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Selectively guanidinylated derivatives of neamine. Syntheses and inhibition of anthrax lethal factor protease

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Abstract—A series of mono-, di-, and tri-guanidinylated derivatives of neamine were prepared via selective guanidinylation of neamine. These molecules represent a novel scaffold as inhibitors of anthrax lethal factor zinc metalloprotease. Methods for the synthesis of these compounds are described, and structure–activity relationships among the series are analyzed. In addition, initial findings regarding the mechanism of LF inhibition for these molecules are presented.

Bacillus anthracis[1] is a spore-forming Gram-positive bacterium that is the causative agent of anthrax infection.[2] Generally, the spores can enter a subject by oral ingestion, through the skin, or by inhalation.[1] The spores are phagocytized and travel to regional lymph nodes where they germinate and release toxins[3] which are crucial for the pathogenesis of anthrax.[4] Although the use of vaccines prior to infection is preventive against anthrax,[5] various factors make mass vaccination impractical. Antibiotics such as ciprofloxacin, penicillins, and tetracyclines may be effective in reducing the bacterial infection itself at the very early stage,[6] but once the toxins are released, such therapy does not significantly arrest the course of the disease because of the continuing action of the toxins. Given the attractiveness of anthrax as a biological weapon, the modification of wildtype *B. anthracis* to provide antibiotic resistant strains is a distinct possibility. Therefore, it is important to provide a post-infection anthrax treatment which is not prone to antibiotic resistance.

After onset of infection, the toxins are released in the form of three plasmid-encoded proteins: protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa).[7] When PA_{83} (83 kDa)[8] specifically binds to a cell surface anthrax toxin receptor,[9] a 20 kDa fragment is cleaved by a furin-like protease,[10] allowing the remaining PA_{63} (63 kDa) to heptamerize. After binding to LF to give lethal toxin (LT) or EF to give edema toxin (ET), the heptamer behaves as a shuttle to translocate LF and EF into an intracellular endosomal compartment.[11] After LT and ET enter the endosomes, dissociation occurs, and LF and EF are released into the cytoplasm to exert their toxic effects.[12] Edema factor is a Ca^{2+}/calmodulin-dependent adenylate cyclase that triggers the synthesis of cAMP, leading to edema.[13] Lethal factor is a Zn^{2+}-dependent metalloprotease that specifically cleaves mitogen-activated protein kinase kinases (MAPKKs) in macrophages.[14] Degradation of MAPKKs interrupts critical signaling pathways, resulting in cell death.[15] The dead cells then release cytokines and NO which cause septic shock and death of the subject. The fact that EF-deficient *B. anthracis* strains are still toxic, while those lacking LF are greatly attenuated,[16] suggests that LF is the dominant virulence factor of anthrax. Therefore, inhibition of LF should offer an

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efficient therapeutic approach for treating anthrax, particularly in late stage infection. During the last few years, progress has been made in the search for specific inhibitors of LF.[17–31]

Our early work in the anthrax project led to the discovery of a series of cationic small molecule LF protease inhibitors. An intermediate stage of this work was the identification of the semi-synthetic LF inhibitors derived from neamine. In this letter, we present the systematic investigation of these guanidinylated neamine derivatives.[32] The significance of this study is twofold. First, the selective synthesis of guanidinylated neamine derivatives remains a significant challenge in organic synthesis. Efficient and practical methods for mono-, di-, and tri-guanidinylation of neamine are presented in this letter. Second, these compounds are shown to be potent inhibitors of anthrax LF protease using an in vitro biochemical assay.[33] Based on this work, we further identified fully synthetic LF inhibitors derived from 2,5-dideoxystreptamine, which were published earlier.[17] Taken together, these studies illustrated the ability to use a biologically active natural product to identify fully synthetic, potent small molecule inhibitors of anthrax lethal factor protease.

The project was initiated by the screening of a focused library of commercially available cationic compounds. It was found that some aminoglycosides, commonly used antibiotics for treatment of Gram-negative and Gram-positive bacterial infections,[34] exhibited strong inhibitory activity against anthrax LF protease.[35] For example, neomycin B (Fig. 1, **1**) showed K_i^{app} of 0.5 μM for inhibition of LF in our in vitro FRET assay.[32] Because of its structural complexity, neomycin B does not represent an attractive lead for a medicinal chemistry program. The decision was then made to investigate structurally simpler neamine (Fig. 1, **2**), a common pseudodisaccharide present in most naturally occurring aminoglycosides, for further optimization because it could be manipulated more easily than neomycin B. Accordingly, we prepared neamine via treatment of commercially available neomycin B with acetyl chloride in methanol.[36] Neamine was found to behave as a weak inhibitor of LF with K_i^{app} of 42.9 μM.

The available X-ray crystal structures of LF reveal that the active site of the protease possesses high negative charge density due to the presence of clusters of negatively charged, acidic Asp and Glu residues.[21,22,29,30,37] The electrostatic interactions between the positively charged amino groups of aminoglycosides and the negatively charged residues of LF have been shown to play a vital role in determining the inhibitory activity.[24,27] We hypothesized that introduction of highly charged guanidinyl groups onto the neamine core might result in better potency by inducing stronger electrostatic interaction with the binding site.[38] To test the hypothesis, tetraguanidinoneamine **4** was synthesized via guanidinylation of neamine with excess of *N*,*N*′-di-(*tert*-butoxycarbonyl)-*N*″-triflylguanidine **5**,[39] followed by TFA deprotection, as a TFA salt (Scheme 1). We were encouraged by the finding that compound **4** showed a K_i^{app} of 0.7 μM for inhibition of LF, which is comparable to that of neomycin B.

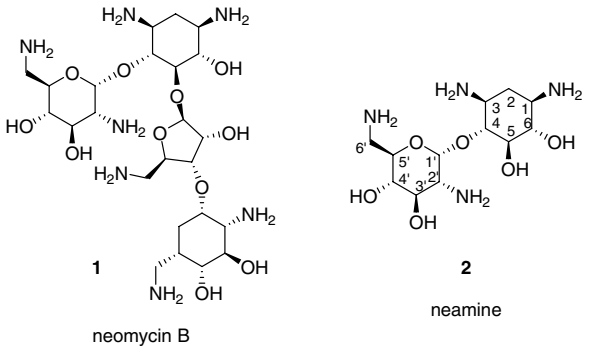


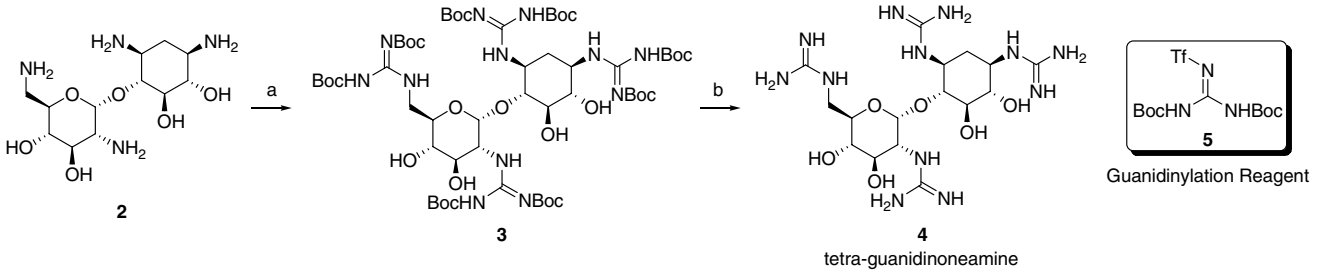
Figure 1. Structures of neomycin B **1** and neamine **2**.

The above result strongly suggested the significant effect of the guanidinyl group on the inhibitory activity against LF.[40] Consequently, our next goal was to determine the minimum number of guanidinyl groups required for potent activity as well as to investigate the structure–activity relationship requirements for the location of these guanidinyl groups. Therefore, a series of mono-, di-, and tri-guanidinylated derivatives of neamine were prepared. In each synthetic strategy described below, only the key steps are highlighted. The last step of the synthesis was the same in each case, deprotection with TFA to give the final products as TFA salts.

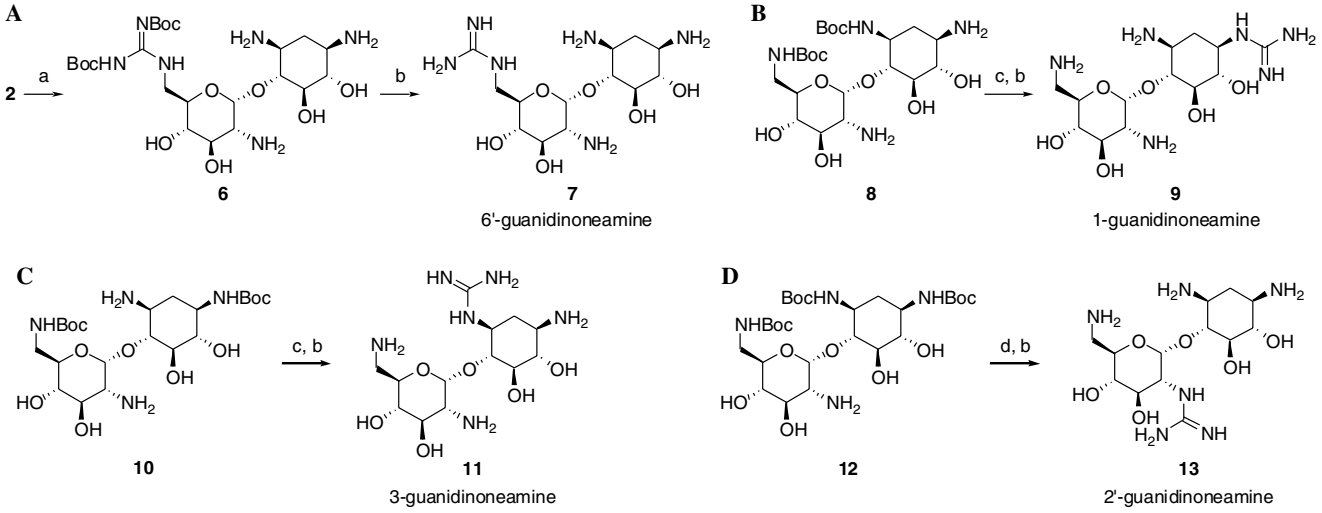
In general, the primary 6′-amino group is the most reactive of the four amino groups of neamine with slight differences in reactivity among the remaining three positions (1 > 3 > 2′) due to their steric accessibility.[41] As a result, the mono-guanidinylated neamines, 6′-guanidinoneamine **7**, 1-guanidinoneamine **9**, and 3-guanidinoneamine **11**, were prepared by selective guanidinylation of neamine **2**, 3,6′-di-(*tert*-butoxycarbonyl)-neamine **8**,[42] and 1,6′-di-(*tert*-butoxycarbonyl)-neamine **10**,[43] respectively, employing a limiting amount of **5** (Schemes 2A–C).[44] The 2′-guanidinoneamine **13** was prepared from 1,3,6′-tri-(*tert*-butoxycarbonyl)-neamine **12**[45] with excess of **5** (Scheme 2D).

The synthesis of the di- and tri-guanidinylated neamines began with either a mono-guanidinylated neamine derivative or selectively N-protected neamine compounds. The di-guanidinylated neamines, 1,6′-diguanidinoneamine **14** and 3,6′-diguanidinoneamine **15**, and tri-guanidinylated analogs, 1,3,6′-triguanidinoneamine **16** and 1,2′,6′-triguanidinoneamine **17**, were prepared via a single guanidinylation reaction of compound **6** (see Scheme 2A) with 1 equiv of **5** (Scheme 3). The four intermediate compounds were readily separated from the mixture by silica gel column chromatography.

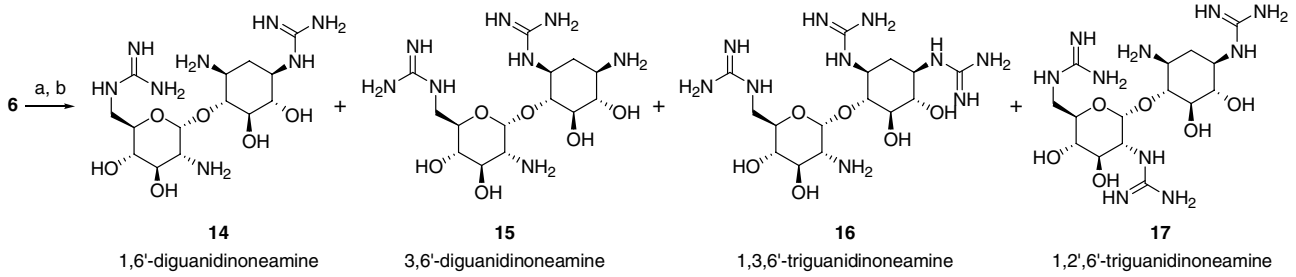
The 1,3-diguanidinoneamine **19** was obtained via selective guanidinylation of 6′-(*tert*-butoxycarbonyl)-neamine **18**[46] with 1.9 equiv of **5** (Scheme 4A), and 1,2′-diguanidinoneamine **20** (Scheme 4B), 3,2′-diguanidinoneamine **21** (Scheme 4C), and 1,3,2′-triguanidinoneamine **23** (Scheme 4E) were prepared from compounds **8**,[42] **10**,[43] and **18**,[46] respectively, by using excess of **5**.



Scheme 1. Reagents and conditions: (a) 6 equiv of **5**, Et_3N, MeOH/H_2O, rt; (b) TFA–DCM (1:1), rt.



Scheme 2. Reagents and conditions: (a) 0.95 equiv of **5**, MeOH–H_2O (1.3:1), rt; (b) TFA–DCM (1:1), rt; (c) 1 equiv of **5**, Et_3N, MeOH, rt; (d) 1.5 equiv of **5**, Et_3N, MeOH, rt.

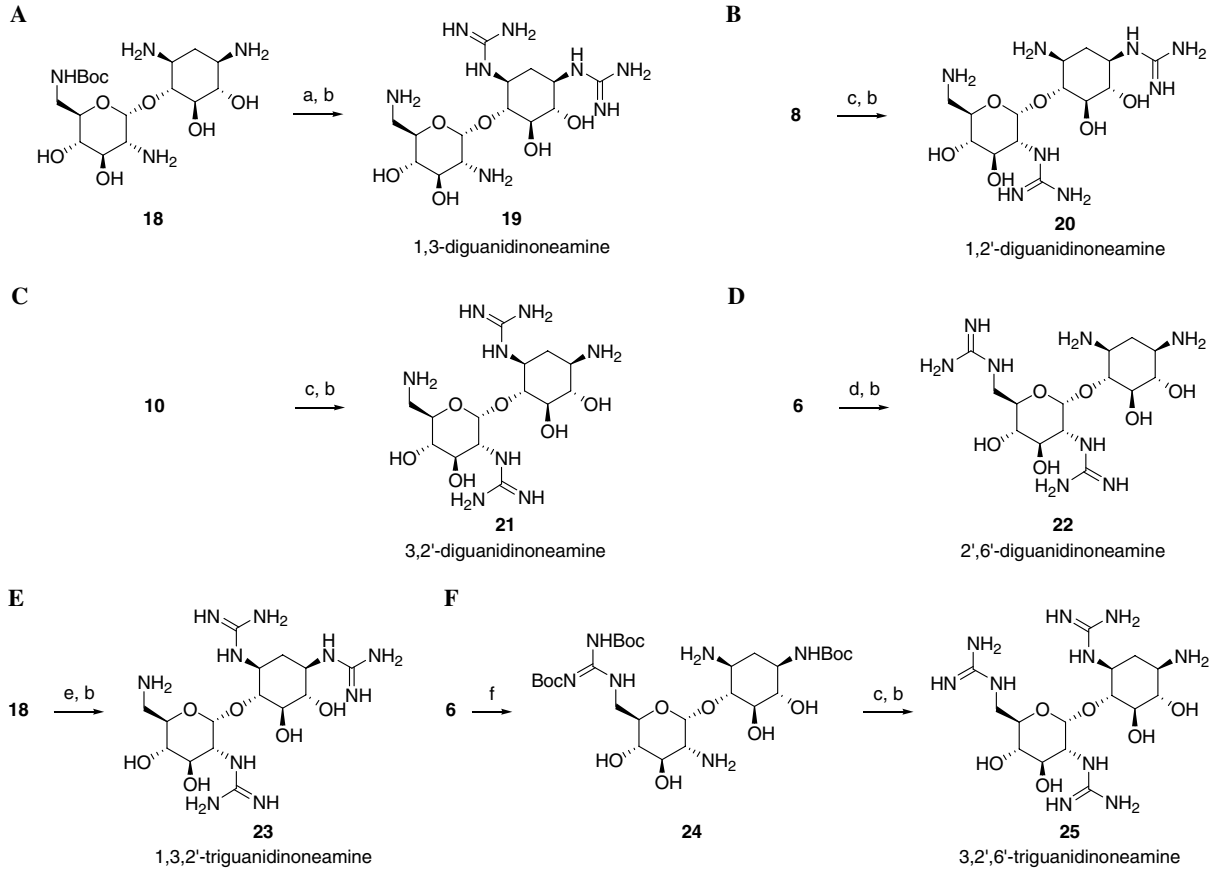


Scheme 3. Reagents and conditions: (a) 1.0 equiv of **5**, Et_3N, MeOH, rt; (b) TFA–DCM (1:1), rt.

A highlight of this work was the application of metal-chelation reactions, which have been used in selective Cbz- or Boc-protection of neamine,[42,43,47] to prepare 2′,6′-diguanidinoneamine **22** and 3,2′,6′-triguanidinoneamine **25**.[48] Guanidinylation in the presence of nickel acetate, which ties up the 1- and 3-positions,[47] resulted in 2′,6′-diguanidinoneamine **22** (Scheme 4D). The intermediate compound **24** was first prepared by using 1 equiv of di-*tert*-butyl-dicarbonate in the presence of zinc acetate, which blocks access of 3-position.[43] Guanidinylation of compound **24** with 3 equiv of **5** then gave 3,2′,6′-triguanidinoneamine **25** (Scheme 4F).

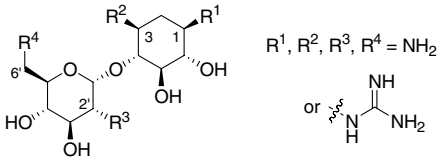
Once the mono-, di-, and tri-guanidinylated neamine derivatives were prepared, their inhibition of LF was evaluated using an in vitro biochemical assay.[33] The results are summarized in Table 1. In general, it was found that guanidinyl groups added to neamine enhanced the potency against LF, but there is no clear linear relationship between the potency and the number of guanidinyl groups. Surprisingly, 3,2′-diguanidinoneamine **21** and 1,3,2′-triguanidinoneamine **23** exhibited comparable potency to 1,3,2′,6′-tetraguanidinoneamine **4** in the sub-micromolar range, which indicates that two guanidinyl groups are sufficient for potent activity. The finding that the most potent derivatives bear guanidinyl groups on both the 3- and 2′-positions suggests that the spatial location of these groups on neamine is preferred for good inhibitory activity against LF.[49]

Finally, to explore the significance of the OH groups on the inhibition of LF activity by neamine, the



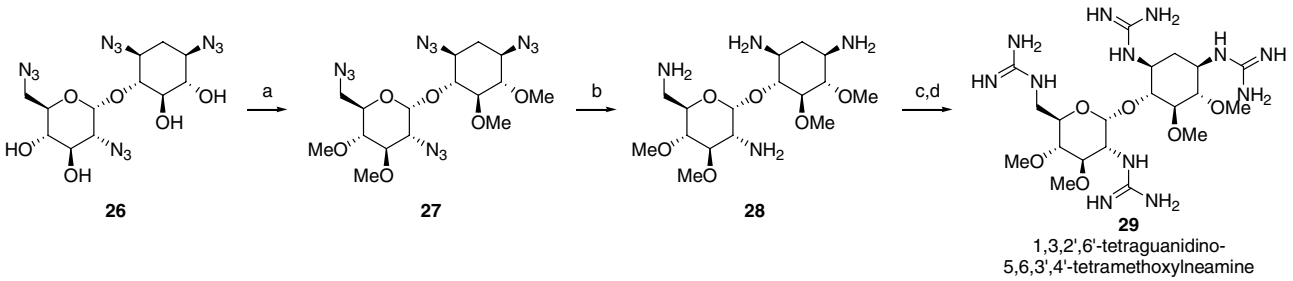
Scheme 4. Reagents and conditions: (a) 1.9 equiv of **5**, Et_3N, MeOH, rt; (b) TFA–DCM (1:1), rt; (c) 3 equiv of **5**, Et_3N, MeOH, rt; (d) 1 equiv of **5**, $Ni(OAc)_2$, MeOH, rt; (e) 4.5 equiv of **5**, Et_3N, MeOH, rt; (f) 1 equiv of Boc_2O, $Cu(OAc)_2$, MeOH, rt.

Table 1. Inhibition constant (K_i^{app}) values for guanidinylated neamine derivatives against lethal factor



Compound	Trivial name	No. of guanidinyl group	K_i^{app} (μM)[a]
1	Neomycin B	0	0.5 ± 0.1
2	Neamine	0	42.9 ± 6.3
4	1,3,2′,6′-Tetraguanidinoneamine	4	0.7 ± 0.1
9	1-Guanidinoneamine	1	5.0 ± 0.7
11	3-Guanidinoneamine	1	5.2 ± 0.7
13	2′-Guanidinoneamine	1	10.9 ± 1.5
7	6′-Guanidinoneamine	1	24.3 ± 0.6
19	1,3-Diguanidinoneamine	2	5.6 ± 0.7
20	1,2′-Diguanidinoneamine	2	5.3 ± 1.1
14	1,6′-Diguanidinoneamine	2	10.2 ± 3.2
21	3,2′-Diguanidinoneamine	2	0.7 ± 0.1
15	3,6′-Diguanidinoneamine	2	8.8 ± 2.4
22	2′,6′-Diguanidinoneamine	2	7.7 ± 1.1
23	1,3,2′-Triguanidinoneamine	3	0.5 ± 0.1
16	1,3,6′-Triguanidinoneamine	3	8.7 ± 1.0
17	1,2′,6′-Triguanidinoneamine	3	3.1 ± 0.8
25	3,2′,6′-Triguanidinoneamine	3	9.2 ± 1.1
29	1,3,2′,6′-Tetraguanidino-5,6,3′,4-tetramethoxylneamine	4	1.5 ± 0.2

[a] The values are means of three experiments.



Scheme 5. Reagents and conditions: (a) MeI, NaH, THF, rt; (b) H_2, Pd/C, MeOH, rt; (c) 6 equiv of **5**, Et_3N, MeOH, rt; (d) TFA-DCM (1:1), rt.

1,3,2′,6′-tetraguanidino-5,6,3′,4′-tetramethoxylneamine **29** was prepared from 1,3,2′,6′-tetraazidoneamine **26**[50] via methylation with methyl iodide, followed by reduction, guanidinylation, and deprotection (Scheme 5). It was found that the OH groups in the guanidinylated neamine derivatives had negligible influence on the potency (**29** vs. **4**, Table 1). This result implies that substitution or replacement of the OH groups might provide an opportunity to modify neamine as a lead structure to improve bioavailability and other pharmacological properties without loss of potency.

Mechanistic studies suggest that the guanidinylated neamine derivatives are mixed-type inhibitors of LF.[51] As illustrated in the double reciprocal Lineweaver–Burk plot of compound **21** (Fig. 2), the primary plot of inhibition gave straight lines with a point of intersection in the second quadrant. Both the slope and the vertical axis intercepts increased with increasing inhibitor concentration, indicating a mixed-type inhibition. It was also observed that the addition of NaCl (up to 40 mM) to the assay buffer solution resulted in dramatic increase of K_i^{app} values for these compounds (ca. 10-fold, data not shown), thus supporting the idea that the interaction between LF and the guanidinylated neamine derivatives is predominantly electrostatic. Since aminoglycosides have been shown to chelate to zinc,[43] we briefly investigated the effect of the nonspecific Zn-chelation on the inhibition of LF. As an example, compound **21** was assayed at various concentrations of $ZnCl_2$ to determine if the added zinc ions would compete with the catalytic zinc of LF for binding to the inhibitor. The results showed that addition of $ZnCl_2$ at concentrations below 16 μM does not significantly affect the K_i^{app} values,[52] indicating that the nonspecific Zn-chelation is unlikely to govern the inhibition of LF. We also conducted counterscreens of the most potent compounds **4**, **21**, **23**, and **29** against several relevant zinc-dependent metalloproteases such as MMP-1, MMP-3. MMP-9, MMP-12, and MMP-14. Very weak or no activity ($K_i^{app} > 300$ μM) was observed against these enzymes, suggesting that the guanidinylated neamine derivatives are selective inhibitors of LF.

In summary, a series of novel guanidinylated neamine derivatives have been synthesized via selective mono-, di-, and tri-guanidinylation of neamine. These molecules were shown to be potent, selective inhibitors of LF. Among them, 3,2′-diguanidinoneamine, 1,3,2′-triguanidinoneamine, and 1,3,2′,6′-tetraguanidinoneamine exhibited the most potent activities in the sub-micromolar range. Our work with this series demonstrates that multiple cationic groups are important for the inhibition of LF, and the full complex neomycin B structure is not needed for potency.

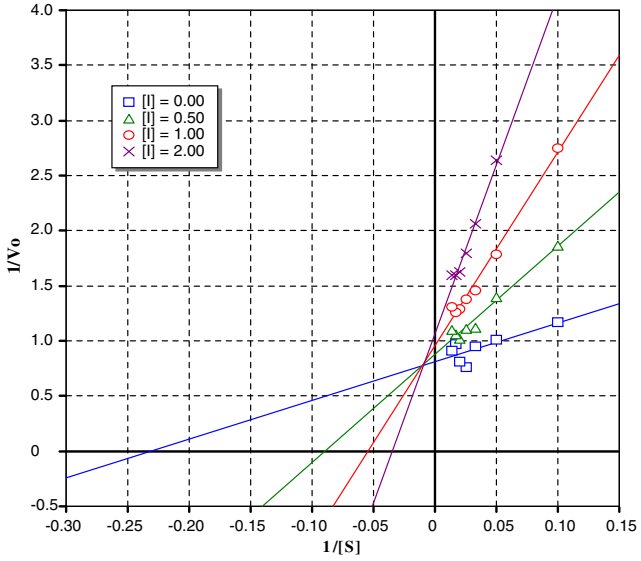


Figure 2. Lineweaver–Burk plot of compound **21**.

Acknowledgments

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33. 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, MAP-KKide™ (*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_i for the peptide substrate under these conditions are 8.6 ± 1.5 and 85 ± 17 μM, respectively. Hydrolysis has been confirmed by HPLC, and the data were presented at the Fifth International Conference on Anthrax, March 30, 2003 in Nice, France, in a poster titled 'Internally Quenched Fluorogenic Substrates for Anthrax Lethal Factor'.
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35. Similar finding has been reported by Wong et al. See Ref. 27.
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37. 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.
38. The guanidinyl group can be found in many drug structures. See: Baker, T. J.; Goodman, M. *Synthesis* **1999**, 1423.
39. Baker, T. J.; Rew, Y.; Goodman, M. *Pure Appl. Chem.* **2000**, *72*, 347.
40. The guanidinyl group has been also found to be crucial for the cell penetration of guanidinylated aminoglycosides. See: Luedtke, N. W.; Carmichael, P.; Tor, Y. *J. Am. Chem. Soc.* **2003**, *125*, 12374.
41. Grapsas, I.; Cho, Y. J.; Mobashery, S. *J. Org. Chem.* **1994**, *59*, 1918.
42. Compound **8** was prepared following the literature procedure. See: Grapsas, I.; Massova, I.; Mobashery, S. *Tetrahedron* **1998**, *54*, 7705.
43. Compound **10** was prepared following the literature procedure. See: Roestamadji, J.; Grapsas, I.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 11060.

44. In each reaction, the desired intermediate compound was isolated as the major product by silica gel column chromatography. Minor diguanidinoneamine derivatives and the starting material were also identified.
45. Compound **12** was prepared following the literature procedure. See Ref. 41.
46. Compound **18** was prepared following the literature procedure. See Ref. 41.
47. Kirst, H. A.; Truedell, B. A.; Toth, J. E. *Tetrahedron Lett.* **1981**, *22*, 295.
48. It is believed that, during the reaction process, intermediate metal chelates are formed between the proximal amino and hydroxyl groups of the aminoglycosides. As a result, the coordinated amino groups are less accessible than the other free ones for electrophilic reagents.
49. Compound **25** appears to be the exception to this statement.
50. Compound **26** was prepared following the literature procedure. See: Alper, P. B.; Hung, S.-C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029.
51. For a detailed study on mixed-type inhibition of LF by cationic molecules. See: Kuzmic, P.; Cregar, L.; Millis, S. Z.; Goldman, M. *FEBS J.* **2006**, *273*, 3054.
52. When the concentration of $ZnCl_2$ is below 16 μM, the effect on ionic strength by $ZnCl_2$ is negligible.