Aminoglycosides

Dual Effect of Synthetic Aminoglycosides: Antibacterial Activity against *Bacillus anthracis* and Inhibition of Anthrax Lethal Factor**

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Anthrax is an infectious disease caused by toxigenic strains of the Gram-positive *Bacillus anthracis*.[1] If inhaled, *B. anthracis* spores rapidly reach the regional lymphonodes of the lungs, where they germinate and release anthrax toxins.[2] These toxins inhibit the adaptive immune response, thereby enabling the bacteria to reach the blood system, where they cause bacteremia and toxemia, which rapidly kill the host. Non-toxigenic strains of *B. anthracis* are poorly pathogenic, thus indicating that the anthrax toxins play a major role from the

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Supporting information for this article (selected procedures and complete analytical data for compounds **14**–**15**, **17**, **18**, and **22a**–**e**, selected data for compounds **5**–**12**) is available on the WWW under http://www.angewandte.org or from the author.

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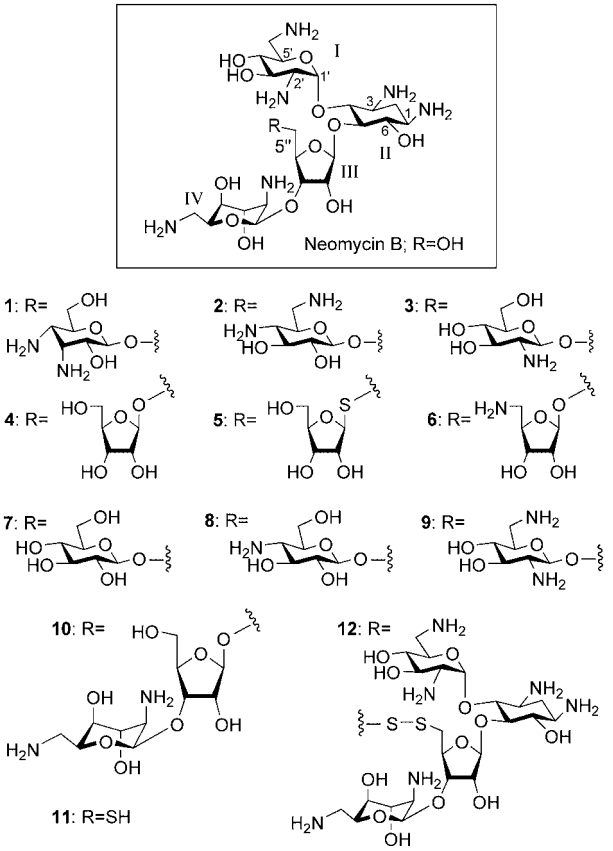
very beginning of infection to death. Since anthrax is asymptomatic until the bacterium reaches the blood,[1,2] the development of antitoxin therapeutic compounds for preventive use or for use in combination with antibiotics is of high urgency.[3] Alternatively, it would be highly beneficial if the developed material were bifunctional, with the ability to inactivate the released toxins and, in parallel, to function as an antibiotic.

The anthrax toxins consist of three proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF).[4] Individually these proteins are nontoxic, so their toxic effects during anthrax infection require cooperation: PA binds to a cell-surface receptor and forms an oligomeric pore that translocates both EF and LF into the cytosol of target cells. Once inside the cell, EF causes edema by Ca^{2+}/calmodulin-dependent adenylate cyclase activity. LF is a zinc-dependent endopeptidase that specifically cleaves most isoforms of mitogen-activated protein kinase kinases, thereby inhibiting one or more signaling pathways of the host macrophage.[5] Although the precise mechanism is not yet well understood, this process results in the death of the host. Strains of *B. anthracis* deficient in EF remain pathogenic, whereas those that lack LF become attenuated. LF is therefore considered the dominant virulence factor of anthrax.[6] Consequently, an intensive search for specific inhibitors of LF has been performed during the last few years.[3,7,8]

To find novel inhibitors of LF, we recently tested a library of approximately 3000 compounds, over 60 of which were synthetic and commercial aminoglycosides.[9] Although a number of the compounds tested demonstrated some level of inhibitory activity, neomycin B, a commonly utilized aminoglycoside antibiotic, was found to be the most potent inhibitor of LF with an apparent inhibition constant (K_i) value in the low nanomolar concentration range.

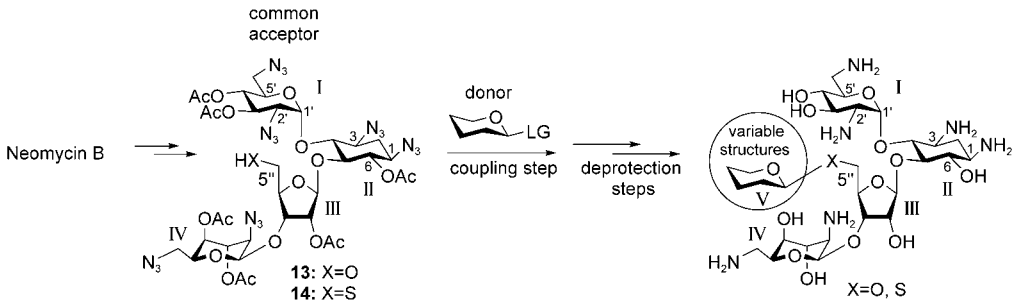
To improve the inhibitory effect of neomycin B derivatives, we focused on the following points. First, aminoglycoside antibiotics such as neomycin B exert their antibacterial activity by selectively recognizing and binding to the decoding A site on the 16S subunit of the bacterial ribosomal RNA (rRNA), thereby causing deleterious misreading of the genetic code.[10] At physiological pH values, aminoglycosides are highly charged and their RNA binding relies on electrostatic interactions.[11,12] Second, examination of the recently determined X-ray crystal structure of LF shows that the active site of the protease consists of a broad, 40 Å groove with a highly negative electrostatic potential.[13] We performed docking experiments and found that neomycin B could reside within the vicinity of the catalytic zinc center and that multiple potential contacts could occur between the negatively charged residues of LF and neomycin B.[9] Based on these data we hypothesized that, since the interaction of neomycin B with both rRNA and LF is mainly determined by electrostatic interactions, it is likely that superior binding to both rRNA and LF, and probably better dual-effect antianthrax performance, would result from maintaining the whole antibiotic constitution intact but adding additional recognition/binding elements. The improved antibacterial activity of the first generation of pseudopentasaccharide derivatives of neomycin B (Scheme 1, compounds **1**–**4**),[14] along with the inhibition of various nucleic acid metabolizing enzymes by aminoglycosides,[15] support this hypothesis.



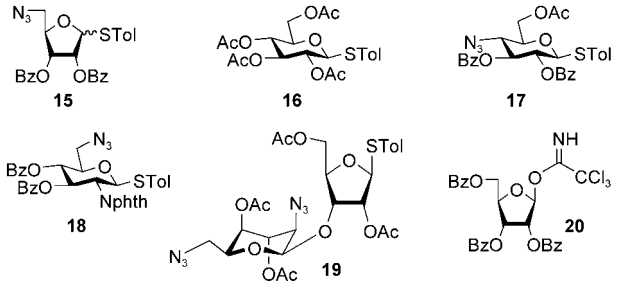
Scheme 1. Structures of neomycin B and the synthetic analogues.

Taking into consideration the relative ease of derivatizing a primary alcohol, we selected position C5″ in neomycin B as a modification site and prepared a series of new derivatives, **5**–**12** (Scheme 1). These structures, along with the previously reported pseudopentasaccharides **1**–**4**, preserve the entire antibiotic domain as a recognition element for both the rRNA and LF. The extended sugar ring systems of each structure were designed in a manner that incorporates different combinations of hydroxy and amino groups as potential functionalities for recognition of the phosphodiester bond of rRNA[16] and, in parallel, the Asp/Glu and Asn/Gln clusters in the active site of LF.[9]

All the new derivatives of neomycin B were synthesized according to the general strategy illustrated in Scheme 2. This strategy involves conversion of neomycin B into the common acceptors **13** and **14**, to which various donor molecules can be attached, followed by a two-step deprotection to yield the target C5″-branched derivatives. The protecting groups used in this study were chosen based on their ease of attachment and removal, and their stability under the reaction conditions. The thioglycoside/*N*-iodosuccinimide (NIS)[17] and trichloroacetimidate/BF_3[18] glycosidation methods proved to be both rapid and efficient. Phthalimido and ester groups at C2 of the donors **15**–**20** (Scheme 3) were designed to allow, through neighboring-group participation, selective β-glycoside bond formation between rings V and III.



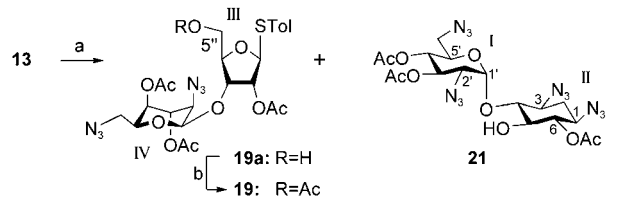
Scheme 2. General synthetic scheme for the assembly of C5″-branched derivatives of neomycin B. LG = leaving group.



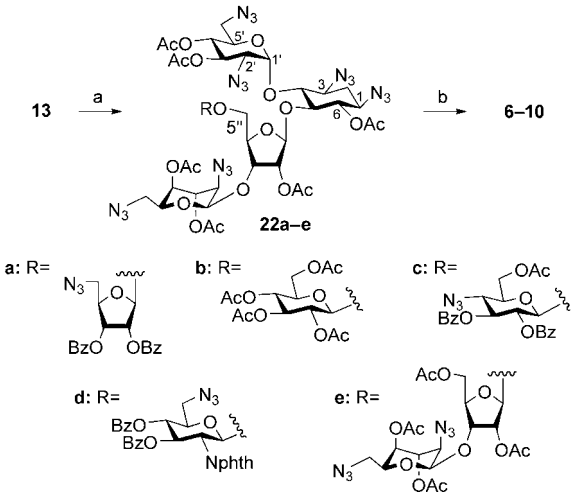
Scheme 3. Structures of the donors **15**–**20**. Bz = benzoyl, Nphth = phthalimido, Tol = tolyl.

The monosaccharide donors **15**–**18** and **20** were prepared by standard methods (see the Supporting Information for experimental details). The disaccharide donor **19** consists of derivatives of rings III and IV of neomycin B and was prepared by direct Lewis acid promoted cleavage of appropriately protected neomycin B according to the procedure of Swayze and co-workers[19] with some modification. As a starting material, instead of the perazidoperacetylneomycin B used in that work, we employed the acceptor **13** (readily accessible in four steps from neomycin B in 57% overall yield).[14] Treatment of **13** with $BF_3{\cdot}Et_2O$ in the presence of TolSH gave a mixture of two fragments, **19a** and **21**, which could be separated easily by chromatography on silica gel. Fragment **19a** was then readily converted into the desired donor **19** by acetylation (Scheme 4).

NIS-promoted coupling of the neomycin acceptor **13** with thioglycoside donors **15**–**19** furnished the designed protected derivatives **22a**–**e** in 47–91% yield (Scheme 5). The structures of **22a**–**e** were confirmed by a combination of various spectroscopic techniques, including HMQC, HMBC, 2D COSY, and 1D TOCSY NMR spectroscopy (see the Supporting Information). These protected compounds were then subjected to a two-step deprotection: removal of all the ester and phthalimido groups by treatment with methylamine (33% solution in EtOH) and reduction of all the azido groups by the Staudinger reaction, to furnish the final products **6**–**10**, which were isolated in excellent purity and yield.

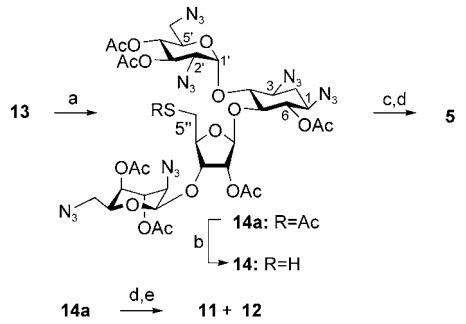


Scheme 4. Reagents and conditions: a) TolSH (1.1 equiv), $BF_3{\cdot}Et_2O$ (3 equiv), CH_2Cl_2, room temperature, 1 h, 32%; b) Ac_2O (1.5 equiv), pyridine, DMAP (cat.), room temperature, 97%. DMAP = 4-dimethylaminopyridine.



Scheme 5. Reagents and conditions: a) NIS, TfOH, CH_2Cl_2; **15**→**22a**: 57%, **16**→**22b**: 85%, **17**→**22c**: 64%, **18**→**22d**: 91%, **19**→**22e**: 47%; b) 1. $MeNH_2$ (33% in EtOH); 2. PMe_3, NaOH (0.1 M), THF/H_2O (3:1); **6**: 57%, **7**: 69%, **8**: 84%, **9**: 79%, **10**: 80%. NIS = *N*-iodosuccinimide, Tf = trifluoromethanesulfonyl.

Several methods[20,21] were examined in attempts to convert the primary hydroxy group in **13** into the corresponding thiol **14**. However, in most cases the yield for **14** was very low and the desired product was often accompanied by multiple by-products, which made the isolation of the target material difficult. The best result was obtained by utilizing the Mitsunobu reaction;[22] **13** was first converted into the corresponding thioacetate **14a** (84%), which after treatment with hydrazinium acetate provided the desired thiol **14** in 65% yield (Scheme 6). Lewis acid promoted coupling of the thiol acceptor **14** with the trichloroacetimidate donor **20** furnished the corresponding protected β-thioglycoside, which after a two-step deprotection as described above provided the designed thioglycoside **5** in 73% yield. When the chromatographically pure thioacetate **14a** was subjected directly to the same two-step deprotection procedure, treatment with methylamine followed by the Staudinger reaction, a mixture



Scheme 6. Reagents and conditions: a) DIAD (3 equiv), PPh_3, $CH_3C(O)SH$ (3 equiv), THF, 0°C→RT, 84%; b) hydrazinium acetate (2 equiv), DMF, room temperature, 65%; c) **20** (4 equiv), $BF_3{\cdot}Et_2O$ (cat.), CH_2Cl_2, 4-Å molecular sieves, −10°C, 90%; d) 1. $MeNH_2$ (33% in EtOH); 2. PMe_3, NaOH (0.1 M), THF/H_2O (3:1); **5**: 73%, **11**+**12**: 88%; e) Biogel P-2 size-exclusion chromatography. DIAD = diisopropylazodicarboxylate, DMF = *N*,*N*-dimethylformamide.

(≈1:3) of **11** and the corresponding disulfide dimer **12** was obtained in an overall yield of 88%. This mixture was purified on a Biogel P-2 column to yield sufficiently pure **11** and **12** for biological tests.

By use of an in vitro fluorescence assay,[8,9] the analogues **1**–**12** were examined for their inhibition of LF protease activity (Table 1). All the compounds tested were found to be competitive inhibitors. From the measured apparent K_i values under low-ionic-strength assay conditions, it turns out that among the 12 analogues tested, 6 compounds (**2**, **6**, **9**–**12**), with K_i values in the range of 0.2–1.3 nM, are significantly better inhibitors than neomycin B itself ($K_i = 37$ nM). Interestingly, the binding affinity of the analogues in the gluco series (with a glucose substituent as ring V) increases gradually with an increasing number of amino groups on the ring: ($2NH_2$)glucose (**2**, **9**) > ($1NH_2$)glucose (**3**, **8**) > glucose (**7**). In this series of compounds, no particular influence of the position of the amino group(s) on the glucose ring is observed. The ring configuration, however, has a more significant effect: The ribosamino derivative **6** binds about 10-fold more strongly than the monoamino derivatives of glucose, **3** and **8**, and the diamino-D-allose derivative **1**, which contains an unusual *cis*-1,2-diamine substitution at ring V, binds about 20-fold more weakly than the diamino-D-glucose derivatives **2** and **9**. These data suggest that, although the number of amino groups on the ligand is in general critical for LF-binding affinity, structural features of the ligand play an important role in the proper recognition of LF.

Table 1: Apparent inhibition constant (K_i) values for commercial neomycin B and the synthetic derivatives **1**–**12** against the protease activity of LF under various assay conditions.

Aminoglycoside	K_i [nM] low salt concentration[a]	K_i [μM] high salt concentration[b]
neomycin B	37 ± 2 (34 ± 0.4)[c]	59 ± 6 (64 ± 8)[c]
1	11 ± 2	50 ± 7
2	0.5 ± 0.1 (17 ± 4)[c]	28 ± 6 (30 ± 5)[c]
3	13 ± 2	66 ± 9
4	28 ± 2	134 ± 17
5	52 ± 5	81 ± 21
6	1.3 ± 0.4	39 ± 6
7	23 ± 2	125 ± 25
8	15 ± 2 (36 ± 5)[c]	85 ± 11 (58 ± 8)[c]
9	0.6 ± 0.1 (15 ± 3)[c]	20 ± 3 (24 ± 3)[c]
10	0.4 ± 0.1	21 ± 4
11	0.2 ± 0.1 (0.3 ± 0.1)[c]	10 ± 2 (12 ± 3)[c]
12	0.7 ± 0.2 (33 ± 6)[c]	1.1 ± 0.2 (1.2 ± 0.2)[c]

[a] Low-salt conditions: potassium 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) buffer (10 mM) at pH 7.4, LF (≈33 nM), a fluorescent substrate (4, 6, 10, and 20 μM), and an inhibitor (the concentrations of **12** were 0, 16.2, 32.5, and 54.1 nM; the concentrations of all other compounds were 0, 165, 330, and 550 nM). The K_i values were estimated from double-reciprocal plots of initial velocities as a function of substrate concentration. [b] High-salt conditions: potassium HEPES buffer (10 mM) at pH 7.4, KCl (150 mM), LF (≈33 nM), a fluorescent substrate (10, 20, 40, and 100 μM), and an inhibitor (the concentrations of **12** were 0, 16.2, 32.5, and 54.1 μM; the concentrations of all other compounds were 0, 165, 330, and 550 μM). The K_i values were estimated as in [a]. [c] The data in parentheses were obtained under the same conditions as those of the parent experiments but with the additional presence of BSA (0.1 mg mL^{-1}). All assays were performed in triplicate and analogous results were obtained in at least two or three experiments.

Since the disulfide dimer **12** has twice as many amino groups as its parent "monomeric" **11**, its binding affinity to LF was expected to be significantly higher. The observed similar extent of inhibition of **12** and **11** was, however, very intriguing and suggested that in the case of dimer **12**, in addition to a "specific" active-site binding, an additional "nonspecific interaction" with the LF protein may occur. Various studies dealing with assorted protein–polyelectrolyte interactions[23] and with the interactions of aminoglycosides with ribozymes[12,21] support this presumption. To test this possibility, the analogues **2**, **8**, **9**, **11**, and **12**, along with neomycin B, were evaluated in the presence of 0.1 mg mL^{-1} of bovine serum albumin (BSA). Whereas the binding affinities of both neomycin B and **11** were not affected significantly, the binding of **12** decreased about 50-fold, which implies that nonspecific protein–ligand association may exist in the case of **12**. The data in the presence of BSA also indicate that this type of nonspecific protein–ligand association increases with an increasing number of amino groups on the ligand.

Although to date no direct structural data on the interaction of aminoglycosides with LF are available, our preliminary investigation of the binding mechanism showed that the inhibitory activity of aminoglycosides is ionic-strength dependent, thus indicating that the predominant interaction between LF and the aminoglycosides is electrostatic in origin.[21] An increase in the ionic strength from 0 to 150 mM KCl drastically shifts the K_i values of all aminoglycosides by a factor of ≈1500–53000 towards higher concentrations (Table 1). These data show that all of the synthetic analogues **1**–**12,** as well as the parent neomycin B, can be displaced from their LF-binding site even at a relatively low ionic strength. A possible reason for the observed different sensitivity of different aminoglycosides towards the ionic strength of the solution could be the different number of amino groups and their individual pK_a values. Furthermore, it is likely that the pK_a values of individual ammonium groups of neomycin B and of the dimer **12** are the same, which results in **12** behaving like a "monomer" and displaying the same sensitivity as neomycin B with respect to the ionic strength of

the solution (≈1500-fold increase, Table 1).[24] Nevertheless, the observed 53-fold higher affinity of **12** relative to that of neomycin B, both at the low and high salt concentrations, indicates that the presence of twice the number of charged groups in **12** is probably responsible for the increased affinity. The high-ionic-strength conditions are also able to overcome the nonspecific LF–**12** association, as evidenced from the very similar K_i values observed for **12** in the presence and absence of BSA. Furthermore, since 150 mM KCl best resembles the physiological ionic strength in many cell types,[25] **12** can be considered the best aminoglycoside inhibitor of LF at putative physiological conditions.

When the new derivatives **1**–**12** were tested by means of surface plasmon resonance (SPR) techniques against an immobilized 27-mer RNA construct (AS-wt),[11] binding constant (K_d) values in the range of 0.4–2.9 μM were determined, with no obvious dependence of the K_d value on the modification type (Table 2). Several of these derivatives, including the dimer **12** ($K_d = 0.4$ μM), displayed a similar binding affinity to that of the parent neomycin B ($K_d = 0.3$ μM), thus showing no significant contribution of the number of amino groups on the ligand–RNA binding. These findings suggest that unlike the binding affinity to LF, whereby an increased number of amino groups on the natural drug leads to improved binding, a more subtle balance of interactions governs the binding affinity of these ligands to RNA. To compare the observed RNA-binding affinities to antibacterial activity, the analogues **1**–**12** were further investigated against *B. anthracis* (Sterne strain),[26] and the minimal inhibitory concentration (MIC) values were determined by using a microdilution assay with neomycin B as a control (Table 2). To our knowledge, no previous studies on the activity of aminoglycoside drugs against *B. anthracis* have been performed.

Table 2: Minimal inhibitory concentration (MIC) values against *B. anthracis* and binding constant (K_d) values with 16S A site RNA for commercial neomycin B and the synthetic derivatives **1**–**12**.

Aminoglycoside	MIC [μg mL^{-1}][a]	K_d [μM][b]
neomycin B	0.25	0.3 ± 0.1
1	8	2.0 ± 0.2
2	2	1.3 ± 0.3
3	2	0.9 ± 0.1
4	1	0.7 ± 0.1
5	2	1.0 ± 0.2
6	2	0.7 ± 0.1
7	8	1.1 ± 0.2
8	2	0.7 ± 0.1
9	1	0.6 ± 0.1
10	2	1.9 ± 0.3
11	8	2.9 ± 0.6
12	2	0.4 ± 0.1

[a] For the MIC value measurements, the concentrated stock solutions of aminoglycosides were prepared to known concentrations in distilled water. The solutions were then diluted twice with BHI (brain heart infusion) broth (100 μL) to concentrations in the range of 0.015–1024 mg L^{-1} and poured into the wells of microtiter plates (Nunc 96-well flat-bottomed microtiter plates; Nunc, Roskilde, Denmark). A 10-μL volume of culture containing *B. anthracis* (Sterne strain, 10^5 cfu mL^{-1}) was then added. Following incubation of the plates for 18 h at 37 °C in air, the MIC values were determined as the lowest concentration of an antibacterial agent that completely inhibited visible growth of the bacteria.[26] [b] The sequence of the 27-mer 16S A-site RNA construct used in this study was 5′-biotinyl-GGCGUCACACCUUCGGGUGAA-GUCGCC-3′. The binding assays were performed as previously described.[11]

From the MIC values, it can be seen that all of the synthetic derivatives possess significant antibacterial activity against *B. anthracis*, with some of them displaying activity levels comparable to that of neomycin B. In spite of the similar binding affinities of neomycin B and the dimer **12** to 16S A-site RNA, their antimicrobial activities differed by a factor of eight, thus suggesting that no direct correlation between rRNA binding and antibacterial activity can be made. Although this is in agreement with earlier reported data on other aminoglycoside analogues,[27] further structure–activity studies with more diverse structural analogues of neomycin B are clearly required to understand this issue in detail.

In conclusion, the neomycin B derivatives synthesized in this study represent a new class of C5″-branched aminoglycoside antibiotics that show a dual effect by inhibiting LF at seemingly physiological conditions and exhibiting activity against *B. anthracis* simultaneously. Thus, this study provides a new direction for the development of novel antibiotics that target both the toxigenic bacterium and its released lethal toxin; this progress may offer promise for the effective treatment of anthrax infections. Cytotoxicity assays to determine the ability of the designed structures to protect macrophages against LF and the rate at which *B. anthracis* is killed are currently underway.

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