Identification of Novel Anthrax Lethal Factor Inhibitors Generated by Combinatorial Pictet–Spengler Reaction Followed by Screening in situ

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Anthrax lethal factor (LF) is a zinc-dependent metalloprotease produced by *Bacillus anthracis*, the causative agent of anthrax. LF and two other plasmid encoded proteins known as edema factor (EF) and protective antigen (PA) are responsible for the virulence of *Bacillus anthracis*.[1,2,3] The cause of LF and EF's toxicity necessitates that they translocate from the extracellular media into the cytosol of the host cell by PA.[4,5] The association LF+PA is known as lethal toxin (LTx). The injection of LTx into the bloodstream of animal models is lethal, whereas the association of EF+PA, known as edema toxin (ETx), is nonvirulent without LF.

When released into the bloodstream, PA binds to two extracellular receptors,[6,7] (Figure 1) the anthrax toxin receptor/tumor endothelial marker 8 (ATR/TEM8) and the capillary morphogenesis protein 2 (CGM2),[8] and becomes localized on the

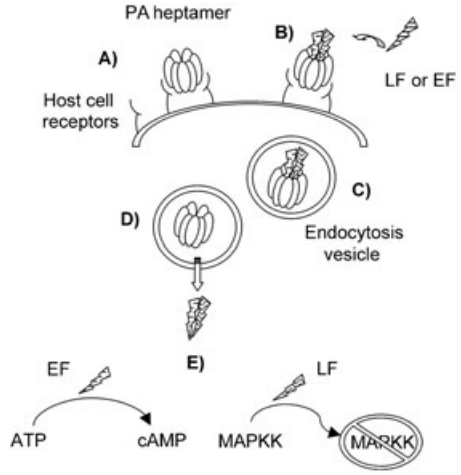

Figure 1. Infection of a macrophage by the anthrax toxins.

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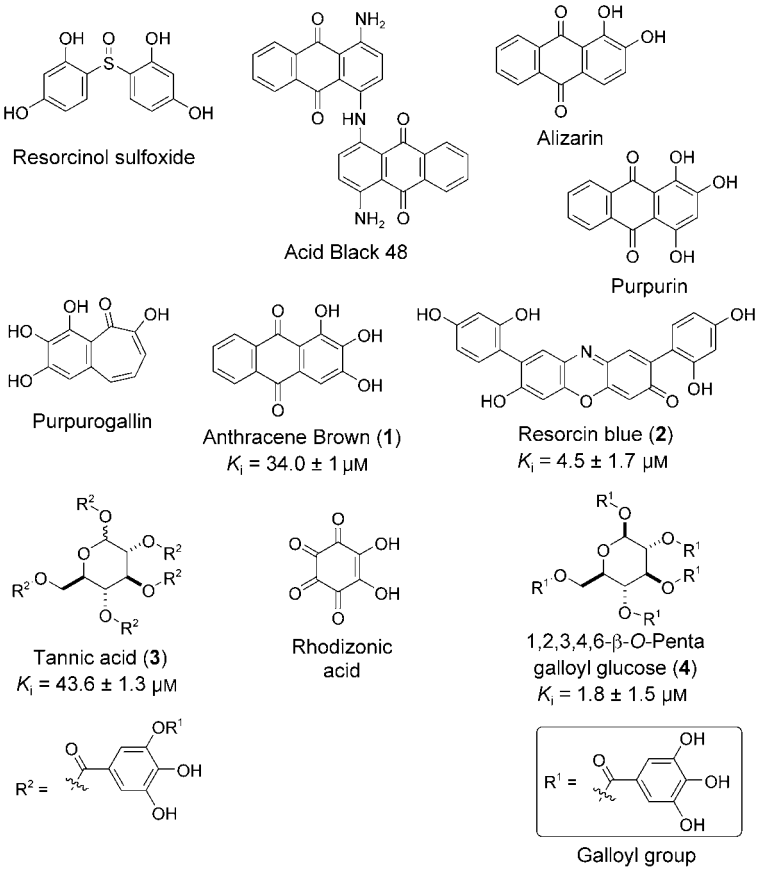
outer surface of the macrophage[9] cell membrane. There a furin-like protease, which is also present on the surface of the cell membrane, activates PA by cleaving its N-terminal 20 kD fragment.[10] The activated PA molecules oligomerize to form a heptamer with a channel-like structure (Figure 1 A), which is then able to bind to LF and EF (Figure 1 B). The ensemble composed of heptameric PA, LF, and EF is absorbed into the cell by endocytosis (Figure 1 C).[4] A pH change then signals PA to mediate the escape of LF and EF from the endocytosis vesicle to the cytosol (Figure 1 D). Once inside the cytosol (Figure 1 E), the presence of EF triggers an increase in the cAMP concentration, which leads to a flow of water into the cell and thus causes edema.[11] LF then cleaves the N-terminal fragment of mitogen-activated protein kinase kinase (MAPKK),[12,13,14] its only known natural substrate, which triggers a cascade of events that lead to the apoptosis of the host cell.[15,16]



Scheme 1. Commercially available compounds screened for activity against the anthrax lethal factor.

Inhalation anthrax is the most severe form of infection. It evolves rapidly to be systemic and leaves almost no chance of survival unless treated in the very early stage with antibiotics.[17] Antibiotics become ineffective and the infection unstoppable once the quantity of LTx produced in the host body reaches a critical threshold. Cutaneous and digestive infections are usually localized but can spread with the same consequences as inhalation anthrax, if left untreated.

An efficient treatment of the *Bacillus anthracis* infection in the late stage requires blocking the activity of LTx, and more specifically that of LF.

The identification of potent inhibitors against LF and the understanding of its unusual mode of action is therefore of primary interest and it has been the focus of several studies. Various structures have been reported to inhibit the activity of LF[18–23] and to prolong the survival of cell cultures or animal models.

A recent report that galloyl (3,4,5-trihydroxy-benzoyl, see Scheme 1 for structure) derivatives extracted from green tea were noncompetitive inhibitors of LF[21] prompted our interest in identifying more potent LF inhibitors that possess a similar polyphenolic motif. We first screened ten commercially available compounds (Scheme 1) and confirmed the importance of the gallate motif for the inhibition of LF (compounds **1**, **3**, and **4**), but also showed that the potency of polyphenols is not restricted to an exact match with the galloyl motif (**2**). Surprisingly, purpurin and purpurogallin did not inhibit LF activity, even though they exhibit very strong structural similarity with **1** and the galloyl group.

Anthracene brown (**1**) and resorcin blue (**2**) are pigments. Tannic acid (**3**) and 1,2,3,4,6-β-*O*-Pentagalloyl glucose (PGG, **4**) are botanical extracts belonging to the tannin family. PGG is a known component of traditional Chinese herbal medicines used to treat various conditions.[24] This interesting compound has been found to inhibit the activity of a wide variety of enzymes such as nitric oxide synthase, cyclooxygenase,[25] and angiotensin converting enzyme.[26] Furthermore, a recent report mentioned that PGG can block the entry of the SARS-CoV into the host cells.[27] PGG has also been reported to have anticancer[28] and anti-inflammatory[29] activities. The high potency of LF inhibitors built around a carbohydrate scaffold is very interesting because a large number of carbohydrate derivatives could be screened for better inhibitors.

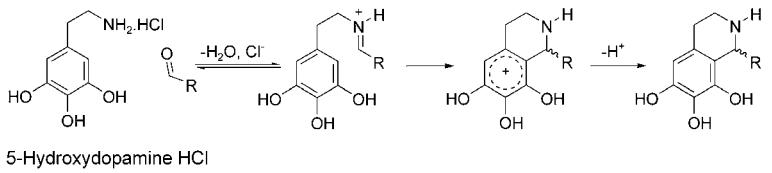
Based on these results we prepared a library of gallate-like tetrahydroisoquinoline polyphenols by diversification of the 5-hydroxydopamine core by using the Pictet–Spengler reaction[30] (Scheme 2). The cyclization occurs between the electron-rich aromatic cycle and the iminium which is formed under acidic aqueous conditions. The commercially available 5-hydroxydopamine core was chosen because it reproduces the 3,4,5-trihydroxy phenyl motif present in the galloyl group.

The reactions were carried out in Eppendorf vials, monitored by LC-MS, and the products were tested for inhibition activity

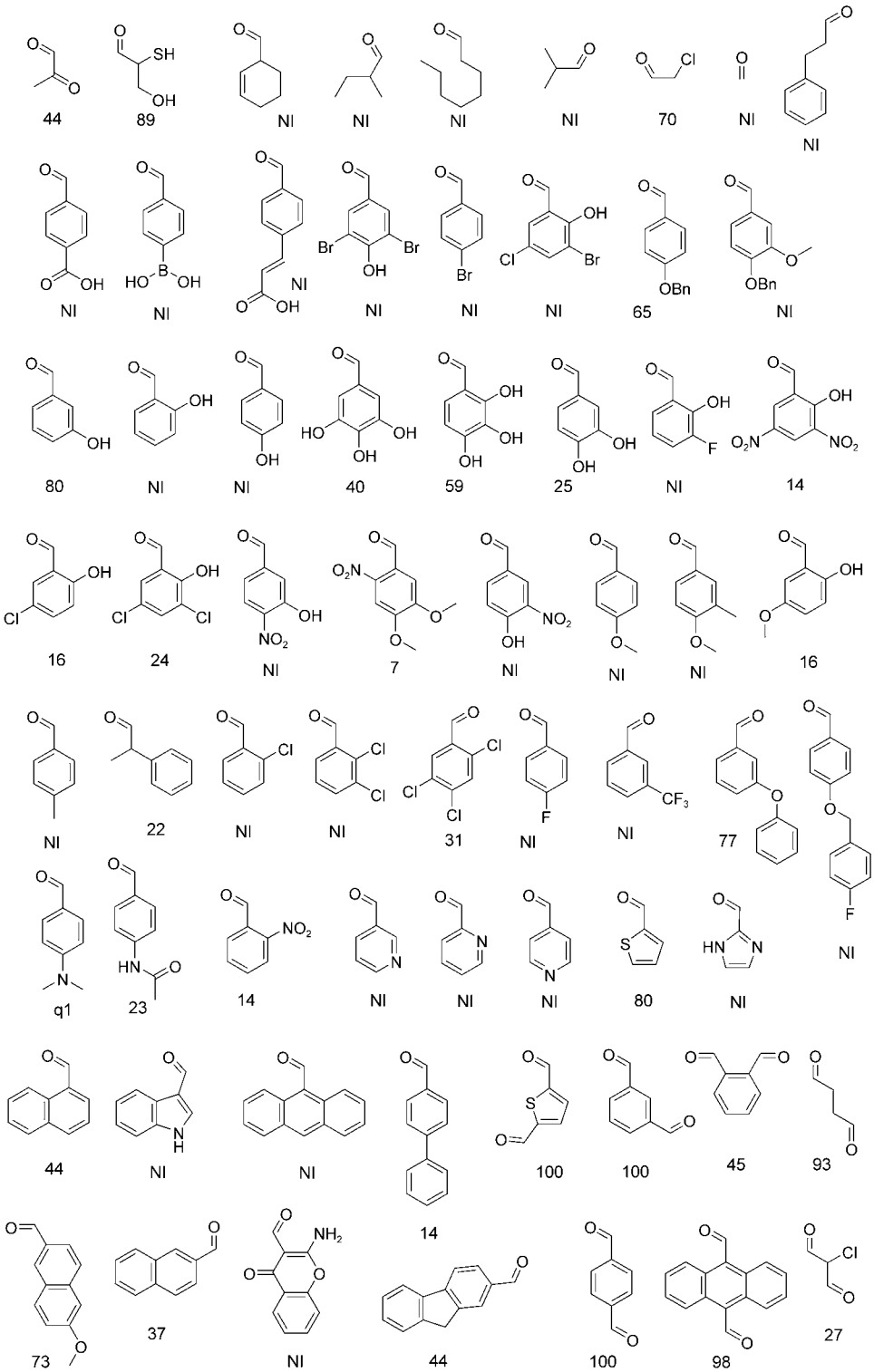
without purification. Each vial was diluted to the desired concentration and used "as is" for the assay. The dilution could be performed by using water only or a water and DMSO mixture—provided that the amount of DMSO does not exceed 0.05% in volume of the final assay solution; the maximum concentration of DMSO under which it has no influence on LF activity is 1% of the final volume. The possibility of false positives was ruled out by monitoring the assays by using LC-MS.

Fifty-nine aldehydes and seven dialdehydes were used. Their structures and the percentage inhibition of the crude products obtained from their reaction with 5-hydroxydopamine are shown in Scheme 3. We observed an overall better inhibition of enzyme activity with library products that were obtained from the reaction with the dialdehydes.

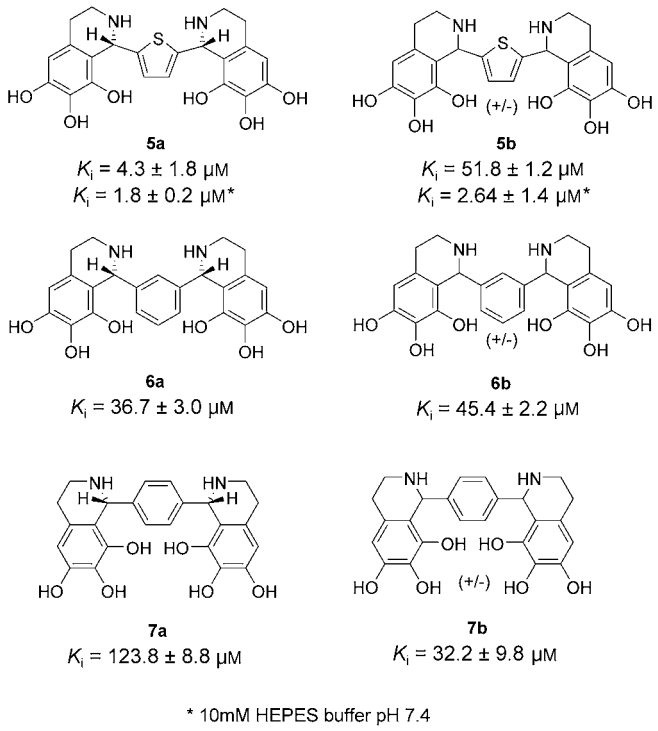
Six compounds (**5a**, **5b**, **6a**, **6b**, **7a**, and **7b**) were purified by preparative HPLC and their kinetic properties were evaluated by using an assay with physiological salt concentration (150 mM; Scheme 4). All compounds are noncompetitive inhibitors of LF. The potency of these compounds is dependent on the ionic strength of the assay condition. They generally display higher inhibition activity of LF under low ionic strength, probably because the electrostatic interactions between the inhibitor and the enzyme are favored under these conditions. For each reaction involving a dialdehyde, the NMR spectroscopy data of the *meso* fraction (fraction **a**) is similar to that from the racemate fraction (fraction **b**). The crystallization of the *meso* products **6a** and **7a** by methanol evaporation provided crystals suitable for X-ray analysis which allowed the unambiguous identification of the fractions.[31]



Scheme 2. Mechanism of the Pictet–Spengler reaction.



Scheme 3. Aldehydes and the percentage of inhibition of their products (NI = no inhibition).



Scheme 4. Structures and K_i of the most potent compounds from the library.

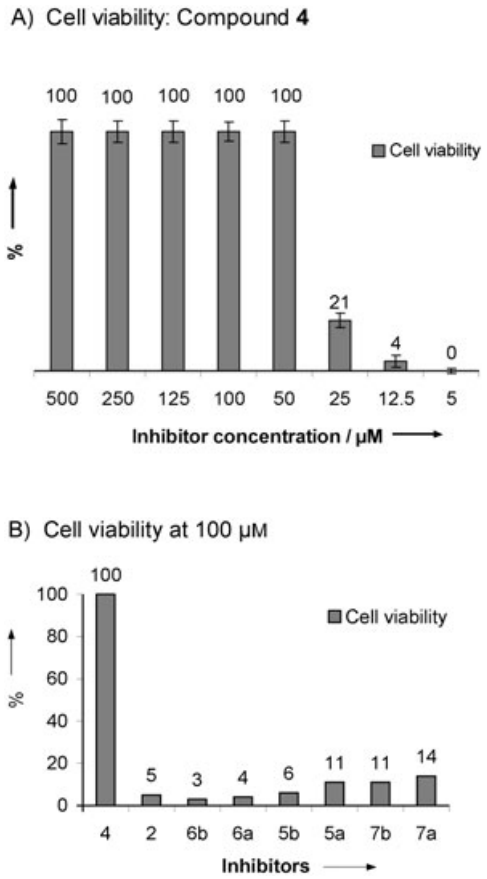


Figure 2. Cell viability assays. A) Compound **4,** B) Comparative assay.

Chiral derivatization led to the identification of **5a** and **5b**. In order to evaluate and compare the potency of the pure enantiomers the chiral resolution of each racemic mixture is one of our future objectives.

Besides inhibiting the enzyme in vitro under high-salt concentration assay, compounds **2**, **4**, **5a**, and **5b, 6b, 7a**, and **7b** provided variable degrees of in vivo cell protection (Figure 2). Compound **4**, which is the most potent in vitro inhibitor, provided the best cell protection (Figure 2A). Compounds **2**, **5a**, **6b**, **7a**, and **7b** provided moderate and similar cell protection (Figure 2B) which does not reflect their in vitro potency.

Green-tea extracts and PGG are known for their instability[32] in the presence of oxygen. The stability of the tetrahydroisoquinolines **5a**, **5b**, **6a**, **6b**, **7a**, and **7b** which were isolated from the library, was monitored by LC-MS. In the assay conditions signs of oxidation and dimerization are observed slowly but steadily after 5 h at room temperature and upon exposure to air. In deionized water, signs of decomposition are observed after two days. In aqueous acidic-media the compounds are apparently fully stable after several days. Interestingly, an aqueous solution of PGG remains unlimitedly potent in the presence of air, which contrasts with the rapid loss of activity of the green-tea extracts under the same conditions.

In conclusion, we screened ten commercially available compounds that bear a polyphenol motif and identified four inhibitors against the anthrax lethal factor. One of them (Compound **4**; K_i 1.8 μM) is very potent under physiological salt concentration and is an encouraging result for the further generation of a carbohydrate-based library of LF inhibitors. We also synthesized a library of tetrahydroisoquinolines by diversifying the 5-hydroxydopamine core by using the Pictet–Spengler reaction, which provided six hits. One of them (**5a**) is a very potent noncompetitive inhibitor and exhibits a K_i at 4.3 μM under physiological salt concentration. This reaction seems to be a good basis for the rapid generation of a large number of LF inhibitors under convenient conditions. However, as for other polyphenols of biological interest the products are reactive in the presence of O_2. The number of derivatives accessible through the Pictet–Spengler reaction is considerable and should not be restricted to simple nonchiral aldehydes. Derivatives of tetrahydroisoquinolines, such as dihydroisoquinolines and isoquinolines, are also interesting targets for the identification of new potent LF inhibitors. The actual potencies of the enantiomers are still unknown. Our current objectives are to synthesize a second-generation library in order to identify new inhibitors with improved activity, to perform structure-activity relationship studies, optimize the hits, purify each hit in pure enantiomeric form, localize the exosite(s) involved, and to screen this library against other types of enzyme.

Experimental Section

General procedure for the library: A solution of 5-hydroxydopamine hydrochloride (125 mg; 0.61 mmol) in TFA-water (2.5 mL; 1:1) was distributed between 50 Eppendorf tubes (50 μl). A 1.1 equiv of mono-aldehyde, or 0.5 equiv of dialdehyde was added to each. The Eppendorf tubes were heated at 90 °C for 10 h. They were

then cooled to RT and diluted with water in order to give fifty 10 mM samples.

General procedure for the pure compounds 6a and 6b: A solution of 5-hydroxydopamine hydrochloride (50 mg; 0.24 mmol) in TFA-water (1 mL; 1:1) was heated at 90 °C for 10 h in the presence of isophtalaldehyde (17 mg; 0.12 mmol; 0.5 equiv). The solvent was removed in vacuo, the crude product was dissolved in water (1 mL, HPLC grade, 0.1 % of TFA *v/v*), and then purified by preparative HPLC. Two main fractions corresponding to 1021 (**6a** 2TFA; 31 mg, 38 %) and 1022 (**6b** 2TFA; 31 mg, 38 %) were isolated.

Condition of separation: Gradient was carried out from acetonitrile/water (5:95) to acetonitrile/water (10:90), over 35 min at 6 mL min^{-1}. **6b** time retention 15 min, **6a** time retention 18 min.

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Keywords: anthrax · carbohydrates · combinatorial chemistry · inhibitors · Pictet–Spengler

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