A rationally designed macrocyclic cavitand that kills bacteria with high efficacy and good selectivity†

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An amphiphilic macrocyclic cavitand that shows good antibacterial activity, comparable to that of peptide-based antibiotics was developed by rational design and its antibacterial selectivity over mammalian cells was examined.

Because of increasing bacterial resistance to conventional antibiotics, there is a surge of interest in developing new antibiotics based on novel chemical scaffolds.[1] To date natural or synthetic peptide-based amphiphiles have demonstrated high potential as emerging antibiotics that may kill drug resistant bacteria below the μM range.[2–5] The antibacterial activity of such amphiphilic peptides has been attributed mainly to the membrane disruption of bacteria as a result of electrostatic and hydrophobic interactions between side chains of the peptides and negatively-charged bacterial membranes.[6,7] Although several synthetic polymer-based disinfectants[8–10] showed excellent antibacterial efficacy, the difficulty in controling their molecular weights limits their applications *in vivo* as antibiotics. Nevertheless, these studies demonstrated the importance of amphiphilicity or the lipophilicity/charge balance in the development of antibacterial agents.[8–10] More recently, antibacterial agents based on amphiphilic dendrimers containing quaternary alkyl ammonium groups at the periphery were developed and their antibacterial activity depends strongly on the alkyl chain length.[11] All these previous findings suggest that both amphiphilicity and multivalency should be considered simultaneously in designing efficient antibacterial agents. With these in mind, we decided to explore new chemical scaffolds that may provide the above mentioned features: a rigid core possessing multiarms to which amphiphilic side chains are attached. Perhydroxycucurbit[6]uril ($(HO)_{12}$CB[6]), a synthetic macrocyclic cavitand, was selected as a scaffold for the purpose because it has a rigid macrocyclic core with twelve identical functional groups at the periphery enabling modification with amphiphilic moieties.[12,13] Herein we report a novel macrocycle-based antibacterial agent that kills a broad spectrum of bacteria from Gram-negative to positive with comparable efficacy to that of natural antibacterial peptides such as magainins.[14] In addition, the interaction of the anphiphilic CB[6] derivative with model membranes of bacterial and mammalian cells has been examined.

The synthesis of macrocyclic amphiphiles **1** and **2** is shown in Scheme 1. The primary and secondary amine-modified CB[6] derivatives were synthesized by photoreaction of $(allyloxy)_{12}$CB[6][12] with 2-aminoethanethiol and 2-(butylamino)ethanethiol, respectively. Both **1** (number of amine attachment, NA = 10–11) and **2** (NA = 10–12) were purified by reverse-phase HPLC and chracterized by ^{1}H NMR and MALDI-TOF mass spectroscopy (see ESI†). Since the chain length of butyl group is known to be enough to interact with bacterial membrane as demonstrated in the previously reported paper,[10] we prepared **2** as a model of amphiphilic cucurbiturils. As illustrated in Scheme 1, **1** contains multiple positively-charged primary amines surrounding the rigid CB[6] core; its interaction with bacterial membranes is thus expected to be mainly electrostatic. On the other hand, the interaction of **2** with bacterial membranes may be enhanced by additional hydrophobic interaction owing to the extra alkyl groups at the N-atom in a similar manner to that of amphiphilic peptide-based antibiotics. In addition, the multiple side chains surrounding the macrocyclic cavitand may show cooperative multivalency effect in action[15,16] as demonstrated in dendrimer amphiphile-based antibacterial agents.[11]

The *in vitro* antibacterial activity of the CB[6] derivatives against three Gram-negative, three Gram-positive, and two drug-resistant bacterial species was determined by broth microdilution assay in Mueller-Hinton broth (Table 1). The minimum inhibitory concentrations (MIC) of both **1** and **2** were determined by measuring the lowest concentration at which bacterial growth is completely inhibited during 24 h incubation at 37 °C. The minimum bactericidal concentrations (MBC) were also obtained by the agar plating method (see ESI†). As anticipated, **2** showed significantly (up to two orders of magnitude) higher antibacterial efficacy than **1** against both Gram-negative and positive species. It is noteworthy that **2** showed 4 μg mL^{-1} of MIC as well as MBC for two bacterial strains (*Pseudomonas aeruginosa* and *Staphylococcus epidermidis*), which is comparable to highly potent peptide-based antibiotics such as magainins.[14] It is further noteworthy that **2** also showed good efficacy in killing drug resistant bacteria, MRSA 3521 and 3514 that show high resistance against various conventional chemical antibiotics including Norfloxacin and Gentamicin (see ESI†). Although **1** has multiple positively-charged primary amine groups which seem to interact with bacterial membrane well, it did not show appreciable antibacterial activity. In contrast, **2** containing additional butyl groups shows a drastically increased activity, indicating that hydrophobic alkyl groups may play a crucial role in killing

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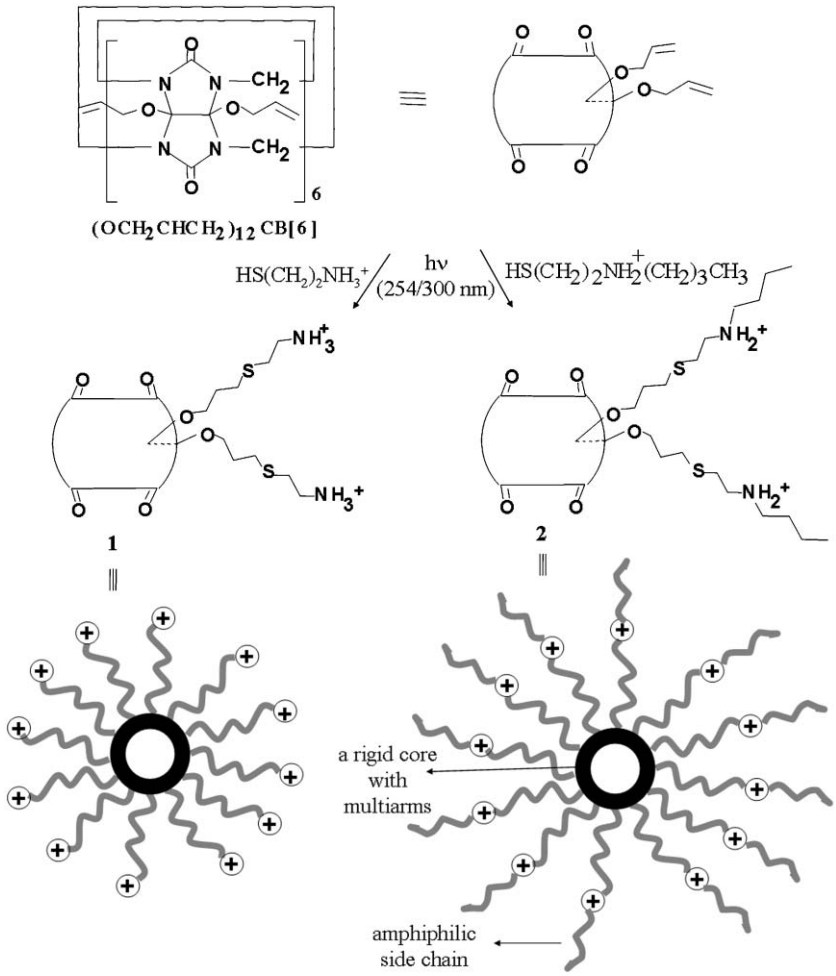

Scheme 1 Synthetic scheme and schematic representation of amine-modified CB[6] derivatives.

Table 1 *In vitro* antibacterial activity of **1** and **2** (MIC and MBC, μg mL^{-1})

	1		**2**	
	MIC[a]	MBC[b]	MIC[a]	MBC[b]
Esherichia coli[c]	>512	>512	32	64
Pseudomonas aeruginosa[c]	>512	>512	32	64
Salmonella typhimurium[c]	>512	>512	4	4
Bacillus subtilis[d]	>512	>512	32	32
Staphylococcus epidermidis[d]	>512	>512	32	64
Staphylococcus aureus[d]	>512	>512	4	8
Staphylococcus aureus[e] *(MRSA3521)*	>512	>512	32	32
Staphylococcus aureus[e] *(MRSA3514)*	>512	>512	32	64

[a] Minimum concentration of compounds required to inhibit the growth of bacterial cells in liquid broth. [b] Minimum concentration of compounds required to kill the bacterial cells. [c] Gram-negative strains. [d] Gram-positive strains. [e] Methicillin-resistant *Staphylococcus aureus*.

bacteria. Whatever the killing mechanism may be, this result implies that **2** interacts with bacterial membranes more strongly than **1** does due to the presence of the extra butyl groups. On the other hand, no detectable antibacterial activity was observed even at the highest concentration (1 mg mL^{-1}) when monovalent primary and secondary amines, the side chains of **1** and **2**, respectively, were tested (data not shown).

To examine how strongly and selectively **1** and **2** interact with bacterial and mammalian cell membranes, we carried out calcein dye release experiments using small unilamellar vesicles (SUVs).[17] Two SUVs systems entrapping calcein dye were prepared from cholesterol, 1-palmitoyl-2-oleylphosphatidyl choline (POPC), and 1-palmitoyl-2-oleylphosphatidyl glycerol (POPG). The SUVs composed of POPC–POPG (3 : 1, w/w) and POPC–cholesterol (10 : 1, w/w) were used as a model bacterial and mammalian cell membrane, respectively. The extent of leakage of entrapped calcein was measured by its fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The dose-response curve for both **1** and **2** denoted as percent calcein leakage is shown in Fig. 1. In the case of **2** (10 μg mL^{-1}) more than 60% of calcein leakage was observed for the negatively charged POPC/POPG SUVs (bacterial membrane model) but less than 20% for POPC–cholesterol SUVs (mammalian membrane model), indicative of high selectivity between bacterial and mammalian cells. As expected, **1** showed much lower membrane-lytic activity (*ca.* 20%) for both SUVs even at concentration of 20 μg mL^{-1}. This result is in good agreement with those obtained from *in vitro* antibacterial activity test. The ability of **2** to selectively disrupt the bacterial membrane model may be attributed to the synergistic interactions of its positive-charged amines and hydrophobic butyl groups with the negatively charged surface and

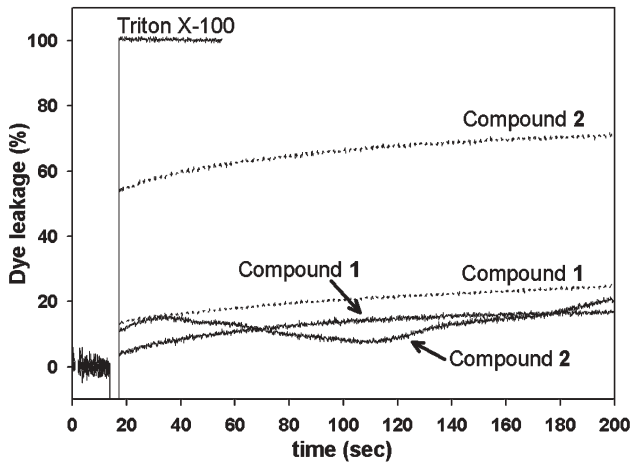

Fig. 1 Percent leakage of calcein from negatively charged POPC–POPG (3 : 1, w/w) SUVs (dotted line) and zwitterionic neutral POPC–Cholesterol (10 : 1, w/w) SUVs (solid line) upon treatment of **1** (20 μg mL^{-1}) and **2** (10 μg mL^{-1}) at pH 7.4.

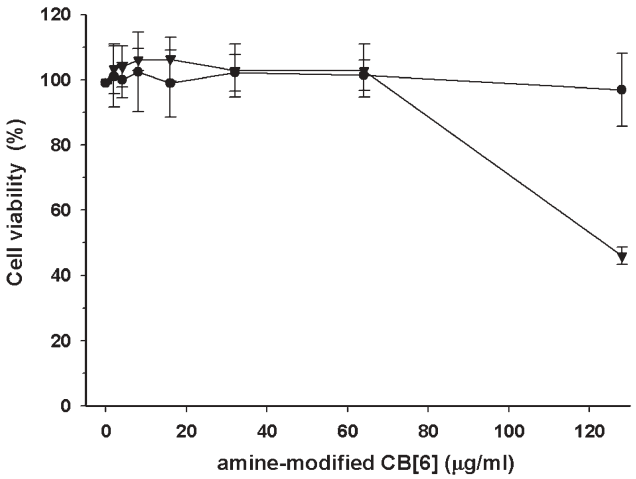

Fig. 2 Viability of NIH3T3 cells relative to control is expressed as a function of concentration of **1**(●) and **2**(▼).

lipid layers of the SUVs, respectively. Since the portal size of CB[6] (3.9 Å) is too small for calcein to pass through and **2** is not likely to self-associate to form a big pore within bacterial membrane, the calcein leakage from SUVs appears to be due to membrane disruption rather than pore-formation.

To further evaluate the toxicity of **1** and **2** against mammalian cells, we carried out an MTT assay (Fig. 2). Compound **2** showed very low cytotoxicity up to about 70 μg mL^{-1} which is much higher than normal MIC of **2** against bacteria (<32 μg mL^{-1}), while revealing some toxicity beyond the concentration. This result suggests that **2** is able to kill bacteria with good selectivity over mammalian cells.

In summary, we have designed and synthesized a novel antibacterial agent containing a CB[6] macrocyclic scaffold. Similar to peptide antibiotics, the amine-modified CB[6] amphiphile **2** exerts its antibacterial activity by disrupting bacterial cell membranes with good selectivity over mammalian counterparts. Unlike peptide antibiotics, however, since the CB[6]-based antibiotic developed here is stable against enzymatic degradation, the present system would be more attractive in clinical applications. Studies of other CB[6] derivatives having either longer alkyl chains or amino acid side chains are currently under way aiming at development of higly efficient antibacterial agents. We anticipate that the design principle developed here can be applied to other macrocycles such as cyclodextrins and calixarenes.

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