Antimicrobial activity of long-chain, water-soluble, dendritic tricarboxylato amphiphiles

André A. Williams1, Eko W. Sugandhi1, Richard V. Macri1, Joseph O. Falkinham III2* and Richard D. Gandour1

1Department of Chemistry MC 0212, Virginia Tech, Blacksburg, VA 24061, USA; 2Department of Biological Sciences MC 0406, Virginia Tech, Blacksburg, VA 24061, USA

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Objectives: To measure the antimicrobial activities of three series of homologous, dendritic tricarboxylato (three-headed) amphiphiles against a battery of bacteria and fungi.

Methods: Three series of homologous dendritic amphiphiles were synthesized containing C13 to C22 fatty chains. Susceptibility of Escherichia coli, Klebsiella pneumoniae, Lactobacillus plantarum, Micrococcus luteus, Staphylococcus aureus, methicillin-resistant S. aureus (MRSA), Mycobacterium smegmatis, Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans and Aspergillus niger to the amphiphiles was measured by broth microdilution and reported as the MIC.

Results: Several amphiphiles from each homologous series, designed and constructed to overcome the low solubility of saturated long-chain fatty acids, had antimicrobial activity against MRSA (MIC 5 36 mg/L), C. albicans (MIC = 4.4 mg/L), S. cerevisiae (MIC = 1.1 mg/L) and M. smegmatis (MIC = 8.9 mg/L). These amphiphiles had considerably better antimicrobial activities than the corresponding saturated fatty acids. Alkyl chain length influenced the values of MIC; longer chains (C18–C22) were generally more antimicrobial, but there was no uniform pattern among the microorganisms tested.

Conclusions: As the antimicrobial activity of the amphiphiles increased with increasing chain length, it is anticipated that maximum activity was not reached with these series. Thus, the identification of the optimal chain length would provide a target compound for development of low-cost, topical microbicides and anti-infectives. Further, these series of dendritic amphiphiles with the very long chains can be used as new water-soluble probes for elucidation of membrane structure and for identification of novel targets for antimicrobial design.

Keywords: synthetic lipids, solubility, fatty acids, mycobacteria

Introduction

Studies of antimicrobial properties of saturated long-chain fatty acids date back more than 80 years.1,2 These studies3–14 have measured the antimicrobial activity of homologous series of fatty acids against various microorganisms—Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans and mycobacteria. Most commonly, measuring microbial susceptibility to fatty acids has involved dissolving the fatty acid in an organic solvent, then diluting it in an aqueous broth. However, measuring antimicrobial activity can be problematic because saturated fatty acids have very low solubility in aqueous solutions.12 Instead, aqueous solutions of long-chain, fatty-acid homologues form colloidal suspensions that are finely dispersed4 or opalescent,6 or that form emulsions with other surfactants.10 Further, as the chain length increases to C18 and beyond, the aqueous solubility of the saturated fatty acids becomes negligible.15 We find no evidence that previous studies determined the solubilities of fatty acids after dilution in the broth. Hence, whether the fatty acids were completely soluble is questionable.

Conversely, determining that fatty acids are completely soluble enables speculation about how chain length affects antimicrobial activity. In a homologous series of compounds, antimicrobial activity often increases up to an optimal point as...
chain length increases; after that, the activity decreases, a phenomenon referred to as the cut-off effect. Unless the amphiphiles fully dissolve in aqueous media, however, we cannot determine whether the decreased activity is due to the 'intrinsic' activity of an amphiphile or to the decreased solubility of an amphiphile in the aqueous broth. Studies with saturated fatty acids typically conclude that the most active compounds range from C8 to C14, except for C18 when tested as solution in dimethylformamide.

Two previous approaches, which might have led to compounds with increased aqueous solubility, were used to attach saturated fatty alkyl chains to molecules that contained two carboxyl groups. First, the synthesized compounds, N-C12-iminodiacetic acid and N-C10-iminodiacetic acid, did not inhibit the growth of E. coli up to a concentration of 2500 mg/L. Second, the fatty-acid derivatives of amino acids, namely N-C18 aspartic acid and N-C18 glutamic acid, exhibited weak antimicrobial activity against E. coli, S. aureus and C. albicans. Unfortunately, neither approach produced effective antimicrobial compounds.

To overcome the challenge of low solubilities of saturated fatty acids, especially C20 and C22, homologous series of very long-chain amphiphiles that are water soluble to 20 000 mg/L greater concentrations have been designed and synthesized. These amphiphiles enable measurement of antimicrobial activity under conditions where even the longest chain (C22) is soluble in aqueous broths. Thereby, these experiments should reveal inherent chain length specificities of antimicrobial activities without resorting to organic solvents, suspensions, or emulsions to deliver the agent. However, because the shortest chain homologues (C13 and C14) might be too soluble to partition into cell walls and membranes, concentrations may be insufficient to yield an accurate measure of the 'intrinsic' antimicrobial activity. The present study reports the synthesis of new, saturated long-chain, dendritic, tricarboxylato amphiphiles, and their antimicrobial activity against a wide range of microorganisms.

Materials and methods

Chemistry

Three different series of dendritic tricarboxylato amphiphiles—3CAmn, 3CUrn and 3CCbn—were tested (Figure 1): 3C, three carboxyl groups; Am, amide linker; Ur, ureido linker;Cb, carbamate linker; and n, the number of carbons in the fatty chain. In the 3CUrn and 3CCbn series, the fatty group was derived from fatty amines and alcohols, respectively. For example, 3CUr20 and 3CCb20 were derived from the C20 fatty amine and alcohol, respectively. Because of the structure and numbering of the 3CAmn series, the fatty group is actually one carbon atom shorter than the fatty acid from which 3CAmn was derived. For example, 3CAm19 was derived from the C20 fatty acid. The synthesis of the amphiphiles 3CAmn (n = 13, 15, 17, 19, 21) was reported and that of 3CUrn (n = 14, 16, 18, 20, 22) was recently submitted (E. W. Sugandhi, R. V. Macri, A. A. Williams et al.). The amphiphiles 3CCbn (n = 14, 16, 18, 20, 22) and 3CAmn (n = 14, 16) were synthesized for this study. General comments about the synthesis and characterization data for 3CCh14, 3CCh16, 3CCh18, 3CCh20, 3CCh22, 3CAm14 and 3CAm16 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

General procedures for the preparation of long-chain carbamate tri-tert-butyl esters, 3ECbn (n = 14, 16, 18, 20, 22)

Alkan-1-ol (3.4 mmol), WeisocyanateTM (3.2 mmol), and triethylamine (Et3N) (3.2 mL) were combined in a 50 mL round bottom flask and stirred at 95°C for 4 h. The mixture was allowed to cool to room temperature, and diluted with diethyl ether (Et2O) (40 mL). The resulting solution was washed with 2 M HCl (3/C25 mL), then saturated NaHCO3 (3/C25 mL), and finally saturated NaCl (1/C25 mL). The solution was dried with MgSO4, filtered, and concentrated to a white solid (92%). The resulting solid was purified via flash column chromatography with 6 : 1 hexane/EtOAc to give a white solid (63–84% yield).

General procedures for the preparation of long-chain carbamate triacids, 3CCbn (n = 14, 16, 18, 20, 22)

Formic acid (15 mL) was added to the tri-tert-butyl ester (5.02 mmol) in tetrahydrofuran (5 mL). The resulting mixture was stirred to give a transparent solution. For some compounds, it was necessary to warm the solution to get the ester to dissolve. The transparent solution was stirred at room temperature for 48 h; the complete reaction was identified by the formation of milky solution. The solution was concentrated to yield a white solid, which was recrystallized with acetonitrile, or methanol/ water to give a white solid (75–96% yield).

Figure 1. Structure of dendritic tricarboxylato amphiphiles.
Strains of inocula for susceptibility testing

Microbial strains, culture conditions, and preparation of 3CAmn (n = 13, 15, 17, 19, 21)

The solubilities of the odd members of 3CAmn series were measured in 42 mM phosphate buffer (pH 7.2). Samples of 3CAmn (n = 13, 15, 17, 19, 21) were placed in a vial and weighed. Buffer solution was added to the vial, which was then placed in a 37°C water bath for 1 h and the appearance of the solution noted (i.e., clear versus turbid, opalescent, or suspended particulates). This procedure was repeated by increasing the amount of an amphiphile (if clear) or the amount of buffer (if turbid, opalescent, or suspended particulates) until the appearance of a clear solution persisted for 48 h. Stock solutions (12 500 mg/L) for all homologues were easily prepared by simply vortexing the triacylcarboxylic acid in the aqueous triethanolamine solution. Final stock concentrations ranged from 20 800 to 27 300 μM depending on the formula weight of the homologue.

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General procedures for the preparation of long chain amido tri-tert-butyl esters, 3EAm14 and 3EAm16

Behera’s amine23 (12.0 mmol) was dissolved in dry phenylhydrazine (60 mL). Triethylamine (14.4 mmol) and the appropriate acid chloride (12.6 mmol) were added to the flask, which immediately resulted in a cloudy solution. After stirring at room temperature for 72 h the reaction mixture was washed with saturated NaHCO₃, water, cold 10% aqueous HCl, and saturated NaCl. The solution was dried with Na₂SO₄. After filtration, the solution was concentrated yielding an off-white solid, which was recrystallized from EtOH/H₂O to yield white needles or a white solid (25–76% yield).

General procedures for the preparation of long chain amido triacids, 3CAm14 and 3CAm16

The tri-tert-butyl esters were dissolved in a minimal amount of 99% HCOOH and stirred at room temperature for 48 h. The milky white solution was concentrated in vacuo. The white solid was recrystallized from glacial HOAc/hexane to yield a white solid (74–80% yield).

Solubility of 3CAmn (n = 13, 15, 17, 19, 21)

For the work reported here, all cultures and suspensions used as inocula were uncontaminated and the colonies had the expected morphologies. All viable, uncontaminated inocula were stored up to 14 days at 4°C until used, without any differences in susceptibility to antimicrobial compounds.

Measurement of MICs

MICs of compounds dissolved in aqueous triethanolamine were measured by broth microdilution in 96-well microtitre plates.24 Preliminary experiments demonstrated that 4% (w/v) triethanolamine/water did not inhibit the growth of any microorganism tested. A 2-fold dilution series of the compounds was prepared in 96-well microtitre plates in a 50 μL volume of 1/10-strength BHIB + S and the dilution series was inoculated with 50 μL of each cell suspension. For E. coli and K. pneumoniae, the volumes of the medium and the inoculum were doubled. The resulting inoculated dilution series were incubated at 30°C and growth, as turbidity, scored visually and recorded on the fourth day. For E. coli and K. pneumoniae, the incubation temperature was 37°C and the duration was 7 days. The MIC of each compound was measured in triplicate and was defined as the lowest concentration of drug resulting in a prominent visible decrease in turbidity (incomplete; i.e. ≥50%) or a complete absence of visible turbidity compared with the drug-free control. In many cases inhibition was incomplete.

Results

Synthesis of 3CCbn (n = 14, 16, 18, 20, 22)

Figure 2 illustrates the synthesis of the 3CCbn series. The addition of alkan-1-ols to Weisocyanate ™ gave tri-tert-butyl esters, 3EChn, in good yields of chromatographed products. The
addition procedure required some optimization as the initial procedures of mixing a long-chain alkan-1-ol and Weisocyanate™ in refluxing dichloromethane, heating in toluene at 95 °C, and heating neat compounds at 95 °C gave poor yields of the desired product. Following the precedent that base catalyses the addition of the alcohol to the isocyanate, heating the mixture in triethylamine as the solvent gave high crude yields (>90%) of a colourless product. Formolysis of the tri-tert-butyl esters, 3ECbn, produced the triacids, 3CCbn, in good yields of recrystallized products. All compounds were fully characterized.

**Synthesis of 3CAmn (n = 14, 16)**

Figure 3 illustrates the synthesis of 3CAm14 and 3CAm16. The condensation of an acid chloride with Behera’s amine gave the amide, 3EAmn. Formolysis of the tri-tert-butyl esters, 3EAmn, produced the triacids, 3CAmn, in good yields of recrystallized products. All compounds were fully characterized.

**Aqueous solubilities of 3CAmn (n = 13, 15, 17, 19, 21)**

Given the limited solubility of 3CAm21 in phosphate buffer (79 μM), the solubility of this amphiphile in aqueous solutions of triethanolamine was determined. The 3CAm21 dissolved in triethanolamine/water [~4% (w/v)], such that the final solution contained ≥6:1 molar equivalents of triethanolamine/tricarboxylic acid. The solubility of 3CAm21 was greater than 12,500 mg/L (22,000 μM), and the solubilities of 3CCb22 and 3CUr22 were similar. We did not pursue maximal solubilities beyond this limit because any compound with an MIC at this concentration is not worth developing as a lead. Table 1 lists a micromolar comparison of the solubilities in phosphate buffer of the 3CAmn series and saturated fatty acids. For both series of compounds, the solubilities fell as the chain length increased. For fatty acids, the drop-off in solubility was substantial with each addition of two carbons. In the 3CAmn series, a slight decrease in solubility occurred for 3CAm13 to 3CAm17, followed by a dramatic decrease in solubility for 3CAm19 and then a modest decrease for 3CAm21. Comparisons of the two series revealed that the tricarboxylato amphiphiles were substantially more soluble in neutral phosphate buffer than the saturated fatty acids.

Because the solubilities of 3CAm19 and 3CAm21 in phosphate buffer were too low for us to feel comfortable about assessing their antimicrobial activity, we chose an alternative aqueous solution to maximize the solubility of the tricarboxylic acids in water. We followed Kaneko et al., who found that, for a dicarboxylic acid (N-C12-L-glutamate), the bis(triethanolammonium) salt dissolved to a much greater concentration than did the dipotassium salt.

Triethanolamine, which is found in several cosmetic and health care products, is a weak base, (pK_a = 7.76). (Stock solutions of the amphiphiles in triaqueous triethanolamine had a pH of 8–9.) Comparable solubility data for fatty acids in triethanolamine are not in the open literature; however, the critical micelle concentrations of the C_3–C_18 homologues range from 24 000 to 92 μM. These micellar data suggest that fatty acids are considerably more soluble in aqueous triethanolamine than...
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Table 1. Comparison of solubilities of long-chain fatty acids with 3CAmn in phosphate buffer

<table>
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<tr>
<th>Fatty group</th>
<th>Common name</th>
<th>Solubility (from ref 15)</th>
<th>3CAmn</th>
<th>Solubility</th>
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<td>C_{12}H_{25}</td>
<td>myristic</td>
<td>20–30 μM</td>
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</table>

in phosphate buffer (Table 1). This improvement in solubility would be expected based on the increase in pH of the aqueous triethanolamine solutions compared with phosphate buffer, as well as the increased solubility of triethanolamine salts relative to potassium salts.

Antimicrobial activity of dendritic tricarboxylato amphiphiles

Table 2 lists the MICs of the 3CAmn, 3CUrn and 3CCbn series. The data are values for three separate measurements; in all instances where variation was observed, all values are provided. Rather than measuring MICs of these amphiphiles against multiple representatives of an individual species (e.g. S. aureus) or type (e.g. MRSA), we measured activity against a wide range of bacteria, yeast and fungi. In so doing, we sought to determine the range of antimicrobial activity of the compounds. Further, we hoped to identify a narrow range of candidate compounds that had activity against a specific group (e.g. pathogenic yeast).

The amphiphiles demonstrated both species and chain-length specificities. Complete inhibition of growth (with exceptions) at specific concentrations was attained against E. coli, M. smegmatis, MRSA, K. pneumoniae, S. cerevisiae and A. niger (Table 2). Against L. plantarum, S. aureus, C. albicans and C. neoformans, complete inhibition was never observed, even at the highest concentration of amphiphiles. The values in Table 2 represent the lowest concentrations that displayed a decrease in turbidity (incomplete; i.e. ≥50%) or a complete absence of visible turbidity compared with the drug-free control. Cells of the two Gram-negative microorganisms, E. coli and K. pneumoniae, and the filamentous fungus, A. niger, were relatively resistant to most compounds, as were the Gram-positive microorganisms M. luteus (totally resistant), S. aureus and L. plantarum. The relative resistance of E. coli and K. pneumoniae was probably due, in part, to the presence of their hydrophilic, lipopolysaccharide-rich outer membrane. The MRSA isolate was completely inhibited by 3CAm19 (MIC = 66 μM) and 3CAm21 (MIC = 125 μM), whereas the unrelated S. aureus strain was resistant to both amphiphiles with MICs of greater than 684 μM. The M. smegmatis strain was moderately susceptible to most of the compounds, particularly 3CAm19 (MIC = 33 μM), 3CUr18 (MIC = 32 μM), 3CCb18 (MIC = 16 μM) and 3CCb20 (MIC = 31 μM) (Table 2). Cells of the pathogenic yeast strain, C. albicans, were most susceptible to 3CAm19 (MIC = 16 μM) and 3CAm21 (MIC = 7.7 μM). Cells of the other pathogenic yeast, C. neoformans, were moderately susceptible to 3CAm19 (MIC = 66–33 μM), 3CUr16 (MIC = 47 μM) and 3CCb16 (MIC = 47 μM). The S. cerevisiae strain was most susceptible to the amphiphiles with the longest chain within each of the three series; namely, 3CAm21 (MIC = 7.7 μM), 3CUr22 (MIC = 30 μM) and 3CCb22 (MIC = 1.8 μM).

Discussion

The carbon-branched dendrons (Figure 1) offer a facile entry into the study of water-soluble amphiphiles which have very hydrophobic groups. These fatty groups, as acids, alcohols and amines, are attached to the tricarboxylic-acid head group. Dissolving these derivatives in aqueous triethanolamine gives the trianionic salt (Figure 1) in relatively concentrated solutions. Having three carboxylates in the head group enables aqueous solubility of saturated C_{22} chains at concentrations sufficient for antimicrobial screening without requiring an emulsion. The highest concentrations of the compounds inhibiting growth were well under the highest soluble concentrations. Thus, the results were not influenced by solubility limits.

The data in Table 2 demonstrate that compounds 3CAm19 and 3CAm21 partially inhibited growth of C. albicans at low concentrations. These results suggest that longer chain lengths could have better activity, possibly complete inhibition, against C. albicans. In the 3CUr and 3CCbn series, 3CUr16 and 3CCb have the lowest MICs (47 μM). This difference in
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*aIncomplete inhibition.*
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chain length specificity among the three series suggests a different mechanism of activity for the 3CAm series than the other series. Several of these amphiphiles had almost no activity against L. plantarum (incomplete inhibition by all compounds at ≥280 mg/L, Table 2). If 3CAm19 and 3CAm21 do not irritate dermal tissue, they could be used in topical formulations for preventing or curing Candida infections (e.g. vaginitis) without inhibiting L. plantarum, a member of the normal vaginal flora.

Topical fatty acid-based microbicides also might help prevent nasal colonization and transmission of infection by S. aureus or MRSA.31 Amphiphiles 3CAm19 and 3CAm21 completely inhibited the growth of the MRSA strain, whereas the MICs against the unrelated S. aureus strain were considerably higher (Table 2). The differences in MICs may relate to some unknown differences in cell surface structure and composition between the unrelated S. aureus and MRSA strains.

Against the S. cerevisiae strain, the longest chain amphiphiles of each series (3CAm21, 3CUr22 and 3CCb22) exhibited strong antimicrobial activity (Table 2). Possibly, amphiphiles with longer chain lengths could have better activity against S. cerevisiae. Because of the existence of an immunocompetent mouse model,32 availability of mutants, and ease of genetic manipulation of S. cerevisiae, the strong antimicrobial activity against S. cerevisiae could be exploited to identify the mechanism of action of these antimicrobial amphiphiles. Further, the results could also guide studies of antibiotic targets in C. albicans, which is related to S. cerevisiae.

Amphiphiles from all series had antimicrobial activity against M. smegmatis strain ATCC 607. Mycobacteria are relatively susceptible to fatty acids, probably because the elevated cell surface hydrophobicity of mycobacteria is the highest among microorganisms.33,34 The tricarboxylato amphiphiles that exhibited the best activity within each series were not the longest chain members. Instead, a specific chain length (i.e. C20) has the most activity. Moreover, activity of these tricarboxylato amphiphiles against M. smegmatis indicates they might be active against pathogenic mycobacteria that cause skin infections (e.g. Mycobacterium marinum and Mycobacterium ulcerans).

Synthesizing, measuring antimicrobial activity, and measuring solubility of longer saturated chains (>C22) as well as unsaturated derivatives are current goals. An additional goal is to identify the intrinsic antimicrobial activity of each amphiphile in a homologous series. Further, evidence that different chain lengths give optimal activity for different microorganisms, e.g. M. smegmatis and S. cerevisiae (Table 2), indicates that this experimental approach may identify chain lengths that are more selective in inhibiting the growth of a given microorganism. Additional goals are to use these water-soluble tricarboxylato amphiphiles as probes to identify the cut-off effect16 and to understand the reasons for it. For example, chain-length selectivity may be due to partitioning effects18 between broth and cell walls or cell membranes or both.

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Transparency declarations

None to declare.

Supplementary data

General comments about the synthesis and characterization data for 3CCb14, 3CCb16, 3CCb18, 3CCb20, 3CCb22, 3CAm14 and 3CAm16 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


