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Synthesis and Antimicrobial Activity of Triazine Dendrimers with DABCO Groups

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Triazine dendrimers and smaller dendritic scaffolds that present 1,4-diazabicyclo[2.2.2]octane (DABCO) on the periphery were prepared and assessed for antimicrobial activity and human cell toxicity. Hydrophilic linkers on the periphery of these multivalent scaffolds were derivatized with 2 to 6 DABCO groups that presented either methyl, benzyl, or dodecyl substituent. All of these derivatives were highly soluble in water. Antimicrobial assays against *Staphylococcus aureus* (Newman), methicillin-resistant *S. aureus* (MRSA; Sanger 252) and *Escherichia coli* (K-12) revealed that antimicrobial activity is influenced by two factors; the alkyl substituent on the DABCO group and the valency of the construct. Antimicrobial activity decreased from dodecyl > benzyl > methyl. Divalent and trivalent compounds showed greater activity than tetravalent and hexavalent compounds.

Introduction

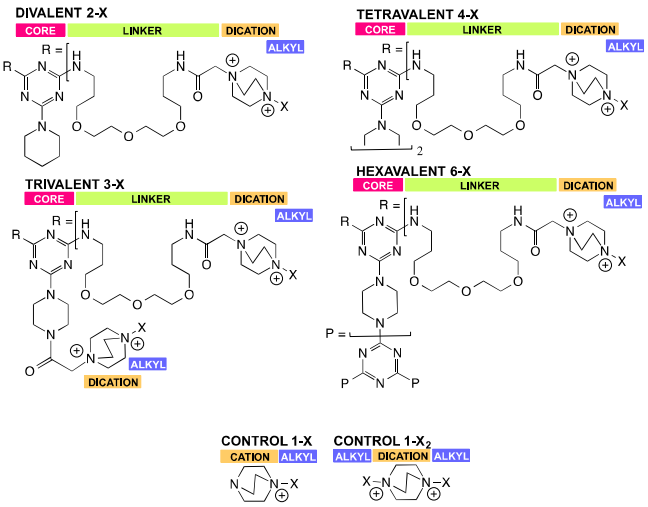
Quaternary ammonium compounds (QAC) are widely used as disinfectants, surfactants and antistatic agents.[1] For example, benzalkonium chloride (BKC), cetyl trimethylammonium chloride and cetrimide are commonly found in first aid antiseptics for use as topical antimicrobial agents. Continued interest in QAC stems from their activity against multi-drug resistant bacteria. Although the exact mechanism of their antimicrobial action is still unclear, QACs are believed to disrupt the integrity of cell membranes and increase cell permeability.[2-4]

In addition to the exploration of small molecule QAC, larger "multivalent" molecules are broadly employed as antimicrobials. One example is Polyquad (Alcon), a polymeric antimicrobial found in contact lens solutions, wherein the QAC is on the polymer backbone.[5] Alternatively, side chains containing a QAC have been incorporated into linear polymers including polycarbonates[6] and polyacrylamides.[7] Dendritic molecules offer an alternative to linear polymers. A variety of dendritic scaffolds have been explored that incorporate both cationic and anionic groups.[8,9] Some of these materials incorporate QAC derived from tetralkylated amines while others rely on protonation. For example, tetraalkylated QACs have been incorporated into carbosilane dendrimers,[10] alkylated poly(propyleneimine)[11] and PAMAM dendrimers displaying DABCO, 1,4-diazabicyclo[2.2.2]octane have been described.[12]

Here, we combine triazine chemistry for the polymer backbone and DABCO as our source of QAC. Triazine chemistry offers easy access to a variety of dendritic, multivalent compounds.[13] In general, triazine dendrimers are resistant to a hydrolysis and degradation across a broad pH range—from 0 to 14—making them candidates for surface use. DABCO-containing compounds show activity across both gram positive and gram negative pathogens as well as other microbes.[14] Installation of DABCO groups is readily accomplished. Additional compositional diversity can be accessed when the DABCO group is present on the periphery because it can also present a variable alkyl substituent.

The molecules examined in this study are shown in Chart 1. All comprise a hydrophobic triazine core with hydrophilic linkers that present the substituted DABCO group. The choice of the hydrophilic linker (4,7,10-trioxa-1,13-tridecanediamine) was based on the solubility it conveys to dendritic structures.[15]

Chart 1 Compounds used in this study. "X" can be Me, Bz, or $C_{12}H_{25}$. The "R" group represents the entire chain comprising linker, dication and alkyl group illustrated to the right of the open parenthetical.



Three different alkyl substituents were chosen for the DABCO groups; methyl, benzyl, and dodecyl. The nomenclature adopted reflects the valency of the dendritic compound and the alkyl substituent presented. For example, the divalent construct presenting two DABCO derivatized with benzyl groups is identified as **2-Bz**, while the hexavalent molecule presenting dodecyl groups is **6-**

C_{12}. In addition, two groups of controls, **1**, were employed that comprised either a mono- or disubstituted DABCO. To differentiate these compounds, the number of alkyl substituents is indicated.

Experimental

Materials

4,7,10-Trioxa-1,13-tridecanediamine (>98%), piperazine (99%) purchased from Sigma Aldrich (St.Louis, MO, USA). N,N-diisopropylethylamine (99%, DIPEA), di-tert-butyl dicarbonate (99%) purchased from AKScientific (Union City, CA USA). Dichloromethane (DCM, 99.9%, HPLC grade), methanol (99.9%, HPLC grade), ethyl acetate (99.9%, HPLC grade), hexane (99.9%, HPLC grade), acetone (99.9%, HPLC grade) all are purchased from Pharmco-AAPER (Brookfield, CT, USA). Acetonitrile (99.9%, HPLC grade) purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). Piperidine (99%), magnesium sulphate ($MgSO_4$, 99.5%) purchased from Alfa Aesar (MA, USA). The Discover microwave-SP purchased from CEM Corporation is used for the reactions. The automated column chromatography, Teledyne Isco CombiFlash® Rf-200 is used for purifying the compounds.

Synthesis

The supporting information contains detailed experimental protocols, summaries of spectral data, and spectra.

Bacterial strains and growth media

Staphylococcus aureus (Newman), Methicillin-resistant *Staphylococcus aureus* (MRSA, Sanger 252), and *Escherichia coli* (K-12) were grown in Mueller-Hinton broth (MHB, Sigma Aldrich) overnight at 37°C in a shaking incubator.

Preparation of compound solutions

Stock solutions (1000 ppm) were made (1mg / 1mL) for each test compound. These solutions were diluted to 200, 100, 50, 25, and 12.5 ppm solutions. The stock solution and dilutions were made with purified Milli-Q DI water.

Minimum inhibitory concentration assay (MIC)[16-18]

The MIC values were determined using the broth microdilution approach in accodrance with clinical laboratory guidelines. Specifically, overnight bacterial cultures were diluted 1:20 and grown to optical density (OD) of approximately 0.4 at a 600 nm wavelength on the day of the assay. Cultures were then diluted to a final concentration of 1:2000 (approximately 2 x 10^5 cfu/ml) in a final volume of 200 ul of MHB at the indicated concentration of each compound (0, 6.25,12.5, 25, 50 and 100 µg/mL) in 96-well plates. The plates were then incubated overnight (18 hours) at 37°C under static conditions. The optical density was read at 600 nm on a plate reader (FLUOstar Omega). MIC assays for each compound were repeated at least two times in triplicate.

Cytotoxicity assay 1[19-21]

Hela cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37 °C in 5% CO_2. Cells were plated at 2 x 10^4 cells per well in 96-well tissue-culture treated plates and incubated for 24 hours. The media was then removed and replaced with serum-free DMEM containing the compound at the indicated concentration and cells were incubated for an additional 24 hours. Twenty microliters of MTS tetrazolium from the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was added to each well and the plates were incubated for 2 h at 37 °C in 5% CO_2. Reduction of the tetrazolium salt by living cells produces a coloured formazan compound that was quantified by measuring absorbance at 490 nm. One hundred percent viability was set as the absorbance of the untreated cells.

Cytotoxicity assay 2

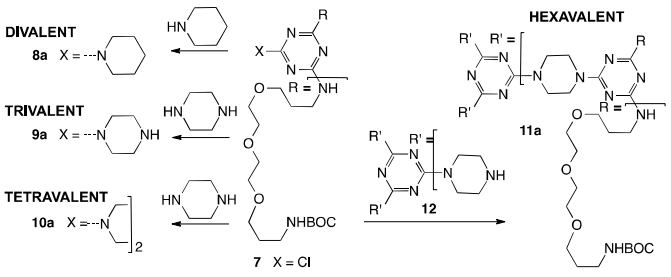
HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37 °C in 5% CO_2. Cells were plated at 2 x 10^4 cells per well in 96-well tissue-culture treated plates and incubated for 24 hours. The media was then removed and replaced with serum-free DMEM containing the compound at the indicated concentration and cells were incubated for an additional 24 hours. Plates were then equilibrated to room temperature for 30 minutes and 100µl of CellTiter-Glo Reagent from the CellTiter-Glo Luminescent Cell Viability Assay (Promega) was added to each well. Plates were placed on an orbital shaker for 2 minutes to mix contents and induce cell lysis. Cell viability was assessed by measuring the amount of ATP present following lysis, which indicates the presence of metabolically active cells. ATP was quantified by recording the luminescent signal generated from the reaction of luciferase in the CellTiter-Glo Reagent. The plates were incubated at room temperature for 10 minutes prior to measuring luminescence using a FLUOstar Omega Microplate Reader (BMG Labtech) (emission filter: blank; gain: 2000; integration time: 0.5 second/well). One hundred percent viability was set as the luminescence of the untreated cells.

Results and Discussion

Synthesis

The synthesis of the compounds described in this study relied on preparation of the multivalent core followed by elaboration of the periphery with DABCO groups. Scheme 1 shows the synthesis of these cores commencing with monochlorotriazine **7**. Intermediate **7** is obtained from reaction of the mono-BOC protected diamine and cyanuric chloride. From **7**, all four cores are available in a single step by reaction with the suitable amine precursor. Divalent core **8a** requires piperidine. Trivalent core **9a** and tetravalent core **10a** require piperazine. Hexavalent core **11a** derives from trispiperazinyltriazine **12** (itself available in two steps). The yields for the preparation of **8a-11a** from **7** are shown in Table 1.

Scheme 1 Syntheses of the multivalent cores.



Elaboration of these cores to the final compounds is shown in Scheme 2. First, the core is deprotected with 3N HCl in MeOH. The resulting amines are treated with chloroacetylchloride. To

complete the syntheses, a suitably derivatized DABCO reagent is employed. Advantageously, the final target compounds can be purified by precipitation. The supporting information provides full experimental details (and spectra) for these compounds

Scheme 2 Elaboration of the core to the final library of compounds.

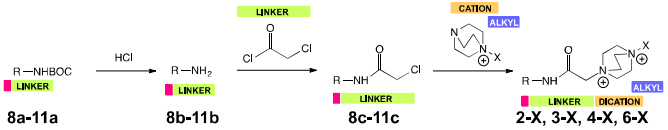


Table 1 summarizes the yields associated with these syntheses. The column labels (a) – (d) correspond to the molecules shown in Scheme 2. These suffixes are appended to compounds when appropriate (ie **8b** of Figure 1). The cumulative yield for the core is given in the first column. For this exploratory work, these reactions were not optimized. Still, the yields in most cases are acceptable. The low yields associated with the chloroacetyl derivatives (c) are attributed to decomposition during chromatographic purification.

NMR spectroscopy is invaluable in characterizing the products of synthesis. Figure 1 shows the spectra derived from intermediates in the synthesis of **2-C$_{12}$**. Control **1-C$_{12}$** and the divalent core, **8a**, are readily assigned. Intermediates derived from treating **8a** with acid to yield amines including **8b** are not characterized. Yields reported in Table 1 correspond to the oil obtained after extraction. Reaction of **8b** with chloroacetyl chloride yields a number of products upon analysis with thin layer chromatography. Column chromatography provides **8c** which is clearly a mixture of products including traces of **8a** as well as other products attributed to hydrolysis or cyclization products. These latter products are best indicated by extraneous singlets of an acetyl methylene appearing near or downfield of 4ppm.

The NMR spectra of **2-C$_{12}$**, in contrast, suggests a single major compound. The purity obtained on extraction is largely due to the to the solubility of sideproducts in the organic phase. These NMR traces provide specific signatures which have prove generally useful when monitoring the syntheses of these compounds. Notably, the methylene derived from the acetyl portion of the linker as well as the significant downfield shifts of the DABCO dication. Integration provides a useful tool for assessing the process. For **2-C$_{12}$**, the ratio of integrations (listed from downfield to upfield shift as indicated with a "*" beneath the trace) of methylene:DABCO:DABCO: piperidine:piperidine:piperidine:aliphatic region:terminal CH_3 is theoretically 2:6:6:2:1:2:18:3. The measured integrations relative to the terminal methylene of the acetyl group is 2:6.3:6.5:2:1.1:2.2: 19.5:3.8, values that are in good agreement with the proposed structure.

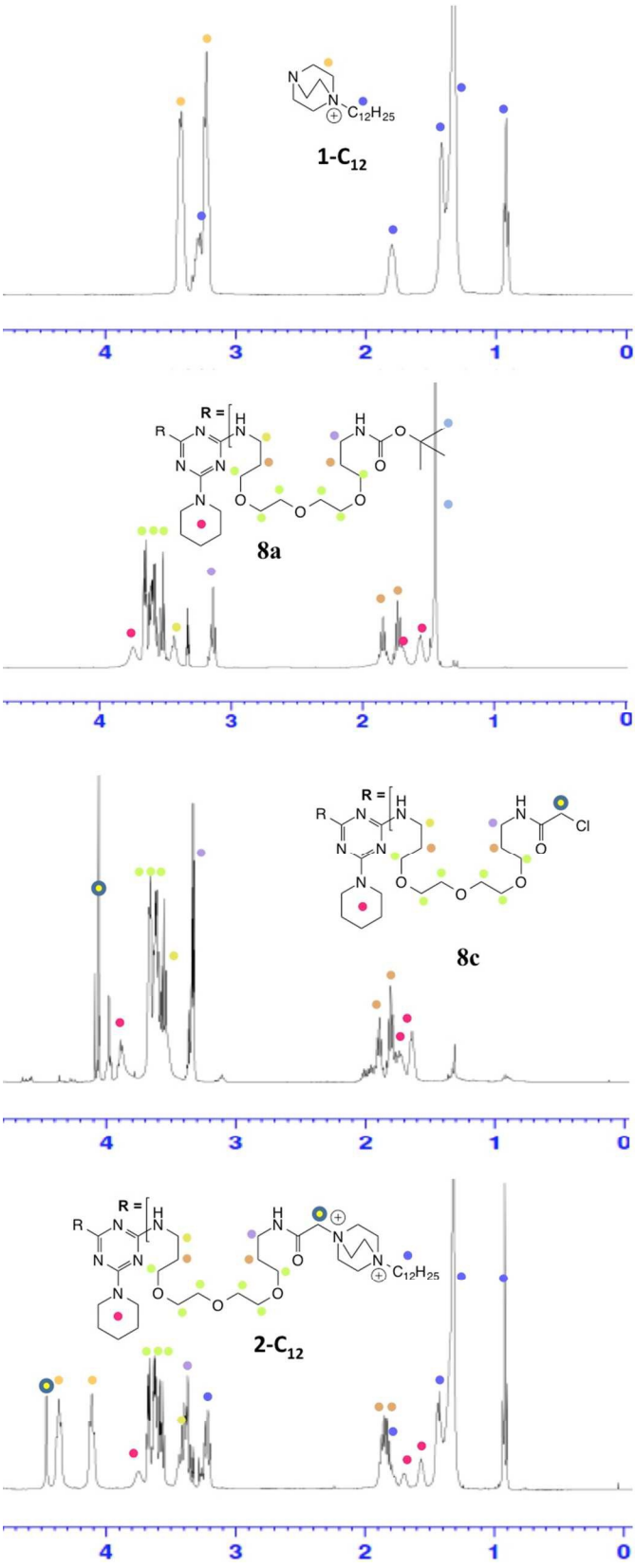
Biological Activity

Antibacterial activity of the compounds were assessed by measuring bacterial growth in the presence of each compound over a concentration range from 1.6 µg/mL – 100 µg/mL. The minimum inhibitory concentration (MIC) is defined as the minimum concentration needed to inhibit bacterial growth. Each compound was assessed at least twice in triplicate and results are shown in Table 2. Graphical data is available in supporting information. Cytotoxicity of the compounds to HeLa cells, an immortalized human cervical cell-line, was assessed by measuring levels of a formazan byproduct that is produced at levels proportional to cellular survival.

Table 1 Yields of synthesized compounds. Column (a) shows the cumulative yield of the core. Columns show the yields for the remaining reactions as indicated in Scheme 2 including the final target. [a]The calculated molecular weight of the final target is given in Daltons. Experimental observation of the polyion was not achieved.

Cmpd	(a)	(b)	(c)	Target	MW[a]
				2-Me 73%	935
8	90%	77%	27%	**2-Bz** 58%	1087
				2-C$_{12}$ 81%	1243
				3-Me 78%	1104
9	91%	72%	27%	**3-Bz** 91%	1332
				3-C$_{12}$ 76%	1566
				4-Me 73%	1784
10	68%	78%	69%	**4-Bz** 79%	2088
				4-C$_{12}$ 79%	2400
				6-Me 77%	2885
11	91%	78%	18%	**6-Bz** 25%	3341
				6-C$_{12}$ 90%	3809

Fig 1. The NMR spectra of the intermediates of **2- C$_{12}$** including a) **1- C$_{12}$**, b) **8a**, c) **8c**, d) **2-C$_{12}$**.



Concentrations reported in Table 2 indicate the highest concentration of compound where greater than 50% of the HeLa cells survived.

Dodecyl derivatives showed the greatest antimicrobial activity when compared with benzyl and methyl derivatives. This observation is consistent with the literature.[1-3] Increasing valency leads to decreasing antimicrobial activity: **2-C_{12}** > **3-C_{12}** > **4-C_{12}** > **6-C_{12}**. Advantageously, these compounds show high activities against both gram-positive and gram-negative pathogens with **1-$(C_{12})_2$**, **2-**C_{12} in particular exhibiting a higher degree of selective toxicity towards bacterial cells.

In comparison to dodecyl derivatives, benzylated materials were less active and less cytotoxic. Indeed, benzyl derivatives showed activities that places them between dodecyl and methyl classes. Controls **1-Bz** and **1-$(Bz)_2$** are not active against these three strains of bacteria at concentrations up to 100 μg/mL. The multivalent derivatives showed similar broad activity against all three strains of bacteria. Interestingly, **4-Bz** showed significant inhibition of growth against gram-negative *E. coli* at 6.25 μg/mL, while at the same time showing no cytotoxicity against HeLa cells at concentrations of up to 100 μg/mL.

In response to concerns that the MTT assay may not be the best choice of assays to assess cytotoxicity for reasons of sensitivity, interference arising from non-enzymatic reduction, its role in measuring cell metabolism as opposed to cell number, and the chance that the targets uncouple electron transport from oxidative phosphorylation, a second cytotoxicity assay based on ATP bioluminescence was performed on a subset of these compounds. Satisfyingly, the results mirrored those of the MTT assay, but reduced the therapeutic index. These activities appear in Table 2. Graphical data is provided in the SI.

Table 2 Summary of MIC values for different bacterial strains and cytotoxicity values (μg/mL) against Hela cells using an MTT and ATP assay. Here, "n/a" reflects assays that were not conducted, and "---" indicates no activity at 100 μg/mL for the MIC assay. The ATP assay reports percent survival at the dose (in μg/mL) indicated.

Cmpd	*S.aureus*	*MRSA*	*E.coli*	MTT	ATP
1-Me	---	---	---	n/a	---
1-$(Me)_2$	---	---	---	n/a	---
1-Bz	---	---	---	n/a	---
1-$(Bz)_2$	---	---	---	n/a	---
1-C_{12}	25	25	100	n/a	---
1-$(C_{12})_2$	<1.56	<1.56	<1.56	> 25	---
2-Me	---	---	---	n/a	100 (82% ± 3)
2-Bz	12.5	50	12.5	>100	100 (86% ± 4)
2-C_{12}	<1.56	<1.56	3.125	>50	12.5 (61% ± 6)
3-Me	---	---	100	n/a	100 (61% ± 8)
3-Bz	100	100	50	>100	---
3-C_{12}	<3.125	<3.125	12.5	>25	---
4-Me	---	---	12.5	>100	---
4-Bz	25	25	<6.25	>100	---
4-C_{12}	50	25	25	>25	---
6-Me	50	50	50	>100	100 (64% ± 8)
6-Bz	50	50	50	>100	50 (76% ± 6)
6-C_{12}	50	25	100	>25	---

Methyl derivatives showed very little activity in any assay. The controls and **2-Me** had no effect on either bacterial or mammalian cells at the concentrations measured. Curiously, the tetravalent construct, **4-Me** showed a similar "hot-spot" of reactivity as **4-Bz** against *E. coli.* This "hot-spot" is inconsistent with the trend of decreasing activity with increasing valency.

Taken in aggregate, the data reveals a correlation between valency and biological activity. Specifically, increasing the number of QAC decreases both antimicrobial activity and HeLa cell toxicity: **2**-X > **3**-X > **4-X ~ 6-X** (where X is any alkyl substituent). The choice of alkyl substituent for these compounds is significant. In the context of the broader literature, the trends are in general agreement with some notable exceptions. An abbreviated discussion of the most relevant examples follows: the field has been recently reviewed.

As it pertains to DABCO-containing QAC, working with cyclodextrins containing either 6 or 7 DABCO groups, Engel found that alkyl substituents varying from methyl to C_{22} showed similar activity.[14] However, significantly greater (>100x) activity was measured in the multivalent construct than in mono and disaccharides functionalized with either 1 or 3 DABCO groups. Using polyacrylates with a DABCO group tethered to the backbone with a 10-methylene spacer, Mathias found a 4x difference in MIC values for butyl and hexyl substituents (250 μg/mL and 62.5 μg/mL, respectively against *S. aureus* and *E. coli*).[7] The impact that polymer size had on bioactivity was not assessed. Both Yang[6] and Tew[22] have shown that decreasing the size of polymers increases their antimicrobial activity. This behavior is more pronounced for *S. aureus* than *E. coli.* Tew proposed a model wherein the peptidoglycan of gram-positive bacteria only allows access of smaller molecules in a process described as "sieving".

The antimicrobial activity of dendrimers and dendritic QACs have been probed. Cai has shown that PAMAM dendrimers of generation 3 and 5 presenting primary amines on the periphery, have little difference in activity.[23] Pegylation, however, reduces both activity and cytotoxicity. Earlier studies by Cooper focused on *E. coli* biocides showed a dependence on both dendrimer generation and nature of the cationic group of the tetralkylammonium ion.[11] Larger dendriemrs were more active than smaller dendrimers. Gomez and de la Mata showed that the activity of carbsilane dendrimers derivatized with tetraalkylammonium ions depending on the nature of cation and showed trends that modest differences in activity could be seen with increasing size (for the dications) and decreasing size (aryl monocation) from small generation one dendrimers to larger generation three constructions.[10]

Conclusion

What emerges from these studies is that highly soluble, low valent, DABCO-derivatized triazine derivatives can act as antimicrobials. The potency measured for the most active of these molecules largely matches that seen in a range of different constructs—from monomeric to polymeric—wherein MIC values hover in the low μg/mL. The general observation that biological activity persists similarly in both gram positive and gram negative pathogens is important. Moreover, activity against methicillin-susceptible and resistant strains of *S. aureas* is conserved in the most potent agents including **2-C_{12}**. The differences in the concentration required for advantageous antimicrobial activity and the onset of human cell toxicity is noteworthy for **2-C_{12}.** We hypothesize that the activity observed derives from the membrane-disrupting properties of the DABCO group, but this belief has not been experimentally validated. Unfortunately, the therapeutic index of these compounds, a value calculated by comparing the cytotoxic dose to MIC value, is relatively low, and approximates 8 across the range of compounds assessed. Accordingly, this molecule—especially in light of its synthetic accessibility—serves as a guidepost for future studies of cation choice and alkyl substituent.

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Notes and references

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† S.R.S. and Z.M.A. contributed equally to this work. Electronic Supplementary Information (ESI) available: detailed synthetic procedures, summaries of characterization data, spectra, and biological data. See DOI: 10.1039/b000000x/

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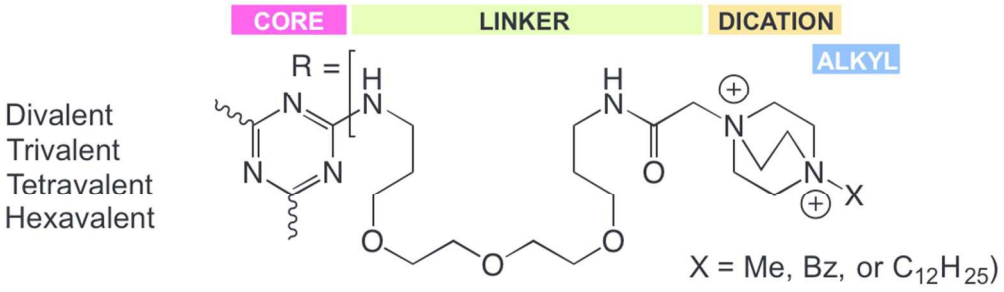

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