3H), 1.27 (s, 38H), 1.54 (m, 2H), 1.72 (m, 2H), 1.98 (m, 2H), 2.16 (m, 4H), 3.91 (t, 2H), 6.78 (s, 1H), 12.06 (s, 2H); ^{13}C NMR (125.8 MHz, DMSO-d_6, δ): 14.9, 23.0, 24.2, 26.3, 29.57, 29.65, 29.67, 29.94, 29.95, 29.96, 29.98, 32.2, 33.9, 54.2, 64.0, 155.6, 175.6; IR: 3424, ~2900, 2847, 1725, 1673, 1520, 1229 cm^{-1}; HRMS–ESI (m/z): [M+Na]$^+$ calcd for $C_{31}H_{44}O_6NNa$, 564.4235; found 564.4246. Anal. Calcd for $C_{31}H_{44}O_6N$: C, 68.72; H, 10.98; N, 2.59. Found: C, 68.58; H, 10.95; N, 2.54.

3.2. CMC measurements

3.2.1. Surface-tension measurements

CMCs for the **4**(*n*) series were measured by the pendent-drop method as described previously.[2]

3.2.2. Fluorescence measurements

Aliquots (10 μL) of 1×10^{-4} M pyrene in MeOH were added to vials and then placed in oven at 60 °C for 10 min to evaporate the MeOH. Solutions (3 mL) of amphiphiles in aq triethanolamine (0.9% w/v) at given concentrations were added to vials, which were vortexed and placed on a rocker at normal speed in an incubator at 30 °C overnight. The emission spectra of pyrene were recorded by a fluorimeter at the excitation wavelength of 334 nm. Excitation and emission bandpasses were set at 5 and 2.5 nm, respectively. The emission intensities of the first (I_1 = 373 nm) and third (I_3 = 385 nm) peaks were used to determine the CMC value. Plots of log concentration of amphiphile versus I_1/I_3 of were made. The CMC was taken as point where a distinct change in the decrease of I_1/I_3 occurred.[27] Measurements were made at 25 °C.

CMC measurements at pH 7.4 and 25 °C were made by using the samples described above. The pH was adjusted by adding 1.0 M H_3PO_4 (70 μL) to each vial, which was vortexed and kept in an incubator at 30 °C overnight.

3.2.3. Dye-release measurements

Sterile full-strength BHIB (100 μL) was added to 10 microcentrifuge tubes (500 μL). Amphiphile (100 μL, 560 μg/100 μL in aqueous triethanolamine) was added to the first tube. A twofold dilution series was made by removing an aliquot (100 μL) from the first tube and transferring it to a second tube. This process was repeated successively for ten tubes, ending with discarding 100 μL. Sterile full-strength BHIB (100 μL) and 1 blue-dye-covered glass bead (Optimizer *blue*BALLS™, G Biosciences®) were added to each tube. The tubes were placed in a test-tube rack, which was placed on a platform mixer and swirled moderately for 2–4 h. The tubes were centrifuged at 14000 rpm for 5 min. An aliquot (150 μL) from each tube was placed in a well on a microtiter plate. Absorbance at 595 nm was measured in a microtiter plate reader. A plateau for the maximum absorbance was not reached at the concentrations tested and the absorbance change was quite small (∼0.04). To be conservative, the CMC was recorded as the first well where the absorbance increased by 0.01 above the background absorbance. All measurements were made in duplicate.

3.3. Hemolysis assay

Hemolytic activities of the dendritic amphiphiles were measured as described[17] with defibrinated, sheep red-blood cells (1.1×10^7 cells/mL), which were washed in phosphate-buffered (pH 7.4) saline (PBS) at 4 °C. Triton® X-100 (0.1% in PBS) served as the reference for complete release of hemoglobin. Dendritic amphiphiles in aqueous triethanolamine solution (5% w/v) were diluted with PBS. All dendritic amphiphile and Triton® X-100 solutions were placed in microcentrifuge tubes and warmed to 37 °C; the suspension of red-blood cells in PBS was also warmed to 37 °C prior to mixing. Suspensions of red-blood cells (500 μL) were added to solutions (500 μL) of amphiphile or Triton® X-100; the mixtures were incubated at 37 °C for 15 min. Then, the mixtures were centrifuged at 5000*g* for 20 min to pellet red-blood cells. Supernatants (100 μL) were transferred to wells of a 96-well plate without disturbing the pelleted red-blood cells; absorbances were recorded at 540 nm by using a microplate reader. Hemolytic activities (EC_{10}s) were reported as the lowest concentrations that produced an absorbance, $\leqslant 0.1$, that was 10% of complete hemolysis ($\geqslant 1.0$) by the reference. Aqueous triethanolamine solution (5% w/v) and pelleted red-blood cells did not show any absorbance.

Measurements of EC_{10} in full-strength BHIB followed the identical procedure except that the cells were suspended in broth instead of PBS. All measurements were made in duplicate.

3.4. Antimicrobial assays

3.4.1. Stock solutions of dendritic amphiphiles and vancomycin

Stock solutions (12,500 μg/mL) for all homologues were prepared by vortexing the di- and tricarboxylic acids in aqueous triethanolamine solution (5% w/v). Final stock-solution concentrations ranged from 20,800 to 31,300 μM depending on the formula weight of the homologue. Vancomycin·HCl, which was used as received, was dissolved in sterile distilled water to a final concentration of 500 mg/L.

3.4.2. Bacterial strains

Staphylococcus aureus strain ATCC 6358 and an unrelated MRSA strain ATCC 43330 were acquired from Danville Community Hospital. Six recent patient isolates of MRSA (523000, 522870, 34864, 36361, 53016, and 34380) were obtained from Georgetown University Medical Center Hospital. The Institutional Review Board of Georgetown University Medical Center approved collection and use of MRSA from adults.

3.4.3. Measurement of MIC

MIC measurements were performed as described,[1] with the single modification of dilution of cultures in growth medium (10- to 100,000-fold). Undiluted cultures and dilutions were used as inocula for MIC measurements. All measurements were made in duplicate.

3.4.4. Quality assurance

All cultures used as inocula were uncontaminated and the colonies had the expected morphologies. Cultures used for inoculation were stored up to 4 d at 4 °C until used without any differences in experimental results.

Acknowledgments

The authors acknowledge the guidance and technical assistance of Ms. Myra D. Williams in growing target microorganisms, measuring MICs, and measuring hemolysis. The authors thank Mr. Aaron Commons for preparing octadecanoyl chloride and Mr. James Satalich for measuring the CMCs of two compounds. Support for this work was provided by a grant from the Georgetown University/Virginia Tech Joint Center for Drug Discovery and Development, the Institute for Biomedical and Public Health Science at Virginia Tech, and the Institute for Critical Technology and Applied Science at Virginia Tech. We thank reviewers on early versions of this manuscript for helpful comments. We especially thank a reviewer, who challenged us to find a way to compare all three properties in the same medium. Finally, we thank the editorial staff for the generous extension of time to submit this revised version.

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