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Identification of a Potent Botulinum Neurotoxin A Protease Inhibitor Using in Situ Lead Identification Chemistry

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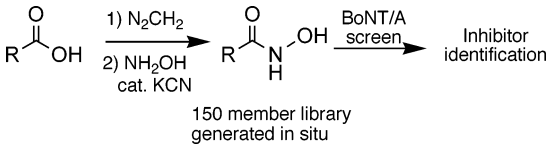
ABSTRACT



Botulinum neurotoxins (BoNTs), etiological agents of the deadly food poisoning disease botulism, are the most toxic proteins currently known. By using in situ lead identification chemistry, we have uncovered the first class of inhibitors that displays nanomolar potency. From a 15 μM lead compound, structure-activity relationship studies were performed granting the most potent BoNT/A inhibitor reported to date that displays an inhibition constant of 300 nM.

Botulinum neurotoxin (BoNT), an agent responsible for the deadly food poisoning disease botulism and a dreaded biological weapon, is one of the most toxic proteins currently known (~100 billion times more toxic than cyanide).[1] *Clostridium botulinum* is classified into seven strains (A−G) each of which can cause flaccid muscle paralysis and subsequent death by blocking the release of a neurotransmitter, acetylcholine, at neuromuscular junctions.[2] Structurally, BoNT consists of three functional domains, catalytic, translocation, and binding; BoNT toxicity results from the catalytic activity of its light chain, a Zn(II) endopeptidase.

The catalytic domain of BoNT is a compact globule consisting of a mixture of α-helices, β-sheets, and strands with a gorgelike zinc-containing metalloprotease active site (15−20 Å deep depending on serotype).[3] The metalloprotease activity is responsible for BoNT's neurotoxicity through the hydrolytic cleavage of one of three SNARE (soluble NSF-attachment protein receptor) proteins that are involved in the neuronal synaptic vesicle function. Moreover, the hydrolytic cleavage sites of these SNARE proteins (SNAP-25, VAMP, Sb-1) differ across the BoNT serotypes; however, any degradation of these SNARE proteins disables the exocytosis of acetylcholine, resulting in paralysis and potentially death.[2] Current therapy for BoNT intoxication involves "passive immunization" with equine antitoxin.[4] Unfortunately, treatment must start shortly after intoxication, and several safety concerns exist[2] over the use of antitoxins in the general population.[5] Therefore, inhibition of the catalytic light chain protease with a small molecule inhibitor may provide an attractive approach to counter the effects of botulism poisoning.

BoNT serotype A (BoNT/A) is the most toxic form of BoNTs and is considered the most threatening for biological attacks due to a prolonged half-life in vivo and ease of its production.[4] Although there are reports of success treating BoNT/A toxicity with multiple monoclonal antibodies[6] as

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antitoxins, this is of limited therapeutic utility because the antibodies must be administered prior to, or shortly after, toxin exposure (<12 h).

Presently, there are only modest small molecule, nonpeptidic, protease inhibitors for BoNT/A with IC_{50} values in the range of >20 μM.[7] We established a high-throughput screen for the identification of inhibitors of BoNT/A LC protease.[8] Using this screen, we have analyzed a library of hydroxamate-based compounds generated using in situ chemistry to reveal the lead structure **1** (Figure 1). Herein, we report on

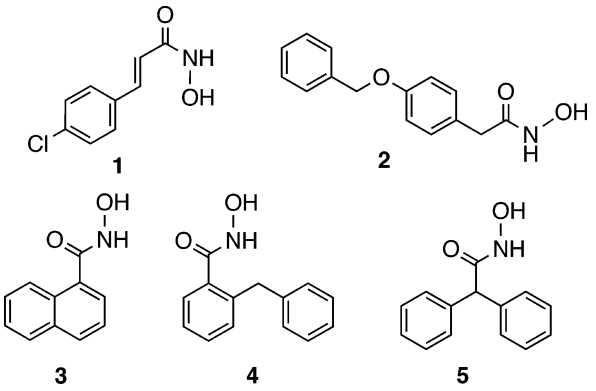


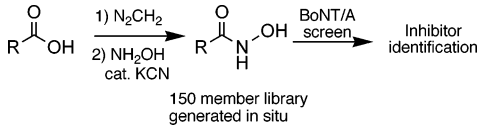
Figure 1. Structures of lead compounds identified from in situ screening.

the synthesis and structure-activity relationship studies of these BoNT/A inhibitors.

Recently, a convenient method for the preparation of hydroxamates from readily available esters was reported.[9] In this general procedure, a diverse array of acids can easily be converted to hydroxamates with hydroxylamine in the presence of a catalytic amount of potassium cyanide. In addition, several reports have been disclosed for the preparation of hydroxamates;[10] however, these procedures have strict substrate requirements.

With these thoughts in mind, we set out to generate a library of diverse hydroxamates from a readily available carboxylic acid library. Namely, 150 carboxylic acids purchased from Aldrich Chemical Co. were randomly chosen and converted to their ester by treatment with diazomethane (Scheme 1). After removal of the solvent, reactions were

Scheme 1. Synthesis and Screening of the in Situ Hydroxamate Library



subjected to a mixture of THF:MeOH:50% aqueous $HONH_2$ 2:2:1 with a catalytic amount of KCN overnight. Again, the solvent was removed and the crude products were reconstituted with DMSO to prepare stock solutions of a final concentration of 10 mM for screening. To check the quality of the library, 30 compounds were randomly selected and analyzed by ES-MS, and in all cases, the expected masses corresponding to the products were found (data not shown).

Using a high-throughput screen,[8] namely, a 13 amino acid substrate fluorescence resonance energy transfer (FRET) assay developed in our laboratory, we analyzed the library of hydroxamates at a concentration of 50 μM. Any inhibitors found to display 50% inhibition were considered "hits" and evaluated further. From the initial screen, five compounds were found to give 50% or more inhibition in the FRET-based assay. These compounds were resynthesized, purified, and validated using the FRET-based assay. Of the five lead compounds, only compound **1** showed potent activity; *para*-chloro-cinnamic hydroxamate (**1**) displayed an IC_{50} value of 15 μM, and thus, this simple high-throughput screen uncovered an interesting lead for further development.

To refine this positive lead, we synthesized a series of 12 compounds so as to further explore the structure-activity relationship of compound **1**. However, the method outlined in Scheme 1 was found to be inefficient for the isolation of the desired compounds because of purification difficulties. Therefore, we sought out a more convenient method for the expeditious generation and purification of the hydroxamate compounds. Solid-phase organic synthesis (SPOS) lends itself to these requirements nicely due to reactions proceeding in high yield and purification being significantly simplified. Several groups have demonstrated successful hydroxamate syntheses using solid-phase routes[11] and hydroxyamine resins.[12] However, we found these procedures to be insufficient for our purposes. 2-Chlorotrityl resin was purchased

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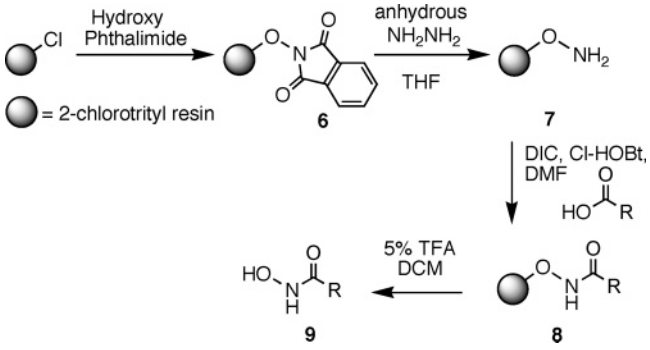
from Chemical and Biopharmaceutical Laboratories of Patras S. A. (CBL) and used in the synthesis of hydroxamate inhibitors as outlined in Scheme 2. It is important to note

Scheme 2. Solid-Phase Synthesis of Hydroxamic Acids



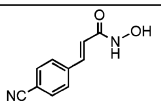
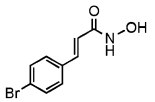
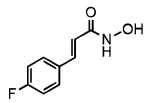
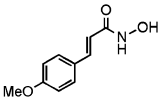
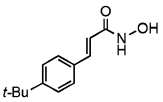
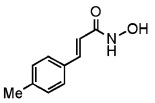
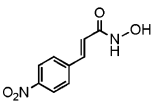
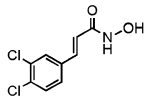
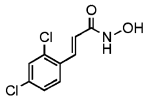
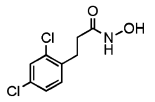
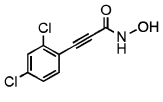
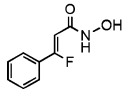
that several resins were explored using this methodology; however, the CBL was the only resin found to give satisfactory results. This resin was treated with *N*-hydroxyphthalimide for 2 days in DMF at room temperature for the quantitative conversion to **6.** The phthalimide was successfully removed with exposure to anhydrous hydrazine to give the hydroxylamine resin **7**, ready for the coupling of a variety of acids. Next, the desired carboxylic acids were coupled to the resin **7** using standard diisopropylcarbodiimide and 6-chlorohydroxybenzotriazole protocols. The desired hydroxamates were recovered by treatment of the resin with 5% TFA and removal of the solvent.

These compounds were further individually purified via silica gel and then reconstituted in DMSO for evaluation of BoNT/A LC inhibition.

To investigate the structure-activity relationship of compound **1**, we first synthesized a group of para-substituted cinnamic hydroxamates. Unexpectedly, the chloro substitution in the para position appears to be highly conserved as seen in Table 1, compounds **10**−**16**. For example, when a bulky functionality such as a *tert*-butyl, compound **14**, is introduced to the para position, inhibition is completely lost. Moreover, inhibition is compromised when introducing electron-withdrawing or donating groups (compounds **10**−**16**). Thus, we next examined other possible chloro substitution patterns of the lead compound **1**. Gratifyingly, when evaluating different chloro substitution patterns (compounds **17** and **18**), the *ortho-para*-cinnamic hydroxamate, **18**, displayed an IC_{50} value of 0.41 μM. Furthermore, it appears that the *trans*-olefin motif is also conserved as seen in compounds **19**−**21**. It is important to note that the *cis*-olefin form of **1** was not examined because of instability in solution.[13] However, strikingly, when the *trans*-olefin was substituted for an alkyne, **20**, inhibition was attenuated by greater than 175-fold. The reduced form of **18** to the alkane, compound **19**, also reduced potency significantly.

(13) *cis*-2,4,Dichloro-cinnamic acid was found to be unstable during coupling.

Table 1. IC_{50} Values[a]

compound	structure	IC_{50} μM
10		61 ± 4
11		40 ± 5
12		54 ± 8
13		79 ± 8
14		>100
15		87 ± 6
16		72 ± 5
17		75 ± 4
18		0.41 ± 0.03
19		3.1 ± 0.9
20		73 ± 5
21		>100

[a] Assays were conducted at various inhibitor concentrations at 22.5 °C, pH 7.4 in 40 mM HEPES 0.01% (W/V) Tween 20, 5 μM SNAPtide substrate, and 200 nM enzyme.

To further kinetically characterize **18**, we evaluated it in an assay system with the native SNAP-25 (141-206) substrate using HPLC for analysis. It is important to note that SNAP-25 (141-206) has no structural modification, thus the structural integrity of the molecule has not been compro-

mised. Using this substrate, a K_i of 300 ± 12 nM was found and also displayed the expected competitive mode of inhibition (data not shown). Moreover, these findings demonstrate that a 10 contiguous carbon chain previously thought to be a strict requirement for BoNT/A LC inhibition[14] is in fact not a prerequisite and small organic molecules can be used for possible drug leads.

In total, a simple in situ synthesis and screen was developed for the identification of nonpeptidic protease inhibitors of BoNT/A LC. The power of using this strategy was that no prior bias was placed on the selection of input acids, yet a desirable lead was uncovered. We note that compound **18** (Table 1) is the most potent protease inhibitor described to date for BoNT/A and is currently being investigated in cell and mouse assays for antibotulism effects and will be reported in due course.

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Note Added after ASAP Publication. A 13 amino acid substrate FRET assay was used, not a 17 amino acid substrate as indicated in the version published ASAP March 17, 2006; the corrected version was published ASAP March 23, 2006.

Supporting Information Available: Experimental synthetic procedures and NMR and HRMS data for the library members and intermediates and screening procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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