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Inhibition of *S. aureus* α-hemolysin and *B. anthracis* lethal toxin by β-cyclodextrin derivatives

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Abstract—Many pathogens utilize the formation of transmembrane pores in target cells in the process of infection. A great number of pore-forming proteins, both bacterial and viral, are considered to be important virulence factors, which makes them attractive targets for the discovery of new therapeutic agents. Our research is based on the idea that compounds designed to block the pores can inhibit the action of virulence factors, and that the chances to find high affinity blocking agents increase if they have the same symmetry as the target pore. Recently, we demonstrated that derivatives of β-cyclodextrin inhibited anthrax lethal toxin (LeTx) action by blocking the transmembrane pore formed by the protective antigen (PA) subunit of the toxin. To test the broader applicability of this approach, we sought β-cyclodextrin derivatives capable of inhibiting the activity of *Staphylococcus aureus* α-hemolysin (α-HL), which is regarded as a major virulence factor playing an important role in staphylococcal infection. We identified several amino acid derivatives of β-cyclodextrin that inhibited the activity of α-HL and LeTx in cell-based assays at low micromolar concentrations. One of the compounds was tested for the ability to block ion conductance through the pores formed by α-HL and PA in artificial lipid membranes. We anticipate that this approach can serve as the basis for a structure-directed drug discovery program to find new and effective therapeutics against various pathogens that utilize pore-forming proteins as virulence factors.

1. Introduction

Recently, using structure-inspired drug design, we demonstrated that persubstituted derivatives of β-cyclodextrin inhibited anthrax lethal toxin (LeTx) action in both in vitro and in vivo models.[1–3] Experiments with the reconstitution of the transmembrane pore formed by the protective antigen (PA) subunit of anthrax toxin into planar lipid bilayers showed that the protective function of the derivatives may well be realized via pore blockage. The oligomeric nature of the central component of the toxin, the PA_{63} heptamer, allowed us to synthesize complementary oligomeric compounds of low molecular weight, which dock to the pore with high affinity. Now, we demonstrate that this approach can be extended to the design of inhibitors of other toxins and protein channels that play key roles in the action of pathogenic bacteria. Here we describe the ability of β-cyclodextrin derivatives (β-CD) to inhibit the activity of α-hemolysin (α-HL) of *Staphylococcus aureus*, a major virulence factor in staphylococcal infection.[4,5] Although the mechanisms of action of α-HL and anthrax toxin are different,[4–8] the common feature is the formation of heptameric transmembrane pores.[9,10] We used β-cyclodextrin as a scaffold in the belief that derivatives of this cyclic molecule consisting of seven identical D-glucose units[11] would be well suited to block the pores, which have the same sevenfold symmetry (Fig. 1). In this study, we describe the synthesis and evaluation of new β-cyclodextrin derivatives with the goal of identifying potent inhibitors of *S. aureus* α-hemolysin and anthrax toxin.

Keywords: Anthrax toxin; α-Hemolysin; *Staphylococcus aureus*; Inhibitors; β-Cyclodextrin derivatives.
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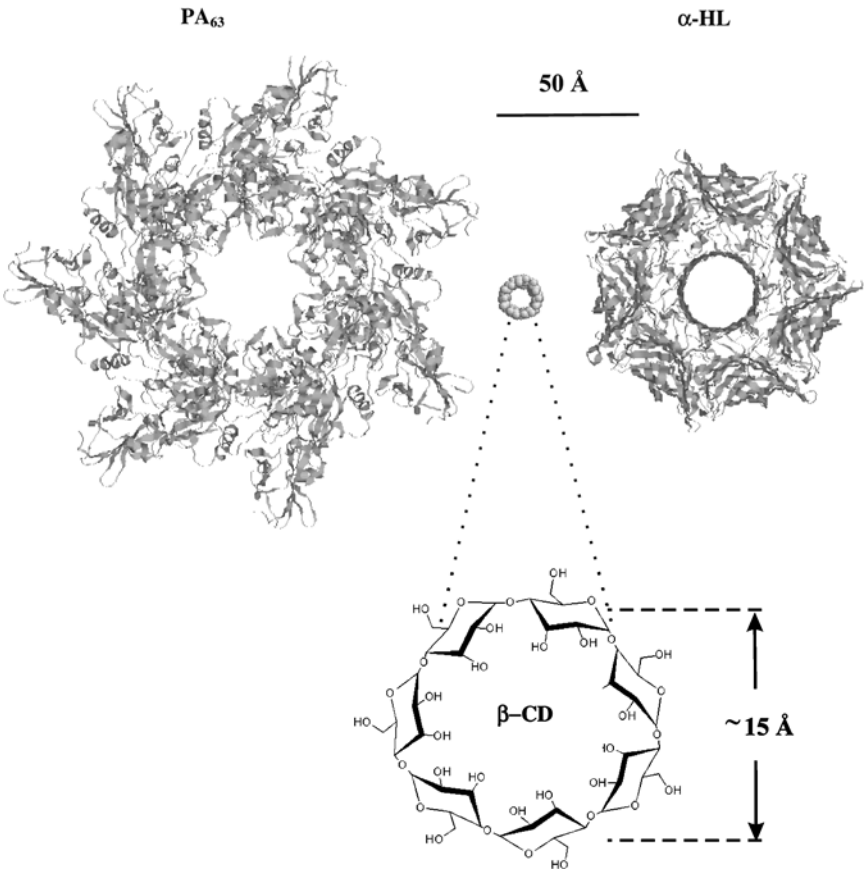


Figure 1. Schematic illustration of β-cyclodextrin (middle and expanded structure at the bottom) in comparison with staphylococcal α-HL (right) channel and anthrax PA_{63} prepore (left).

2. Results and discussion

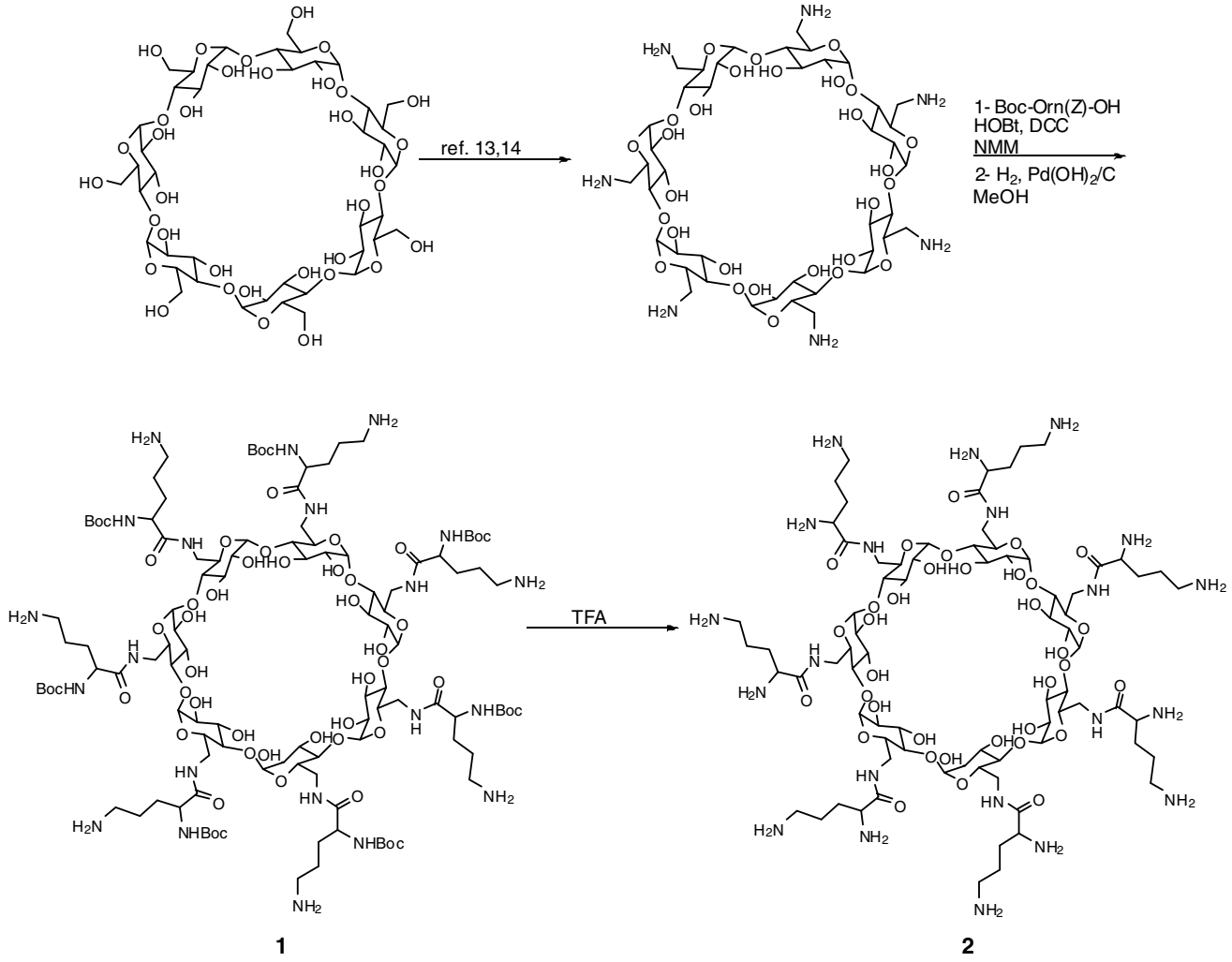
Recently, we synthesized a library of hepta-6-substituted β-cyclodextrins and surveyed it with the goal of finding inhibitors of anthrax toxin. The effectiveness of the approach was demonstrated by the fact that numerous compounds displayed inhibitory activity at low- and sub-micromolar concentrations in cell-based assays.[1–3] These experiments also showed that in most cases the compounds were not toxic to RAW 264.7 cells up to 100 μM concentration, while their IC_{50} values were as low as 0.6 μM. Although the library was designed to block the pore formed by anthrax protective antigen, we decided to screen it against α-hemolysin of *S. aureus*, which forms a similar heptameric transmembrane pore. Earlier, using bilayer lipid membrane technique Gu et al. showed that the ion conductance through the α-HL channel can be partially blocked by β-cyclodextrin itself.[12]

2.1. Chemistry

The synthetic approach to the synthesis of amino acid derivatives involved the attachment of selectively protected amino acids to per-6-amino-β-CD by nucleophilic substitution via dicyclohexylcarbodiimide (DCC) coupling (Schemes 1 and 2). The per-6-amino-β-CD was synthesized by treating per-6-iodo-β-CD[13] with sodium azide in DMF, followed by reduction of the azido cyclodextrin with triphenylphosphine and aqueous ammonia to afford the free amine.[14] Next, one of the several protected amino acids was treated with DCC and HOBt in DMF at 0 °C to afford the symmetrical anhydride. The per-6-amino-β-CD was then treated with the anhydride to yield the desired hepta-substituted cyclodextrins. The amino acid protecting groups were removed using either neat trifluoroacetic acid for the Boc and trityl groups or hydrogenation with Pearlman's catalyst for the benzyl protecting groups. In each case, the free amines isolated were treated with 1 N aqueous HCl and triturated to give the hepta hydrochloride salt. The structures of the final compounds were characterized by ^{13}C NMR and MALDI-MS, since the ^{1}H NMR spectra showed broad and unresolved signals in DMSO-d_6.

2.2. Inhibition of α-HL and LeTx activity

Currently, we have a diverse library of prepared numerous β-CD derivatives persubstituted at position 6 with various neutral, positively or negatively charged groups including amino, *S*-aminoalkyl, *N*-aminoalkyl, *S*-alkylguanidyl, *N*-alkylguanidyl, *N*-alkyl, arylalkyl, aryl, heterocyclic rings, OSO_3Na, and others. Many of these compounds were presented in our previous publications.[1–3] To survey the library for inhibitors of α-HL activity, we used a standard hemolysis assay utilizing rabbit erythrocytes[15] adapted to a 96-well plate format (Fig. 2). The survey revealed three

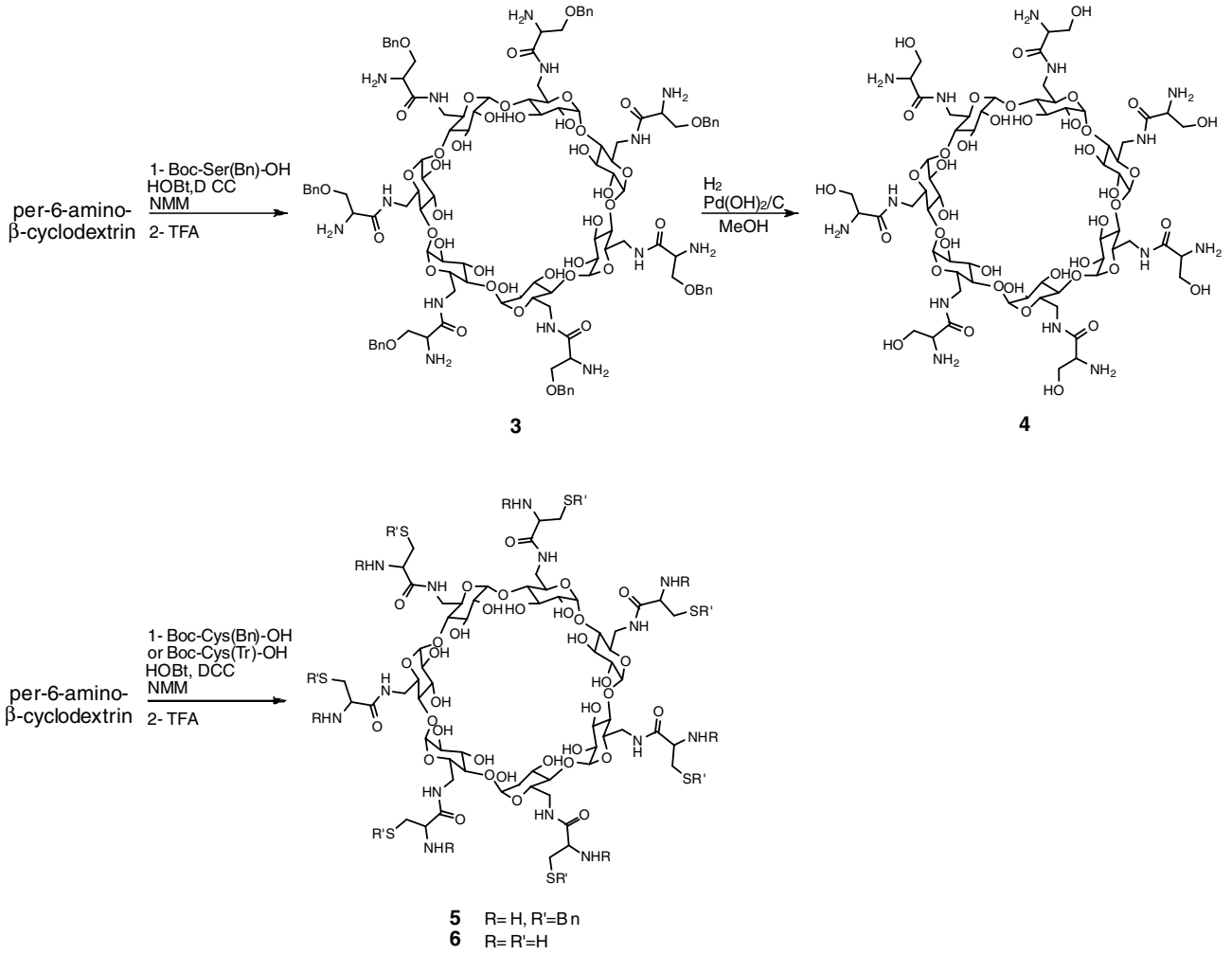


Scheme 1.

compounds that inhibited the hemolytic activity of α-HL at low micromolar concentrations (Table 1). It is interesting to note that all of them are amino acid derivatives carrying protective groups (cyclodextrins **1**, **3**, and **5**), while the structurally related compounds with fully de-protected amino acids (cyclodextrins **2**, **4**, and **6**) did not display potent inhibitory activity in this assay. Thus, while the positive charges on the 6-substituent may well be important for pore binding,[1–3] the additional positive charges in compounds **2**, **4**, and **6** actually resulted in a diminution of the potency of the compounds. Plausibly, the lipophilic and aromatic substituents in compounds **1**, **3**, and **5** may contribute to the affinity of these compounds for the bacterial pores. Of these compounds, one derivative (**1**) was surprisingly active against both staphylococcal α-HL and anthrax LeTx (Fig. 3) in cell-based assays despite the fact that α-HL and PA do not have any structural homology. To obtain a more detailed picture of the pore-compound complexes and provide a more complete analysis of the structure–activity relationship, further studies utilizing computer docking, cross-linking, and X-ray analysis are required.

2.3. Ion conductance studies

Cyclodextrin **1** was assayed for its ability to block ion conductance through the pores formed in artificial membranes by PA (Fig. 4a) and α-HL (Fig. 4b). In both cases, there was a profound inhibition of channel conductance. Fig. 4 illustrates typical recordings of an ion current through single PA_{63} (A) and α-HL (B) pores before and after modification by cyclodextrin **1**. It is seen that even before the addition of **1**, the PA_{63} conductance level was rather noisy (Fig. 4a, topmost track). This noisiness represents the well-known 'gating' of PA_{63} channels,[16,17] which appears as fast flickering between the open and completely closed conformations. The addition of about 5 μM compound **1** to the *cis*-side of the membrane (the side of toxin addition) caused additional step-wise closures of 6 ms average duration (Fig. 4a, middle). Just as gating, these fluctuations are fast transients between the fully open and non-conducting channel. Even though the amplitude of these events coincides with the amplitude of the regular PA_{63} gating (complete channel closure), the difference in the residence times allowed us to sort all observed events into



Scheme 2.

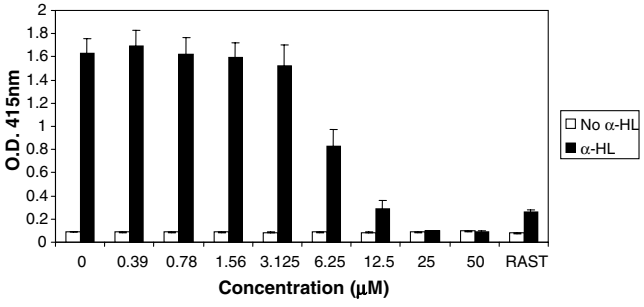


Figure 2. Protection of rabbit erythrocytes from α-HL action by cyclodextrin **1**. Rabbit erythrocytes were incubated with different concentrations of β-CD derivatives with or without α-HL. Each experiment was performed in triplicate. Hemolysis was determined colorimetrically at 415 nm. Error bars represent standard deviations.

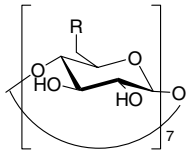
the two different processes. At higher cyclodextrin **1** concentrations channel blockades are more frequent (Fig. 4a, bottom).

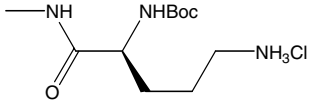
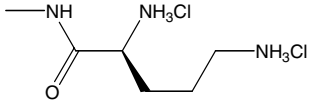
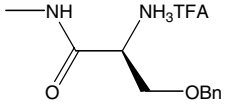
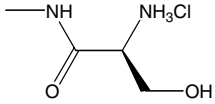
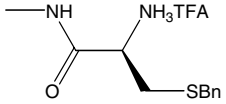
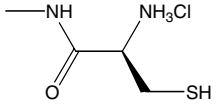
In contrast to PA_{63}, the α-HL–cyclodextrin **1** interaction was irreversible within the limits of the time period of our experiment (Fig. 4b). It was observed that the current through the single α-HL pore was stable; no gating events were seen under applied 50 mV. Addition of about 2 μM **1** to the *cis*-side of the membrane switched the channel to a closed state similar to the 'voltage-gated closed state' seen commonly for α-HL at 100 mV and higher voltages. The residual conductance of the closed state was between 1% and 15% of the total channel conductance. It is remarkable that the introduction of positive charges to the cyclodextrin molecule leads to its ability to block the α-HL channel from the *cis*-side. It is known[12] that unmodified β-cyclodextrin binds only weakly to the heptameric α-HL channel when added from the trans (intracellular) side of the membrane.

3. Conclusions

The present results demonstrate a broader applicability of the idea that compounds designed to block the pore of a pore-forming toxin can serve as inhibitors of the action of the toxin. Originally suggested for the blocking of anthrax toxin, this approach works well with another heptameric pore-forming toxin, staphylococcal α-HL, which does not have any structural homology with the protective antigen of anthrax and acts through a completely different mechanism. Blocking the pore with

Table 1. Activities of the compounds in this study



Compound	R	Inhibition of LeTx cytotoxicity IC_{50} (μM) RAW 264.7 cells	Inhibition of α-HL cytotoxicity IC_{50} (μM) red blood cells
1		3.5 ± 2.2	5.6 ± 1.8
2		>25	>25
3		>25	6.1 ± 2.4
4		>25	>25
5		>25	10.6 ± 3.0
6		>25	>25

molecules having comparable dimensions and the same symmetry as the pore itself appears to be very effective. From our library of β-cyclodextrin derivatives, three compounds were found to inhibit α-HL action at low micromolar concentrations. The efficiency of our approach can be explained by the fact that, due to the sevenfold symmetry, each interaction of a functional group of the blocker molecule with a site within the pore multiplies sevenfold, increasing the blocker-pore affinity.

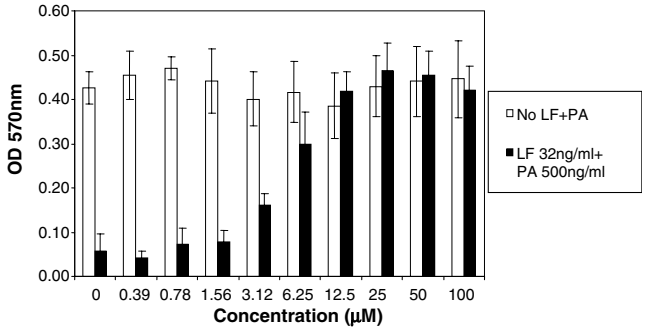


Figure 3. Protection of RAW 264.7 cells from LeTx-induced cell death by cyclodextrin **1**. RAW 264.7 cells were incubated with different concentrations of the β-CD derivatives with or without LeTx. Each experimental condition was performed in triplicate. Cell viability was determined by MTS colorimetric assay. Error bars represent standard deviations.

We believe that the same approach can be utilized in the search for inhibitors of other pore-forming toxins. For example, heptameric β-CD derivatives could bind to heptameric pores formed by *Vibrio cholerae* cytotoxin, *Clostridium perfringens* ε-toxin, α-toxin of *Clostridium septicum* or hepatitis C virus p7 protein. Derivatives of hexameric α-cyclodextrin may also find utility against targets such as *Helicobacter pylori* VacA toxin, which forms hexameric channels.

Although we used persubstituted cyclodextrins in our studies, it is logical to think that cyclic molecules such as cyclic peptides and peptidomimetics, crown ethers or other symmetrical chemical structures could be potentially utilized in an analogous fashion.

It is anticipated that this approach can serve as the basis for a structure-directed drug discovery program to find new and effective therapeutic agents against pathogens that utilize pore-forming proteins as virulence factors.

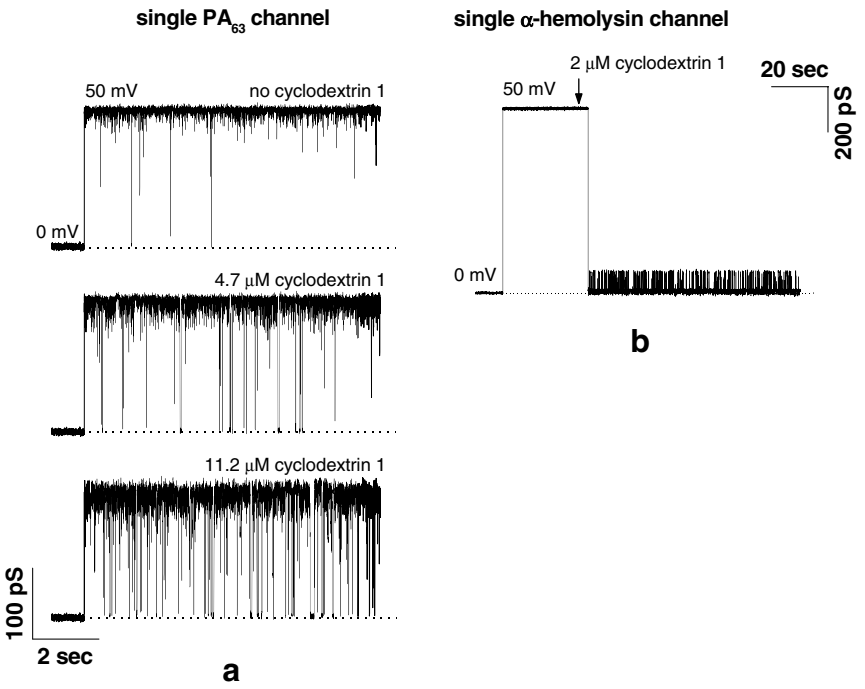


Figure 4. Modulation of an ion current through a single PA_{63} channel (a) and a single α-HL channel (b) by cyclodextrin **1**. (a) In the absence of **1** the ion movement is mainly determined by the geometry and surface properties of the pore (topmost track). Fast flickering between open and closed states inherent to PA_{63} channels (so-called gating) was mainly removed by averaging over a time interval of 5 ms. In the presence of 4.7 μM **1** in the *cis* half of the chamber, the channel gets spontaneously blocked (middle), and at higher cyclodextrin **1** concentrations channel blockages are more frequent (bottom). (b) In the absence of **1**, the current through the single α-HL channel is rather stable (no significant current fluctuations at 10 ms time resolution are seen). Cyclodextrin **1** addition leads the channel to switch to the weakly conductive blocked sub-state. For both channels, a transmembrane voltage of 50 mV was positive from the side of protein addition. Diphytanoylphosphatidylcholine planar lipid membranes were bathed by 1 M KCl solution at pH 6.6.

4. Experimental

4.1. Chemistry

4.1.1. General methods. ^{1}H NMR and ^{13}C NMR spectra were recorded on a General Electric QE-300 or a Varian 300 spectrometer. Moisture sensitive reactions were conducted under argon in oven-dried glassware. All chemical reagents and solvents were purchased from Aldrich Chemicals, NovaBiochem or Fisher Scientific and used without further purification. Analytical thin-layer chromatography was performed on Merck $60F_{254}$ precoated silica gel plates. Visualization was performed by ultraviolet light or by staining with phosphomolybdic acid or sulfuric acid. Flash chromatography was performed using (40–60 μm) silica gel. Melting points were taken with a Mel-Temp melting point apparatus and are uncorrected. Per-6-amino-β-cyclodextrin was prepared according to the methods of Defaye[13] and Stoddart.[14]

4.1.2. Per-6-[(N^{α}-Boc-L-ornithinyl)amino]-β-cyclodextrin trifluoroacetic acid salt (1). N^{α}-Boc-N^{δ}-benzyloxy-L-ornithine (0.587 g, 1.60 mmol) was dissolved in 10 mL of dry DMF. HOBt (0.249 g, 1.63 mmol) was added and the solution was cooled to 0 °C in an ice bath. DCC (0.330 g, 1.60 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was allowed to warm to room temperature and was stirred for an additional 1 h. To the reaction vessel were added 250 mg (0.22 mmol) of per-6-amino-β-cyclodextrin and 0.2 mL of *N*-methylmorpholine. The resulting mixture was stirred for an additional 20 h. The precipitated dicyclohexylurea was filtered off and the filtrate was concentrated under diminished pressure at 60 °C to yield a slightly colored oil. Saturated aqueous sodium bicarbonate was added, resulting in a white suspension which was stirred for 1 h and filtered. The precipitate was washed with water and dried under vacuum. To remove the N^{δ}-protecting group, the solid was dissolved in MeOH and 200 mg (50% wet) of palladium hydroxide on carbon was added with stirring. A hydrogen atmosphere (balloon pressure) was applied to the reaction mixture which was stirred at room temperature for an additional 20 h. The suspension was filtered,and the filtrate was concentrated under diminished pressure. The residue was dissolved in 5 mL of water, diluted with 75 mL of acetone, and the resulting suspension was sonicated. The precipitate was filtered and dried under vacuum to yield **1** as an off-white foam: yield 0.501 g (86%); ^{13}C NMR (DMSO-d_6) δ 21.2, 24.4, 27.9, 30.6, 33.3, 40.9, 50.3, 72.2, 77.7, 101.2, 155.3, and 170.1; mass spectrum (MALDI), *m*/*z* 2651.72 $[M+2H+Na]^{+}$ (theoretical 2651.41).

4.1.3. Per-6-[(L-ornithinyl)amino]-β-cyclodextrin hydrochloride (2). Per-6-[(N^{α}-Boc-ornithinyl)amino]-β-cyclodextrin hydrochloride **(1)** (0.300 g, 0.114 mmol) was dissolved in 3 mL of neat TFA and stirred at room

temperature for 1 h. The reaction mixture was concentrated under diminished pressure to obtain a pale orange oil. Diethyl ether was added and the suspension was sonicated. The precipitate was filtered, dried under vacuum, and re-dissolved in 5 mL of 1 N aqueous HCl. Acetone (65 mL) was added and the resulting suspension was again sonicated. The precipitate was filtered and dried under vacuum to yield **2** as a tan foam: yield 0.180 g (65%); ^{13}C NMR (DMSO-d_6) δ 22.3, 28.25, 38.0, 51.4, 72.0, 72.6, 102.3, and 169.4; mass spectrum (MALDI), *m*/*z* 1950.26 $[M+Na]^+$ (theoretical 1950.11).

4.1.4. Per-6-[(*O*-benzyl-L-seryl)amino]-β-cyclodextrin trifluoroacetic acid salt (3). *N*-Boc-*O*-benzyl-L-serine (0.473 g, 1.60 mmol) was dissolved in 10 mL of dry DMF. HOBt (0.249 g, 1.63 mmol) was added and the solution was cooled to 0 °C in an ice bath. DCC (0.330 g, 1.60 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was allowed to warm to room temperature and was stirred for an additional 1 h. To the reaction vessel were added 250 mg (0.22 mmol) of per-6-amino-β-cyclodextrin and 0.2 mL of *N*-methylmorpholine, and the resulting mixture was stirred for an additional 20 h. The precipitated dicyclohexylurea was filtered and the filtrate was concentrated under diminished pressure at 60 °C to yield a slightly colored oil. Saturated aqueous sodium bicarbonate was added, resulting in a white suspension which was stirred for 1 h and filtered. The precipitate was washed with water and dried under vacuum. To remove the N-protecting group, the solid was dissolved in 3 mL of neat TFA and stirred at room temperature for 1 h. The reaction mixture was concentrated under diminished pressure to obtain a pale yellow oil. Diethyl ether was added and the suspension was sonicated. The precipitate was filtered and dried under vacuum to yield **3** as a light brown solid: yield 0.312 g (45%); mp 148–149 °C (dec); ^{13}C NMR (DMSO-d_6) δ 34.0, 53.4, 66.7, 68.4, 69.4, 70.4, 73.4, 82.1, 102.8, 128.3, 128.5, 128.8, 138.4, and 169.3; mass spectrum (MALDI), *m*/*z* 2371.61 $[M+3H]^+$ (theoretical 2371.51).

4.1.5. Per-6-[(L-seryl)amino]-β-cyclodextrin hydrochloride (4). Per-6-[(*O*-benzyl-L-seryl)amino]-β-cyclodextrin hydrochloride (0.500 g, 0.155 mmol) was dissolved in MeOH and 200 mg of palladium hydroxide on carbon was added with stirring. A hydrogen atmosphere (balloon pressure) was applied to the reaction mixture, which was stirred at room temperature for an additional 20 h. The suspension was filtered, and the filtrate was concentrated under diminished pressure. The residue was dissolved in 5 mL of 1 N aqueous HCl, diluted with 50 mL of acetone, and the resulting suspension was sonicated. The precipitate was filtered and dried under vacuum to yield **4** as a light brown foam: yield 0.310 g (96%); ^{13}C NMR (DMSO-d_6) δ 30.6, 52.3, 54.3, 60.4, 69.4, 72.1, 72.6, 82.9, 102.1, 167.4; mass spectrum (MALDI), *m*/*z* 1759.82 $[M+Na]^+$ (theoretical 1759.70).

4.1.6. Per-6-[(*S*-benzyl-L-cysteinyl)amino]-β-cyclodextrin trifluoroacetic acid salt (5). N^{α}-Boc-*S*-benzyl-L-cysteine (0.498 g, 1.60 mmol) was dissolved in 10 mL of dry DMF. HOBt (0.249 g, 1.63 mmol) was added and the solution was cooled to 0 °C in an ice bath. DCC (0.330 g, 1.60 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was allowed to warm to room temperature and stirred for an additional 1 h. To the reaction vessel were added 250 mg (0.22 mmol) of per-6-amino-β-cyclodextrin and *N*-methylmorpholine, and the resulting mixture was stirred for an additional 20 h. The precipitated dicyclohexylurea was filtered and the filtrate was concentrated under diminished pressure at 60 °C to yield a slightly colored oil. Saturated aqueous sodium bicarbonate was added, resulting in a white suspension which was stirred for 1 h and filtered. The precipitate was washed with water and dried under vacuum. To remove the N-protecting group, the white solid was dissolved in 3 mL of neat TFA and stirred at room temperature for 1 h. The reaction mixture was concentrated under diminished pressure to obtain a pale yellow oil. Diethyl ether was added and the suspension was sonicated. The precipitate was filtered and dried under vacuum to afford **5** as a tan solid: yield 0.273 g (39%); mp 198–200 °C (dec); ^{13}C NMR (DMSO-d_6) δ 32.2, 36.5, 52.5, 52.8, 70.3, 72.9, 82.8, 102.7, 127.8, 129.1, 129.5, 138.5, and 168.7; mass spectrum (MALDI), *m*/*z* 2503.65 $[M+2H+Na]^+$ (theoretical 2503.88).

4.1.7. Per-6-[(L-cysteinyl)amino]-β-cyclodextrin hydrochloride (6). N^{α}-Boc-*S*-triphenylmethyl-L-cysteine (0.742 g, 1.6 mmol) was dissolved in 10 mL of dry DMF. HOBt (0.249 g, 1.63 mmol) was added and the solution was cooled to 0 °C in an ice bath. DCC (0.330 g, 1.60 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was allowed to warm to room temperature and was stirred for an additional 1 h. To the reaction vessel were added 250 mg (0.22 mmol) of per-6-amino-β-cyclodextrin and 200 mL of *N*-methylmorpholine, and the resulting mixture was stirred for an additional 20 h. The precipitated dicyclohexylurea was filtered and the filtrate was concentrated under diminished pressure at 60 °C to afford a slightly colored oil. Saturated aqueous sodium bicarbonate was added, resulting in a light yellow suspension which was stirred for 1 h and filtered. The precipitate was washed with water and dried under vacuum. To remove the acid-labile protecting groups, the solid was dissolved in a solution of 5 mL of TFA, 5 mL of CH_2Cl_2, and 0.5 mL of triethylsilane, and then stirred at room temperature for 6 h under an inert atmosphere. The reaction mixture was concentrated under diminished pressure to obtain a pale yellow oil. Diethyl ether was added and the suspension was sonicated. The precipitate was filtered and dried under vacuum to yield **6** as a tan solid: yield 0.152 g (33%); ^{13}C NMR (DMSO-d_6) δ 21.3, 37.5, 51.3, 67.4, 68.5, 72.4, 82.4, 102.4, and 169.0.

4.2. Cell-based assays

4.2.1. Cells and cell culture. The murine RAW 264.7 monocyte–macrophage cell line (ATCC TIB-71) was obtained from American Type Culture Collection (Manassas, VA). Raw 264.7 cells were cultured in phenol-red free Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Herndon, VA) supplemented with

10% heat-inactivated fetal bovine serum, 100 U/mL:100 μg/mL penicillin–streptomycin, 0.1 mM non-essential amino acids, and 0.5 mM 2-mercaptoethanol at 37 °C in a 5% CO_2 atmosphere. The cells were harvested by gentle scraping with a cell scraper and were then washed once with media. RAW 264.7 cells were plated in 96-well flat-bottomed tissue culture plates from Becton–Dickinson (San Jose, CA) at a concentration of 10^5 cells/well in DMEM and incubated overnight at 37 °C in 5% CO_2.

Rabbit whole blood in Alsevers anti-coagulant was acquired from Hemostat Laboratories (Dixon, CA). The whole blood was diluted (1:10) in PBS and centrifuged at 1500 rpm for 5 min. After discarding the supernatant, a 10% red blood cell (RBC) suspension in PBS was prepared from the pellet.

4.2.2. LeTx cytotoxicity neutralization assay. Recombinant *B. anthracis* lethal factor (rLF) and protective antigen (rPA) were acquired from List Biological Laboratories, Inc. (Campbell, CA). RAW 264.7 cells were pre-incubated with different concentrations of the tested compounds in DMEM at 37 °C for 1 h in a 5% CO_2 atmosphere. Then, DMEM or LeTx (LF, 32 ng/mL; PA, 500 ng/mL) in the medium was added, and the plate was incubated under the same conditions for 4 h. Cell viability was estimated using a MTS kit from Promega (Madison, WI). A micro Quant spectrophotometer from Bio-Tek Instruments, Inc. (Winooski, VT) was used to make OD_{570} readings.

4.2.3. α-HL rabbit erythrocytes hemolysis assay. α-Hemolysin from *S. aureus* and rabbit anti-staphylococcal α-toxin (α-HL) antibody (RAST) were both purchased from Sigma–Aldrich (St. Louis, MO). A 10% solution of rabbit erythrocytes was diluted (1:4) in PBS and added (100 μL/well) to the wells of a round-bottomed 96-well plate. The test compounds (in PBS) were added to the wells. Ten microliters of α-HL (80 U/mL) was added to each well on the bottom half of the plate. The contents of the plate were mixed with a multi-channel pipettor and the plate was incubated at room temperature for 1 h. After incubation, the plate was centrifuged and 40 μL of the supernatant was added to 160 μL of PBS in a flat-bottomed 96-well plate. The samples were mixed and the plate was read at 415 nm with a spectrophotometer.

4.3. Channel reconstitution assay

Channel reconstitution experiments were performed as described previously.[18] To form 'solvent-free' planar lipid bilayers with the lipid monolayer opposition technique,[19] a 5% solution of diphytanoylphosphatidylcholine in pentane was used. Membranes were formed on a 60 μm (for single-channel measurements) aperture in the 15 μm thick Teflon film that separated two compartments. PA_{63} was purchased from List Biological Laboratories, Inc. (Campbell, CA) in the purified form. α-HL was purchased from Calibiochem. For all experiments, a symmetrical aqueous solution of 1 M KCl, 1 mM EDTA at pH 6.6 was used.

Under this protocol, PA and α-HL channel insertions were always directional. The electrical potential difference across the bilayer lipid membrane was applied with a pair of Ag–AgCl electrodes in 2 M KCl, 1.5% agarose bridges.

Conductance measurements were done using an Axopatch 200B amplifier (Axon Instruments, Inc., Foster City, CA) in the voltage–clamp mode. Signals were filtered by a low-pass 8-pole Butterworth filter (Model 9002, Frequency Devices, Inc., Haverhill, MA) at 15 kHz and saved directly into the computer memory with a sampling frequency of 50 kHz.

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