Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 1527–1531

# Guanidinylated 2,5-dideoxystreptamine derivatives as anthrax lethal factor inhibitors

Guan-Sheng Jiao,[a,*] Lynne Cregar,[b] Mark E. Goldman,[b,†] Sherri Z. Millis[b] and Cho Tang[a,‡]

[a]*Department of Chemistry, Hawaii Biotech, Inc., 99-193 Aiea Heights Dr., Suite 200, Aiea, HI 96701, USA*
[b]*Department of Lead Discovery, Hawaii Biotech, Inc., 99-193 Aiea Heights Dr., Suite 200, Aiea, HI 96701, USA*

Received 29 October 2005; revised 9 December 2005; accepted 9 December 2005
Available online 4 January 2006

**Abstract**—Anthraxlethal factor is a $Zn^{2+}$-dependent metalloprotease and the key virulence factor of tripartite anthrax toxin secreted by *Bacillus anthracis*, the causative agent of anthrax. A series of guanidinylated 2,5-dideoxystreptamine derivatives were designed and synthesized as inhibitors of lethal factor, some of which show strong inhibitory activity against lethal factor in an in vitro FRET assay. Preparation and structure–activity relationships of these compounds are presented.
© 2005 Elsevier Ltd. All rights reserved.

Anthrax[1] is an infectious disease induced by the Gram-positive bacterium *Bacillus anthracis* which is usually found in nature as environmentally resistant and durable spores.[2] Once inhaled, *B. anthracis* spores travel to regional lymph nodes and subsequently to the bloodstream where they multiply and release anthrax toxins[3] which play a critical role in the pathogenesis of anthrax.[4] There is an urgent need for efficient and specific therapies[5] to combat anthrax because of its threat as a biowarfare and bioterrorism agent. At present, vaccination[6] prior to infection and administration of antibiotics[7] such as ciprofloxacin soon after infection prove to be effective in minimizing fatality due to anthrax. Unfortunately, there is no effective means available for treating late-stage, established infection, particularly neutralizing the toxin which may continue to damage the host even after the bacteria are killed by antibiotics.

Three plasmid-encoded proteins, protective antigen (PA, 83 kDa), edema factor (EF, 89 kDa), and lethal factor (LF, 90 kDa), form the anthrax toxins.[8] While each of these proteins shows no toxicity by itself, their toxic effect is exerted with the cooperation of each other.[9] $PA_{83}$ (83 kDa)[10] specifically binds to the anthrax toxin receptor (ATR)[11] on the cell surface, where a 20 kDa N-terminal fragment of PA is cleaved by furin.[12] The remaining $PA_{63}$ (63 kDa) still bound to its receptor, heptamerizes, and binds 1 to 3 molecules of LF and/or EF.[13] The complexes translocate to the endosomes. A pH change then mediates the release of LF and EF into the cytosol.[14] EF is a $Ca^{2+}$/calmodulin-dependent adenylate cyclase that synthesizes cAMP, leading to edema;[15] LF is a $Zn^{2+}$-dependent metalloprotease[16] that specifically cleaves mitogen-activated protein kinase kinases (MAPKKs) near their amino termini, leading to the disruption of one or more signaling pathways.[17] Given that *B. anthracis* strains lacking LF are greatly attenuated,[9] LF is considered the key virulence factor of anthrax. Accordingly, inhibition of LF might provide a promising therapeutic option to treat post-exposure anthrax infection. Herein we report a series of guanidinylated 2,5-dideoxystreptamine derivatives which exhibit inhibitory activity against anthrax lethal factor protease.

The search for specific small molecule inhibitors of LF has been extensively conducted in both academia and industry during the last few years.[18–27] One significant discovery is that some aminoglycosides, commonly utilized as antibiotics, show strong inhibition of LF activity.[22,23] An initial screen in our laboratory identified neomycin B (Fig. 1A) and other aminoglycosides as

*Keywords*: Lethal factor inhibitors; Anthrax; Metalloprotease; Guanidinylated 2,5-dideoxystreptamine derivatives.
* Corresponding author. Tel.: +1 808 792 1367; fax: +1 808 792 1348; e-mail: gjiao@hibiotech.com
† Present address: John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii, USA.
‡ Present address: Shanghai Hengrui Pharmaceuticals, Shanghai, China.

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.12.038

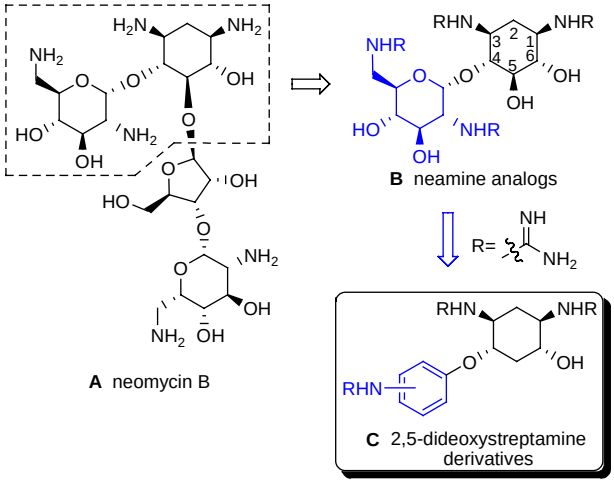
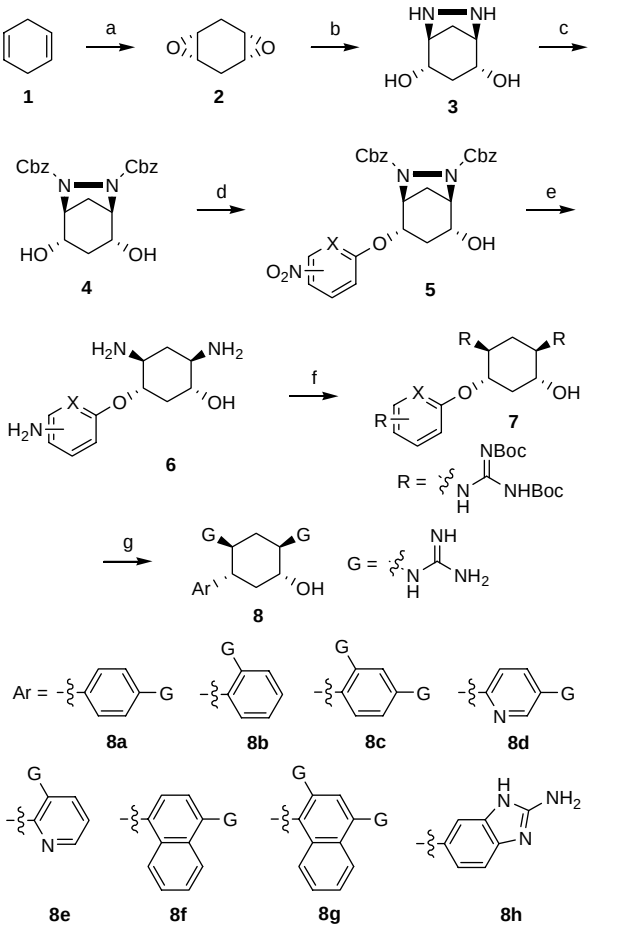



**Figure 1.** (A) Neomycin B, (B) guanidinylated neamine analogs, and (C) guanidinylated 2,5-dideoxystreptamine derivatives.

compounds able to block LF protease activity.[28] However, the structural complexity of neomycin B renders itself an unattractive lead structure in drug discovery. We then turned our attention to a structurally simpler neamine (Fig. 1B, $R$ = NH) which is the core structure of neomycin B and the aminoglycoside derivative of 2-deoxystreptamine.[29] It was found that neamine acted as a weak inhibitor of LF.[30] To improve the potency of neamine, we synthesized a library of selectively guanidinylated neamine analogs (Fig. 1B) and discovered that the guanidinyl groups added to neamine dramatically enhanced the potency.[31,32] Although guanidinylated neamine analogs showed promising inhibitory activity against LF, the use of these compounds as anti-anthrax drugs seems to be an unattainable goal due to the poor oral bioavailability and potential toxicity associated with aminoglycosides.[33] Therefore, we set out to search easily accessible mimetics of guanidinylated neamines which avoid the undesirable pharmacological profile and maintain the potency against LF.

A literature search revealed that a planar aryl group could function as a surrogate of the pyranose ring in neamine mimetics designed for RNA recognition.[34,35] Since the presence of guanidinyl groups in the neamine analogs proved to be crucial for their potency, similar replacement of the amino sugar with a guanidinylated aryl ring seemed plausible. Also, to simplify analog synthesis, a related core structure 2,5-dideoxystreptamine, which preserves the same unique spatial arrangement of amino-groups as 2-deoxystreptamine, was used. In addition, we envisioned that these planar non-sugar groups would increase the lipophilicity of molecules and enhance bioavailability. Therefore, the guanidinylated 2,5-dideoxystreptamine aryl ethers (Fig. 1C) were selected as the initial target compounds.

The synthesis shown in Scheme 1 starts from commercially available 1,4-cyclohexadiene **1**. Epoxidation with *m*CPBA at low temperature gave the desired *cis*-epoxide **2** as major product, along with minor *trans*-isomer.[36,37] Bifunctional hydrazine was employed to open the *cis*-epoxide to give a single product **3** which precipitated from the reaction mixture in high purity.[36,37] In this single step, the correct stereochemistry was set up for the subsequent synthesis of the 2,5-dideoxystreptamine derivatives. Cbz-protection of amino-groups to give **4** allowed the further elaboration of OH-groups. A number of aryl ethers **5** were formed via $S_NAr$ coupling with various nitro-substituted aryl halides, the key step which allowed diversity to be introduced in the molecules. The efficiency of this route is shown by the fact that reduction of the nitro-group, removal of the Cbz-group, and cleavage of the N–N bond were achieved in a single high pressure hydrogenation step. The resulting amino-compounds **6**, though not very stable, could be used directly in the next step without purification. Guanidinylation with *N*,*N*′-di-(*tert*-butoxycarbonyl)-*N*″-triflylguanidine,[38] followed by TFA deprotection, furnished the final products **8** as a racemic mixture of TFA salts.[39]




**Scheme 1.** Reagents and conditions: (a) *m*CPBA, DCM, 0 °C then rt; (b) hydrazine, *n*-BuOH, reflux; (c) Cbz-Cl, dioxane, $H_2O$, rt; (d) 1 equiv of 1-fluoro-4-nitrobenzene for **5a**, 1-fluoro-2-nitrobenzene for **5b**, 2,4-dinitrofluorobenzene for **5c**, 2-chloro-5-nitropyridine for **5d**, 2-chloro-3-nitropyridine for **5e**, 1-fluoro-4-nitronaphthalene for **5f**, 1,3-dinitronaphthalen-4-yl trifluoromethanesulfonate for **5g**, 1,2-dinitrofluorobenzene for **5h**, NaH ($K_2CO_3$ for **5c**), DMF, 25 °C; (e) $H_2$, Pd/C, MeOH, rt; (f) TfON=C(NHBoc)$_2$, pyridine, rt; (g) TFA, DCM, rt.

The biological activities of the compounds were evaluated for inhibition of LF by using an in vitro FRET assay.[40]

**Table 1.** Apparent inhibition constant ($K_i^{app}$) values for guanidinylated 2,5-dideoxystreptamine derivatives against LF

| Compound | No. of guanidinyl group | $K_i^{app}$ [a] (μM) |
|---|---|---|
| **8a** | 3 | 14.9 ± 1.6 |
| **8b** | 3 | 30.6 ± 2.2 |
| **8c** | 4 | 0.6 ± 0.2 |
| **8d** | 3 | 6.6 ± 0.6 |
| **8e** | 3 | 4.1 ± 0.5 |
| **8f** | 3 | 31.4 ± 5.6 |
| **8g** | 4 | 10.7 ± 1.9 |
| **8h** | 2 | 153.7 ± 4.5 |
| **10** | 3 | 24.8 ± 4.5 |
| **12** | 4 | 0.5 ± 0.1 |
| **14** | 6 | 0.065 ± 0.006 |

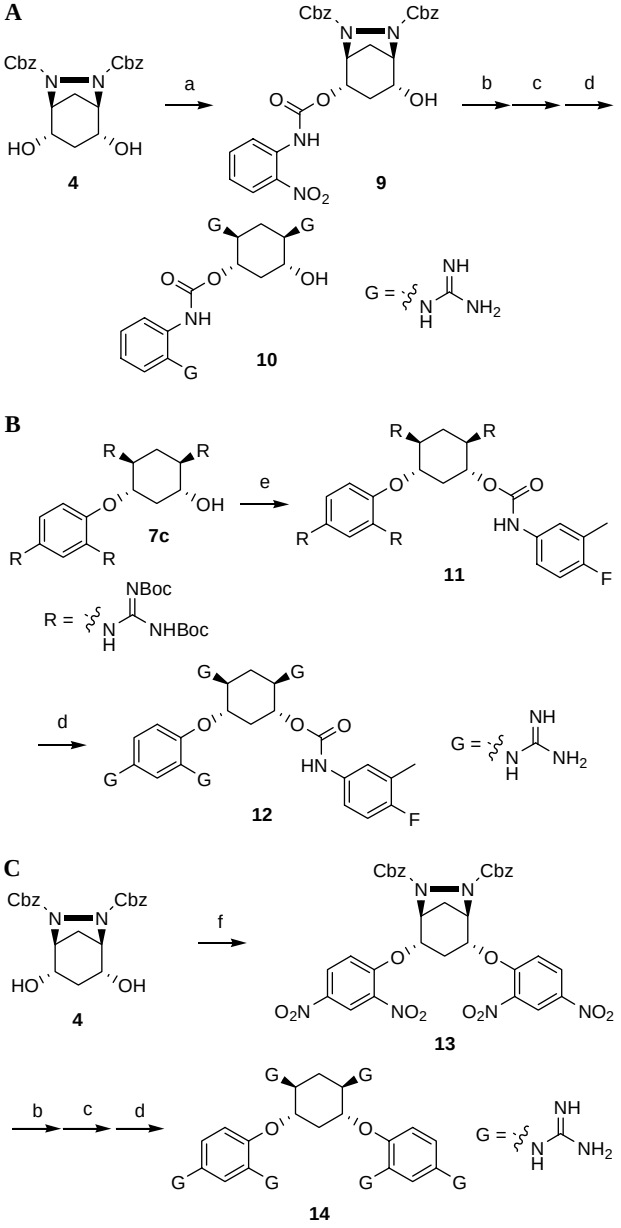
[a] 20 mM Hepes + 0.05% Tween 20 + 0.02% $NaN_3$, pH 7.4, 20 nM LF, 12.5 nM MAPKKide™ substrate, and inhibitor. The $K_i^{app}$ values were calculated using the program BatchKi (BioKin, Ltd., Pullman, WA). The values are means of three separate experiments with each performed in triplicate.

While the corresponding amino-precursors showed very weak or no activity against LF (data not shown), the guanidinylated 2,5-dideoxystreptamine derivatives exhibited strong inhibition of LF. The results shown in Table 1 revealed that the guanidinyl group plays a crucial role for the inhibitory activity of the compounds: the more guanidinyl groups present, the better the activity observed (**8c** vs **8a** and **8b**, and **8g** vs **8f**). For example, compound **8c** with four guanidinyl groups achieved comparable activity to neomycin B against LF.[28] In contrast, compound **8h** with the guanidinyl group on the phenyl group replaced by the benzoimidazolamine group showed greatly reduced activity compared to compound **8a**. In addition, the position of the guanidinyl groups on the aromatic moiety and the aromatic group itself exhibit subtle effects on the inhibitory activities; the *para*-position is better than the *ortho*-position (**8a** vs **8b**). Also, compounds containing a pyridyl group show better activity than those with a phenyl group (**8d** vs **8a** and **8e** vs **8b**), and a naphthyl group was found to be inferior to a phenyl group (**8f** vs **8a** and **8g** vs **8c**).

Encouraged by the above results, we next explored the influence of the linker between the aromatic fragment and 2,5-dideoxystreptamine on inhibitory activity by replacement of the ether (–O–) with carbamate (–NHCOO–). Consequently, compound **10** was prepared from **4** via carbamation as the key step with the remaining transformations following the aryl ether synthesis (Scheme 2A). It was found that the linker, either ether (–O–) or carbamate (–NHCOO–), has no significant effect on the inhibition (**10** vs **8b**).

We also investigated the effect of the free 6-OH group by substitution of it with a carbamate group. Compound **12**[39] was thus obtained by carbamation of **7c**, followed by deprotection (Scheme 2B). The biological data shown in Table 1 revealed that replacement of the 6-OH group with a large carbamate group had little effect on the potency (**12** vs **8c**).

Finally, we generated the symmetric *meso*-compound **14**[39] by replacing the free 6-OH group with a 2,4-diguanidino-phenoxy moiety. Compound **14** was prepared following the same synthetic strategy shown in Scheme 1, except that two equivalents of 2,4-dinitrofluorobenzene were employed in the $S_NAr$ step (Scheme 2C). We were pleased to find that *the symmetric meso-compound **14**, which bears six guanidinyl groups, exhibited nano-molar range activity against LF*, further indicating the importance of the guanidinyl group.




**Scheme 2.** Reagents and conditions: (a) 2-nitro-phenyl isocyanate, pyridine, rt; (b) $H_2$, Pd/C, MeOH, rt; (c) TfON=C(NHBoc)$_2$, pyridine, rt; (d) TFA, DCM, rt; (e) 4-fluoro-3-methylphenyl isocyanate, DCM, rt; (f) 2 equiv of 2,4-dinitrofluorobenzene, NaH, DMF, rt.

The recently determined X-ray crystal structure of anthrax lethal factor provides a clue for interpreting the observed structure–activity relationships.[19,20,25,26,41] The active site of the protease has been found to be a deep, broad, 40 Å groove with a region of highly nega-

tive electrostatic potential due to the presence of acidic Asp and Glu residues. Once the 2,5-dideoxystreptamine derivatives reside within the active site, multiple electrostatic and H-bonding interactions would occur between the positively charged guanidinyl groups of the 2,5-dideoxystreptamine derivatives and the negatively charged residues of LF. Therefore, the guanidinyl groups, which might mimic the positively charged arginine- and lysine-rich section of LF substrates such as MAPKKs,[17] have a primary effect on the inhibitory activity of these molecules against LF. The more guanidinyl groups, the stronger the interactions, leading to better inhibition of lethal factor protease.

In summary, a series of guanidinylated 2,5-dideoxystreptamine derivatives have been designed and synthesized, some of which showed strong inhibition of anthrax lethal factor protease activity. Our future work will focus on further optimization of the lead compounds with emphasis on cellular activity and desirable pharmacological properties.

## Acknowledgments

We thank Dr. Alan T. Johnson for editorial comment on the manuscript, Drs. Alan McClelland, Sean O'Malley, Sina Sareth, and Dominique Nguyen for helpful discussion, and Ms. Linda McKasson and Mr. Anthony Mehok for analytical assistance. This work was funded by the U.S. Department of Defense, U.S. Army Medical Research and Materials Command, Ft. Detrick, MD, and administered by the Pacific Telehealth & Technology Hui, Honolulu, HI, contract # V549P-6073. The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of Defense, U.S. Department of the Army (Department of Veterans Affairs, if applicable), or the U.S. Government. The appearance of name-brand products in this article does not constitute endorsement by the U.S. Department of the Army, Department of Defense (Department of Veterans Affairs, if applicable) or the U.S. Government of the information, products or services contained therein.

## References and notes

1. Dixon, T. C.; Meselson, M.; Guillemin, J.; Hanna, P. C. *N. Engl. J. Med.* **1999**, *341*, 815.
2. Mock, M.; Fouet, A. *Annu. Rev. Microbiol.* **1998**, *225*, 13.
3. Collier, R. J.; Young, J. A. *Annu. Rev. Cell. Dev. Biol.* **2003**, *19*, 45.
4. Moayeri, M.; Leppla, S. H. *Curr. Opin. Microbiol.* **2004**, *7*, 19.
5. Friedlander, A. M. *Nature* **2001**, *414*, 160.
6. Friedlander, A. M.; Welkos, S. L.; Ivins, B. E. *Curr. Top. Microbiol. Immunol.* **2002**, *271*, 33.
7. Bartlett, J. G.; Inglesby, T. V., Jr.; Borio, L. *Clin. Infect. Dis.* **2002**, *35*, 851.
8. Ascenzi, P.; Visca, P.; Ippolito, G.; Spallarossa, A.; Bolognesi, M.; Montecucco, C. *FEBS Lett.* **2002**, *532*, 384.
9. Pezard, C.; Berche, P.; Mock, M. *Infect. Immun.* **1991**, *59*, 3472.
10. Petosa, C.; Collier, R. J.; Klimpel, K. R.; Leppla, S. H.; Liddington, R. C. *Nature* **1997**, *385*, 833.
11. Bradley, K. A.; Mogridge, J.; Mourez, M.; Collier, R. J.; Young, J. A. *Nature* **2001**, *414*, 225.
12. Molloy, S. S.; Bresnahan, P. A.; Leppla, S. H.; Klimpel, K. R.; Thomas, G. *J. Biol. Chem.* **1992**, *267*, 16396.
13. Mogridge, J.; Cunningham, K.; Collier, R. J. *Biochemistry* **2002**, *41*, 1079.
14. Menard, A.; Altendorf, K.; Breves, D.; Mock, M.; Montecucco, C. *FEBS Lett.* **1996**, *386*, 161.
15. Leppla, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3162.
16. Klimpel, K. R.; Arora, N.; Leppla, S. H. *Mol. Microbiol.* **1994**, *13*, 1093.
17. Duesbery, N. S.; Webb, C. P.; Leppla, S. H.; Gordon, V. M.; Klimpel, K. R.; Copeland, T. D.; Ahn, N. G.; Oskarsson, M. K.; Fukasawa, K.; Paull, K. D.; Vande Woude, G. F. *Science* **1998**, *280*, 734.
18. Kocer, S. S.; Walker, S. G.; Zerler, B.; Golub, L. M.; Simon, S. R. *Infect. Immun.* **2005**, *73*, 7548.
19. Forino, M.; Johnson, S.; Wong, T. Y.; Rozanov, D. V.; Savinov, A. Y.; Li, W.; Fattorusso, R.; Becattini, B.; Orry, A. J.; Jung, D.; Abagyan, R. A.; Smith, J. W.; Alibek, K.; Liddington, R. C.; Strongin, A. Y.; Pellecchia, M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9499.
20. Shoop, W. L.; Xiong, Y.; Wiltsie, J.; Woods, A.; Guo, J.; Pivnichny, J. V.; Felcetto, T.; Michael, B. F.; Bansal, A.; Cummings, R. T.; Cunningham, B. R.; Friedlander, A. M.; Douglas, C. M.; Patel, S. B.; Wisniewski, D.; Scapin, G.; Salowe, S. P.; Zaller, D. M.; Chapman, K. T.; Scolnick, E. M.; Schmatz, D. M.; Bartizal, K.; MacCoss, M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7958.
21. Numa, M. M. D.; Lee, L. V.; Liu, C.-C.; Bower, K. E.; Wong, C.-H. *ChemBioChem* **2005**, *6*, 1002.
22. Fridman, M.; Belakhov, V.; Lee, L. V.; Liang, F.-S.; Wong, C.-H.; Baasov, T. *Angew. Chem. Int. Ed.* **2005**, *44*, 447.
23. Lee, L. V.; Bower, K. E.; Liang, F.-S.; Shi, J.; Wu, D.; Sucheck, S. J.; Vogt, P. K.; Wong, C.-H. *J. Am. Chem. Soc.* **2004**, *126*, 4774.
24. Dell'Aica, I.; Dona, M.; Tonello, F.; Piris, A.; Mock, M.; Montecucco, C.; Garbisa, S. *EMBO Rep.* **2004**, *5*, 418.
25. Panchal, R. G.; Hermone, A. R.; Nguyen, T. L.; Wong, T. Y.; Schwarzenbacher, R.; Schmidt, J.; Lane, D.; McGrath, C.; Turk, B. E.; Burnett, J.; Aman, M. J.; Little, S.; Sausville, E. A.; Zaharevitz, D. W.; Cantley, L. C.; Liddington, R. C.; Gussio, R.; Bavari, S. *Nat. Struct. Mol. Biol.* **2004**, *11*, 67.
26. Turk, B. E.; Wong, T. Y.; Schwarzenbacher, R.; Jarrell, E. T.; Leppla, S. H.; Collier, R. J.; Liddington, R. C.; Cantley, L. C. *Nat. Struct. Mol. Biol.* **2004**, *11*, 60.
27. Tonello, F.; Seveso, M.; Marin, O.; Mock, M.; Montecucco, C. *Nature* **2002**, *418*, 386.
28. Neomycin B shows $K_i^{app}$ of 0.5 μM for inhibition of LF in our in-house assay. See: Tang, C.; Simo, O.; Nagata, M.; Jiao, G.-S.; O'Malley, S.; Goldman, M.; Cregar, L.; Nguyen, D.; Hemscheidt, T. *Abstracts of Papers,* 228th ACS National Meeting, Philadelphia, PA; American Chemical Society: Washington, DC, 2004; MEDI 261.
29. Busscher, G. F.; Rutjes, F. P. J. T.; van Delft, F. L. *Chem. Rev.* **2005**, *105*, 775.
30. Neamine exhibits inhibitory activity against LF with $K_i^{app}$ of 42.9 μM. See Ref. 28.
31. Guanidinylated neamine analogs inhibit LF with $K_i^{app}$ of 0.5–24.3 μM. See Ref. 28.
32. Guanidinyl group has been also shown to be crucial for the uptake of guanidinylated aminoglycosides by eukaryotic cells. See: Luedtke, N. W.; Carmichael, P.; Tor, Y. *J. Am. Chem. Soc.* **2003**, *125*, 12374.

33. Umezawa, H., Hooper, I. R., Eds.; *Aminoglycoside Antibiotics*; Spring-Verlag: New York, Herdelberg, 1982.;
34. Ding, Y.; Hofstadler, S. A.; Swayze, E. E.; Griffey, R. H. *Org. Lett.* **2001**, *3*, 1621.
35. Vourloumis, D.; Takahashi, M.; Winters, G. C.; Simonsen, K. B.; Ayida, B. K.; Barluenga, S.; Qamar, S.; Shandrick, S.; Zhao, Q.; Hermann, T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3367.
36. Kavadias, G.; Velkof, S.; Belleau, B. *Can. J. Chem.* **1978**, *56*, 404.
37. Suami, T.; Ogawa, S.; Uchino, H.; Funaki, Y. *J. Org. Chem.* **1975**, *40*, 456.
38. Baker, T. J.; Rew, Y.; Goodman, M. *Pure Appl. Chem.* **2000**, *72*, 347.
39. Characterization data for compounds **8c**, **12**, and **14**: compound **8c**. $^1$H NMR (500 MHz, $CD_3OD$) $\delta$ 7.30 (d, $J$ = 9.0 Hz, 1H), 7.29 (s, 1H), 7.25 (d, $J$ = 9.0 Hz, 1H), 4.46 (td, $J$ = 11.5, 4.0 Hz, 1H), 3.95 (td, $J$ = 12.5, 4.0 Hz, 1H), 3.66 (td, $J$ = 11.5, 4.0 Hz, 1H), 3.47 (td, $J$ = 12.0, 4.0 Hz, 1H), 2.48 (dt, $J$ = 13.0, 4.0 Hz, 1H), 2.23 (dt, $J$ = 13.0, 4.0 Hz, 1H), 1.70–1.59 (m, 2H); $^{13}$C NMR (125.5 MHz, $CD_3OD$) $\delta$ 163.3 (q, $J$ = 35.7 Hz), 159.2, 158.7, 158.5, 158.4, 153.1, 129.6, 128.5, 127.6, 126.3, 118.1 (q, $J$ = 295.4 Hz), 116.7, 78.4, 71.1, 56.5, 54.3, 37.8, 34.3; LC–MS: $t_R$ = 10.5 min; $m/z$ 421 $(M+H)^+$; compound **12**. $^1$H NMR (500 MHz, $CD_3OD$) $\delta$ 7.34 (d, $J$ = 8.5 Hz, 1H), 7.28–7.20 (m, 4H), 6.91 (t, $J$ = 8.5 Hz, 1H), 4.86 (td, $J$ = 10.5, 4.0 Hz, 1H), 4.57 (td, $J$ = 10.5, 4.0 Hz, 1H), 4.06 (td, $J$ = 12.0, 4.0 Hz, 1H), 3.85 (td, $J$ = 10.5, 4.0 Hz, 1H), 2.65 (dt, $J$ = 12.5, 4.5 Hz, 1H), 2.35 (dt, $J$ = 13.0, 4.5 Hz, 1H), 1.82–1.71 (m, 2H); $^{13}$C NMR (125.5 MHz, $CD_3OD$) $\delta$ 163.4 (q, $J$ = 34.8 Hz), 158.7, 158.5 (2C), 158.4, 154.7, 152.8, 135.5, 129.8, 128.5, 127.4, 126.4, 126.1, 126.0, 123.0, 119.0, 118.1 (q, $J$ = 293.5 Hz), 116.8, 115.8 (d, $J$ = 23.8 Hz), 78.0, 72.6, 53.9, 53.7, 34.7, 34.3, 14.6; LC–MS: $t_R$ = 12.2 min; $m/z$ 572 $(M+H)^+$; compound **14**. $^1$H NMR (500 MHz, $CD_3OD$) $\delta$ 7.38 (d, $J$ = 8.5 Hz, 2H), 7.27 (d, $J$ = 2.5 Hz, 2H), 7.23 (dd, $J$ = 8.5, 2.5 Hz, 2H), 4.65 (td, $J$ = 12.5, 4.0 Hz, 2H), 4.05 (td, $J$ = 13.0, 4.0 Hz, 2H), 2.71 (dt, $J$ = 13.0, 4.0 Hz, 1H), 2.37 (dt, $J$ = 13.0, 4.0 Hz, 1H), 1.85 (q, $J$ = 12.0 Hz, 1H), 1.76 (q, $J$ = 12.0 Hz, 1H); $^{13}$C NMR (125.5 MHz, $CD_3OD$) $\delta$ 163.3 (q, $J$ = 34.7 Hz), 158.7, 158.5, 158.4, 153.0, 129.7, 128.5, 127.6, 126.1, 118.0 (q, $J$ = 194.3 Hz), 116.6, 77.6, 54.2, 34.3, 34.1; LC–MS: $t_R$ = 11.3 min; $m/z$ 611 $(M+H)^+$.
40. Lethal factor protease (20 nM) and inhibitor were briefly incubated at room temperature in the assay buffer (25 μl, 20 mM Hepes, 0.05% Tween 20, and 0.02% $NaN_3$, pH 7.4), and the reaction started by the addition of 12.5 μM final of the fluorogenic peptide substrate, MAPKKide™ (*o*-aminobenzoic acid on N terminus and 2,4-dinitrophenol on C terminus, List Biological Laboratories, Inc, Campbell, CA). Fluorescence intensity ($E_x$: 320 nm, $E_m$: 420 nm) was monitored for 15 min at room temperature and the $K_i^{app}$ values were calculated using the program BatchKi (BioKin Ltd., Pullman, WA). The $K_m$ and $K_{cat}$ for the peptide substrate under these conditions are 10 and 103 μM, respectively. Hydrolysis has been confirmed by HPLC, and the data were presented at the 5th International Conference on Anthrax, March 30, 2003 in Nice, France, in a poster titled ''Internally Quenched Fluorogenic Substrates for Anthrax Lethal Factor''.
41. Pannifer, A. D.; Wong, T. Y.; Schwarzenbacher, R.; Renatus, M.; Petosa, C.; Bienkowska, J.; Lacy, D. B.; Collier, R. J.; Park, S.; Leppla, S. H.; Hanna, P.; Liddington, R. C. *Nature* **2001**, *414*, 229.