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A Rationally Designed Macrocyclic Cavitand That Kills Bacteria with High Efficacy and Good Selectivity

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Synthesis of primary amine-modified CB[6] (1). To a solution containing $(allyloxy)_{12}$CB[6] (100 mg, 0.06 mmol) in methanol (1 mL) was added 2-aminoethanethiol (327.2 mg, 2.88 mmol). After degassing the solution under N2 gas stream for 10 min, UV light (254 nm + 300 nm lamps) was irradiated to the solution for 24 h. After solvent was evaporated under a reduced pressure, the remained crude product was dissolved in water. To remove excess amount of unreacted free thiol reagent, dialysis was carried out in distilled water using a 1000 MWCO dialysis membrane for 2 days. After freeze-drying the dialyzed solution, primary amine-modified CB[6] (**1**) was obtained in white powder form. Further purification was done by reverse phase HPLC. ^{1}H NMR (300 MHz, D_2O): δ 5.59 (br, 12H), 4.34 (br, 12H), 3.68 (br, 24H), 3.11 (br, 24H), 2.77 (br, 24H), 1.79 (br, 24H); MS (MALDI-TOF): *m/z* 2519.3 calcd for $C_{94}H_{161}N_{35}O_{24}S_{11}$ 2518.2 (n = number of modification at a parent CB[6]; n=11), 2443.4 calcd for $C_{92}H_{154}N_{34}O_{24}S_{10}$ 2441.0 (n=10), 2366.7 calcd for $C_{90}H_{147}N_{33}O_{24}S_9$ 2363.9 (n=9).

Synthesis of secondary amine-modified CB[6] (2). 2-(Butylamino)ethanethiol was dissolved in ethyl ether with stirring and then 35% aqueous HCl was added to get a protonated form of the aminothiol. After observation of white precipitation, the solvent was removed by evaporation. The remained solid was washed with ethyl ether and then

dried in vacuum. To a solution containing $(allyloxy)_{12}CB[6]$ (100 mg, 0.06 mmol) in methanol (1 mL) was added 2-(butylamino)ethanethiol•HCl salt (408 mg, 2.88 mmol). The following reaction and purification procedure is as same as that of synthesis of **1** described above. ^1H NMR (300 MHz, D_2O): δ 5.56 (br, 12H), 4.36 (br, 12H), 3.25 (br, 24H), 3.18 (br, 24H), 3.16 (br, 24H), 2.87 (br, 24H), 2.70 (br, 24H), 1.96 (br, 24H); MS (MALDI-TOF): *m/z* 3265.1 calcd for $C_{144}H_{264}N_{36}O_{24}S_{12}$ 3268.6 (n = number of modification at a parent CB[6]; n=12), 3132.4 calcd for $C_{138}H_{249}N_{35}O_{24}S_{11}$ 3002.1 (n=11)

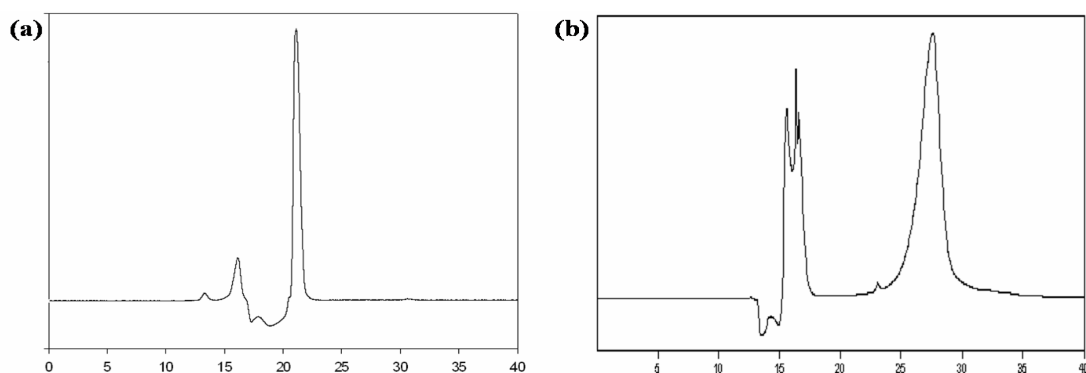

Figure S1. HPLC profiles of (a) compound **1** and (b) **2**

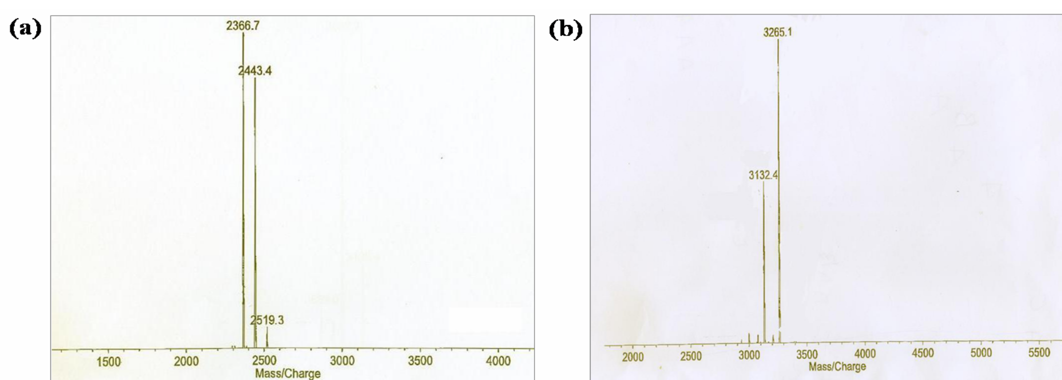

Figure S2. MALDI-TOF analysis of (a) compound **1** and (b) **2**

Microorganisms. *Escherichia coli* KCTC 1682, *Pseudomonas aeruginosa* KCTC 1637, *Salmonella typhimurium* KCTC 1926, , *Bacillus subtilis* KCTC 3068, *Staphylococcus epidermidis* KCTC 1917, and *Staphylococcus aureus* KCTC 1916 were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB) (Daejon, Korea). The clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were supplied from Culture Collection of Antibiotics Resistant Microbes (CCARM) (Seoul, Korea).

Antimicrobial activity. The bacterial strains were grown at 37℃ overnight in 5 mL of a Luria-Bertani (LB) medium. An aliquot of this culture was inoculated into 5ml of the fresh LB medium and incubated for additional 3-5 h at 37℃ to obtain mid log phase (absorbance at 600 nm (A_{600}) = 0.5) cells. The antimicrobial activities were examined as follows. A 2-fold dilution series of primary and secondary amine-modified CB[6] in culture medium (Mueller-Hinton broth) was prepared in 96 well plates (Nunc F96 microtiter plates). Aliquots (100 μL) of bacterial suspension in culture medium were added to 100 μL of aqueous amine-modified CB[6] solution. After incubation for 18-20 h at 37℃, the inhibition of bacterial growth was determined by naked eyes. The minimal inhibitory concentration was defined as the lowest concentration of amine-modified CB[6] that completely inhibited growth of the bacteria.

Bacterial suspension over MIC was spread on LB agar plate and incubated for 20 h to allow full colony development. The colonies were counted and MBC (minimal bactericidal concentration), the lowest concentration of amine-modified CB[6] that completely killed bacteria.

Table S1. MIC of conventional antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) (μg/mL)

	Cephalothin	**Erythromycin**	**Norfloxacin**	**Gentamicin**	**Oxacillin**	**Vancomycin**
MRSA3521	**128**	**>128**	**128**	**128**	**>128**	**2**
MRSA3514	**128**	**>128**	**>128**	**128**	**>128**	**≤1**

Preparation of small unilamellar vesicles (SUVs). Calcein entrapped small unilamellar vesicles were prepared for the dye leakage experiment. Appropriate amounts of phospholipids were dissolved in chloroform and dried under a stream of nitrogen to form a thin lipid film and subsequently placed in a vacuum chamber overnight to remove residual traces of the organic solvent. The dried thin lipid films were hydrated with 2 mL Tris-HCl buffer (10 mM Tris-HCl pH 7.4, 154 mM NaCl, 0.1mM EDTA) containing 70 mM of calcein by vigorous vortex-mixing at 25 combined with 5 freeze and thaw cycles using liquid nitrogen. The resulting lipid dispersions were sonicated for 20-30 min using a titanium-tipped sonicator until the solution was clear. Calcein-entrapped vesicles were separated from free calcein by gel filtration chromatography on a Sephadex-50 column using Tris-HCl buffer.

Calcein leakage experiment. The fluorescence intensity of calcein released from liposomes was monitored at 520 nm (excited at 490nm) on a Shimadzu RF-5301 spectrofluorometer. To measure the maximum fluorescence intensity for 100% dye leakage, 20 μL of Triton X-100 (10% in Tris buffer) was added to dissolve the vesicles. The average percent leakage caused by the amine-modified CB[6] was calculated using the equation :

$$\% \text{ leakage} = 100 \quad (F-F_0) / (F_t-F_0)$$

where F_0 and F_t are the initial fluorescence intensities observed without treatment of the amine-modified CB[6] and after Triton X-100 treatment, respectively, and F is the fluorescence intensity observed with treatment of each amine-modified CB[6].

Cell culture and cytotoxicity assay. NIH3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. Cells were grown at 37℃ in humidified air containing 5% CO_2 and passaged every 3~4 days.

The cytotoxicity of the amine-modified CB[6] was measured by MTT assay. Cells were seeded in a 96-well tissue culture plate at an initial seeding density of $5x10^3$ cells per well in 200 μL of growth medium. Cells achieving 70-80% confluence after 24 h were exposed to 100 μL of serially diluted amine-modified CB[6] solution and then incubated for 12 h. Thereafter 20μL of stock solution of MTT (5 mg/mL in PBS buffer) were added to each well. After 4 h of incubation at 37℃, each medium was removed and 100 μL of DMSO was added to each well. The optical densities of each well were measured at 570 nm by using a microplate reader and expressed as a percentage relative to control cells.