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Chirality Holds the Key for Potent Inhibition of the Botulinum Neurotoxin Serotype A Protease

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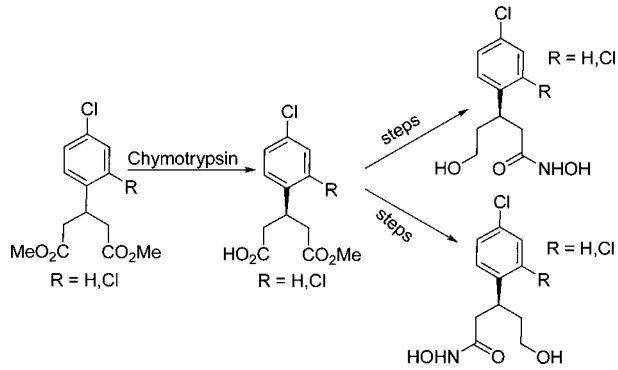
ABSTRACT



Botulinum neurotoxin serotype A (BoNT/A) is the most toxic protein known to man and also a bioterrorism agent. As defined by our previous research targeting the etiological agent responsible for BoNT/A intoxication, a protease, we now report on the asymmetric synthesis of four new BoNT/A inhibitors; the most potent of this series is roughly 2-fold more active than the best small molecule inhibitor currently known.

The seven serotypes (A−G) of botulinum neurotoxins (BoNTs) are proteins produced by bacteria of the genus *Clostridium*.[1] BoNTs are synthesized as ∼150-kDa proteins consisting of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) linked by disulfide bonds.[2] The HC is responsible for translocation and binding, while the LC is a zinc-dependent endopeptidase that cleaves soluble *N*-ethylmaleimide-sensitive fusion proteins (SNARE) located at nerve endings.[3] Cleavage of SNARE proteins by the BoNT LC leads to flaccid paralysis and subsequent death due to

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inhibition of the release of acetylcholine from synaptic terminals.[4]

Current treatment for BoNT intoxication employs an antitoxin to sequester the toxin followed by removal of the complex from the body. However, this treatment option is dependent upon prompt diagnosis of intoxication and must be administered rapidly before the toxin can enter the cell.[5] The antitoxin also suffers from the risk of allergic reaction,[6] long-term effects of unknown origin,[7] and insufficient available quantities for treatment of a large-scale attack.[8]

BoNT serotype A (BoNT/A) is considered to be the most deadly BoNT serotype based on the following: First, and most importantly, BoNT/A has an extremely long duration of action, i.e., weeks to months. Second, there is no antidote for BoNT/A intoxication, with severe intoxication cases requiring mechanical ventilation due to respiratory paralysis.[9] Third, BoNT/A, together with serotype B, is responsible for ~1000 cases of human BoNT poisoning per year.[1] Finally, BoNT/A has a high potential for bioterrorism use due to its extreme toxicity and ease of dissemination through the food or water supply. Thus, the inhibition of BoNT/A LC is an attractive target for nonpeptidic small molecules that can target the enzyme within an intoxicated cell.

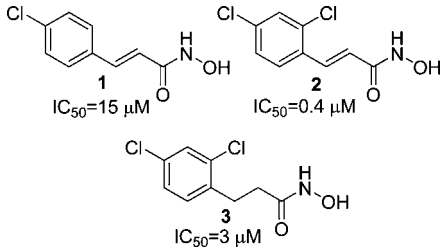
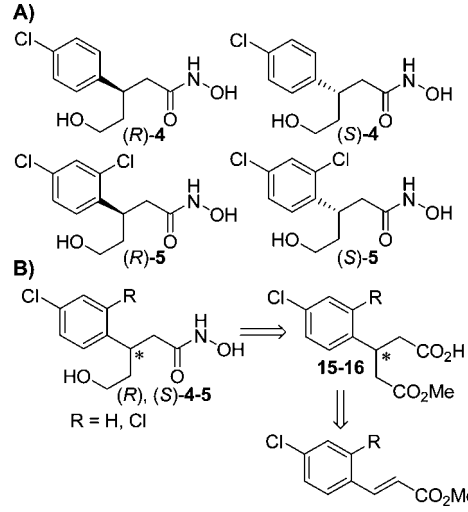


Figure 1. Structure and IC_{50} values of previously identified BoNT/A inhibitors.

Previous research from one of our laboratories screened a diverse library of small molecules and discovered several inhibitors of BoNT/A, with *trans*-cinnamic hydroxamate **1** being our initial lead (Figure 1). Hydroxamate **2** stemmed from structure−activity studies, and is one of the most potent inhibitors known, having a K_i value of 300 ± 12 nM.[10] From these studies several lessons were learned, including the following: (1) all structures required the hydroxamate functionality, a monoanionic bidentate chelator of zinc that has been previously used for the inhibition of metalloproteinases,[11] (2) a *para*-halogenated phenyl ring was needed,[12] and (3) an *ortho*,*para*-dihalogenated ring was superior to *para*-substition alone[10] (Figure 1). Indeed, X-ray crystallographic analysis of the bound inhibitors revealed hydroxyl coordination of the hydroxamate to the Zn(II) ion in the active site in addition to phenyl ring interaction with a hydrophobic portion of the enzyme.[2] Addition of the *ortho* chlorine substituent created an additional favorable interaction with an active site arginine, making this the most potent inhibitor reported to date.[2]

While our initial research has been critical to lending credence that small nonpeptidic molecules can be tailored to the BoNT/A protease, it is readily apparent that more potent inhibitors will be required if a therapeutic is the final goal. As such, we sought to synthesize a series of molecules containing additional chemical diversity for potential interaction(s) with the enzyme active site. We envisioned the preparation of hydroxy ethyl hydroxamates (*R*)- and (*S*)-**4** and -**5**, which would not only extend within polar space of the enzyme's combining site but also explore the importance of molecular chirality and its impact on enzyme inhibition.

Scheme 1. (A) Structures of Hydroxamates (*R*)- and (*S*)-**4** and -**5**; (B) Retrosynthetic Analysis



We viewed these structures as rational choices based on active-site molecular modeling wherein a key goal was to displace a nacent water molecule within the enzyme active site, which on the basis of previous protease inhibitors targeting active site water molecules could improve K_i up to 100-fold.[13] To access such structures required an asymmetric synthetic strategy that would construct both enantiomers of the final hydroxamate products from a single, readily

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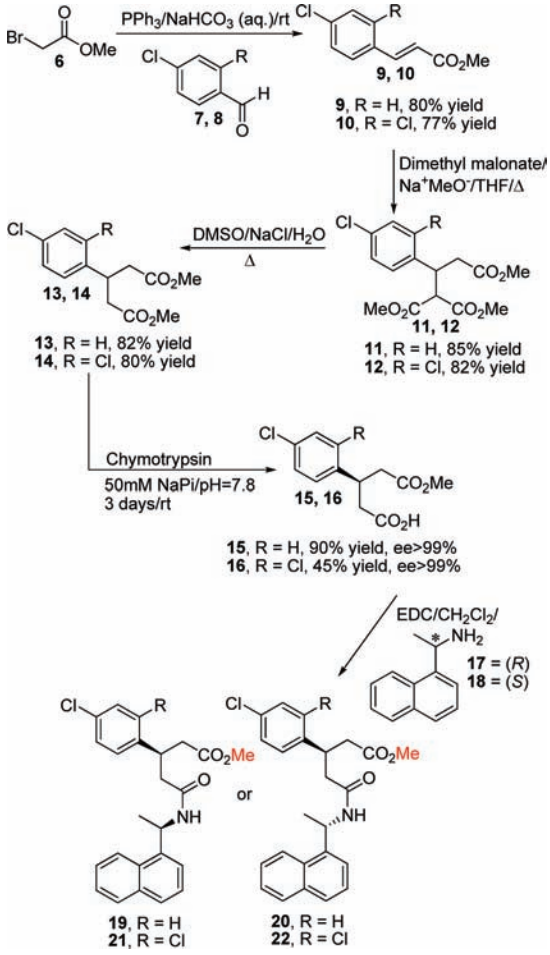
available, starting material (Scheme 1). Herein, we report the successful asymmetric synthesis and inhibitory evaluation of hydroxamates (*R*)- and (*S*)-**4** and -**5** with BoNT/A protease.

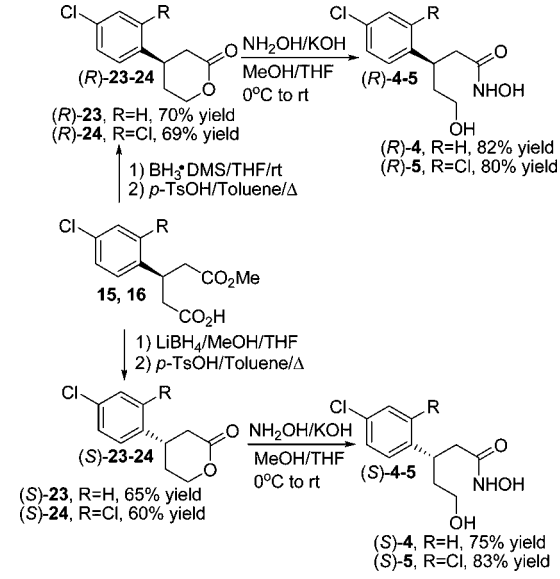
Scheme 2. Synthesis of Acids **15** and **16** and Derivitization to Determine ee and Stereochemistry



Our envisioned hydroxamate synthesis involved ring-opening of the corresponding lactones (*R*)- and (*S*)-**23** and -**24**, which could be independently synthesized from acid/esters **15** and **16** via chemoselective lactonization.[14] Acid/ester **15** was previously synthesized in high enantiomeric excess (>98%) and yield (85%) by chymotrypsin-mediated enantioselective hydrolysis of diester **13**,[15] and we reasoned that the previously unreported hydrolysis of diester **14** using a similar chemoenzymatic approach could be successful due to the similar nature of the two structures. We planned to synthesize diesters **13** and **14** from readily available starting materials as shown in Scheme 2.

Thus, our synthesis of diesters **13** and **14** started with the synthesis of alkenes **9** and **10** using the aqueous Wittig reaction of methyl bromoacetate with the appropriate aldehyde[16] followed by Michael-type addition of dimethyl malonate to yield triesters **11** and **12** in good yield.[15] Selective triester decarboxylation[17] yielded diesters **13** and **14**, the substrates for enzymatic hydrolysis. Diester **13** was hydrolyzed with chymotrypsin to give (*R*) acid/ester **15** with high enantioselectivity and chemical yield. Hydrolysis of *o*-,*p*-dichloro diester **14** using chymotrypsin also proceeded with high enantioselectivity (>99%) and respectable yield (45%) to give (*R*) acid/ester **16**. The enantioselectivity and absolute stereochemistry of these hydrolytic enzymatic resolutions was determined by acid derivitization using (*R*)- and (*S*)-1-(1-naphthyl)ethylamine followed by comparison of the difference in chemical shift of the diastereomeric methoxy ester protons **19** and **20** and **21** and **22** according to the procedure of Hoye et al. (Scheme 2).[18]

Scheme 3. Synthesis of Hydroxamate Enantiomers (*R*)- and (*S*)-**4** and -**5**



With acids **15** and **16** in hand, we sought to prepare both enantiomers of lactones **23** and **24** via chemoselective lactonization.[14] Thus, ester reduction with $LiBH_4$ followed by cyclization under acidic conditions yielded (*S*) lactones **23** and **24**. Conversely, (*R*) lactones **23** and **24** were prepared by carboxylic acid reduction with BH_3·DMS followed by

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lactonization under acidic conditions. The lactones were then smoothly converted to hydroxamates (*R*)- and (*S*)-**4** and -**5** employing hydroxylamine under basic conditions[12] (Scheme 3).

Table 1. IC_{50} and K_i Values for Hydroxamates (*R*)- and (*S*)-**4** and -**5**

compd	IC_{50} (μM)	K_I(μM)
(*S*)-**4**	36	n.d.
(*R*)-**4**	8	1.7 ± 0.3
(*S*)-**5**	21	n.d.
(*R*)-**5**	1	0.16 ± 0.02

Assays were conducted at 22.5°C, pH 7.4 in 40 mM HEPES buffer at 10 μM inhibitor concentration, 0.075 nM enzyme (BoNT/A) concentration using SNAP-25 (66-mer) substrate. An entry of n.d. denotes value was not determined.

All hydroxamates were screened for activity against BoNT/A using our LC/MS-based assay that employs a truncated version of SNAP-25, the SNARE protein cleaved by the BoNT/A LC.[19] Our results clearly show the importance of chirality; in both cases, the (*R*) hydroxamate IC_{50} was less than the (*S*) hydroxamate. The K_i of (*R*) *p*-chloro and *o*-,*p*-dichloro hydroxamates (*R*)-**4** and (*R*)-**5** was determined to be 1.7 ± 0.3 and 0.16 ± 0.02 μM, respectively (Table 1), with inhibition being competitive in nature (data not shown). Importantly, hydroxamate (*R*)-**5** is roughly 2-fold more effective than the best small molecule nonpeptidic BoNT/A inhibitor known. We did not determine K_i values for hydroxamates (*S*)-**4** and -**5** since they were inferior inhibitors as shown by IC_{50} values.

In summary, a concise asymmetric synthesis of four new inhibitors of the highly toxic BoNT/A LC has been devised. As discovered previously, the *o*-,*p*-dichloro hydroxamates are more potent inhibitors than the simple *p*-chloro hydroxamates. We have also clearly shown that control of stereochemistry is crucial for optimum inhibition. Finally, we note that a crystal structure of hydroxamate (*R*)-**4** within the BoNT/A active site has been solved,[20] supporting our hypothesis that the hydroxyethyl moiety of (*R*)-**4** and -**5** replaces an active-site water molecule. Additional research using a combination of crystallography, synthesis, and kinetic analysis is envisioned to discover more potent inhibitors of this unique metalloprotease and will be reported in due course.

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Supporting Information Available: Experimental synthetic procedures and NMR and HRMS data for all previously unreported compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

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