



## Short communication

# The synthesis of 2,5-bis(4-amidinophenyl)thiophene derivatives providing submicromolar-range inhibition of the botulinum neurotoxin serotype A metalloprotease

Igor Opsenica<sup>a</sup>, Vuk Filipovic<sup>a</sup>, Jon E. Nuss<sup>b</sup>, Laura M. Gomba<sup>b</sup>, Dejan Opsenica<sup>c</sup>, James C. Burnett<sup>d,\*\*</sup>, Rick Gussio<sup>e</sup>, Bogdan A. Solaja<sup>a,\*</sup>, Sina Bavari<sup>f,\*\*\*</sup>

<sup>a</sup> Faculty of Chemistry, University of Belgrade, Studentski trg16, P.O. Box 51, Belgrade, Serbia

<sup>b</sup> United States Army Medical Research Institute of Infectious Diseases, Department of Bacteriology, 1425 Porter Street, Frederick, MD 21702, USA

<sup>c</sup> Institute of Chemistry, Technology, and Metallurgy, University of Belgrade, 12 Njegoseva 12, 11001, P.O. Box 473, Belgrade, Serbia

<sup>d</sup> SAIC-Frederick, Inc., National Cancer Institute at Frederick, Target Structure-Based Drug Discovery Group, P.O. Box B, Frederick, MD 21702, USA

<sup>e</sup> National Cancer Institute at Frederick, Developmental Therapeutics Program, P.O. Box B, Frederick, MD 21702, USA

<sup>f</sup> Target Discovery and Experimental Microbiology, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, MD 21702, USA

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## ABSTRACT

Botulinum neurotoxins (BoNTs), composed of a family of seven serotypes (categorized A–G), are the deadliest of known biological toxins. The activity of the metalloprotease, light chain (LC) component of the toxins is responsible for causing the life-threatening paralysis associated with the disease botulism. Herein we report significantly more potent analogs of novel, lead BoNT serotype A LC inhibitor 2,5-bis(4-amidinophenyl)thiophene ( $K_i = 10.88 \mu\text{M} \pm 0.90 \mu\text{M}$ ). Specifically, synthetic modifications involved simultaneously replacing the lead inhibitor's terminal bis-amidines with secondary amines and the systematic tethering of 4-amino-7-chloroquinoline substituents to provide derivatives with  $K_i$  values ranging from  $0.302 \mu\text{M} (\pm 0.03 \mu\text{M})$  to  $0.889 \mu\text{M} (\pm 0.11 \mu\text{M})$ .

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## 1. Introduction

Botulinum neurotoxins (BoNTs), a family of seven serotypes (categorized A–G), are secreted by *Clostridia* species *botulinum*, *baratii*, and *butyricum* [1, 2], and are listed among the highest priority of bioterrorism agents [3].

The enzymes are composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC), which are tethered by a reducible disulfide bridge [4,5]. The HC binds to neuronal receptors and releases the LC into the cell cytosol [5–7]. The LC, a zinc<sup>2+</sup> (Zn<sup>2+</sup>) metalloprotease, cleaves neuron soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins [6,7].

This proteolytic activity inhibits the release of acetylcholine into neuromuscular junctions, resulting in the disease state botulism [6,7]. The BoNT serotype A LC (BoNT/A LC), which is the target enzyme of this study, cleaves SNARE protein synaptosomal associated protein of 25 kDa (SNAP-25) [6,7], and is known to cause human botulism [8,9].

We have previously reported the discovery of a variety of non-Zn<sup>2+</sup> chelating BoNT/A LC lead inhibitor chemotypes [10–15], many of which possess terminal di-cationic moieties [11–14]. With respect to di-cationic inhibitors, the synthesis of more potent derivatives of leads possessing bis-amidine and bis-imidazoline functional groups has been described [16–19]. Importantly, the synthetic modification of one of these leads, bis [3-amide-5-(imidazolino)phenyl]terephthalamide-based inhibitor **I** ( $K_i = 8.52 \pm 0.53 \mu\text{M}$ ) [18] (Fig. 1), via the incorporation of terminal  $-(\text{CH}_2)_3-4,7\text{-ACQ}$  components, resulted in derivative **II** (Fig. 1), which possesses a  $K_i = 0.572 \mu\text{M} \pm 0.041 \mu\text{M}$  [18]. To the best of our knowledge, **II** is the most potent, non-hydroxamic acid-based BoNT/A LC inhibitor reported to date.

As part of an ongoing research program to discover novel BoNT/A LC inhibitor chemotypes for development, a variety of di-amidine

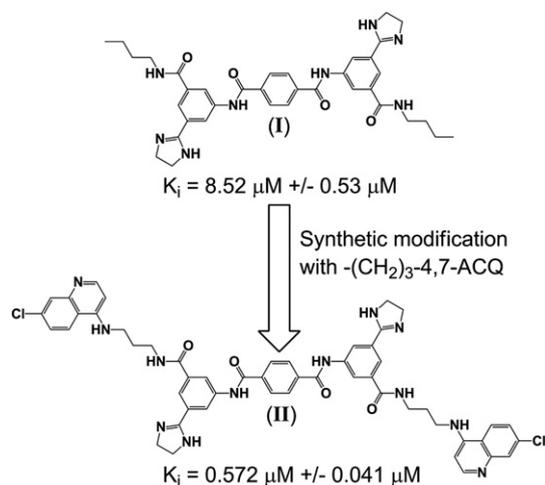
Abbreviations: BoNT/A LC, Botulinum neurotoxin serotype A light chain; 4,7-ACQ, 4-amino-7-chloroquinoline.

\* Corresponding author. Tel.: +381 11 263 86 06; fax: +381 11 263 60 61.

\*\* Corresponding author. Tel.: +1 804 225 0527; fax: +1 301 828 8566.

\*\*\* Corresponding author. Tel.: +1 301 619 8484; fax: +1 301 619 2348.

E-mail addresses: [burnettjames@mail.nih.gov](mailto:burnettjames@mail.nih.gov) (J.C. Burnett), [bsolaja@chem.bg.ac.rs](mailto:bsolaja@chem.bg.ac.rs) (B.A. Solaja), [sina.bavari@us.army.mil](mailto:sina.bavari@us.army.mil) (S. Bavari).



**Fig. 1.** Adding  $-(\text{CH}_2)_3-4,7\text{-ACQ}$  components translated lead BoNT/A LC inhibitor **1** into nM-range derivatives, **II** was the most potent of the congeneric series [18].

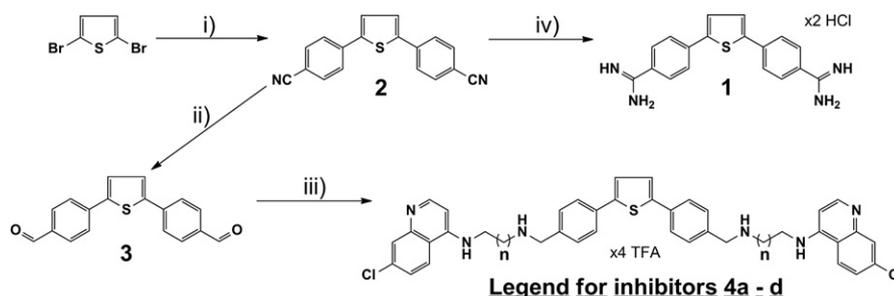
substituted compounds obtained from the National Cancer Institute's Open Repository were screened. Subsequently, 2,5-bis(4-amidinophenyl)thiophene **1** (NSC 300510) (Scheme 1) was found to provide 78% BoNT/A LC inhibition when tested at  $20\ \mu\text{M}$  concentration. Following, **1** was synthesized to ensure purity, and subsequent *in vitro* testing indicated that it possesses a  $K_i = 10.88\ \mu\text{M} \pm 0.90\ \mu\text{M}$  (Table 1).

Interestingly, **1** has previously been shown to possess anti-trypanosomal activity [20, 21], and provided 100% survival when administered as a single, 320 mg/kg dose during *Trypanosoma rhodesiense* infection [21]. Additionally, **1** has been analyzed for antineoplastic activity, and based on a dosing regimen of three 100 mg/kg injections (administered on day one, and then every fourth day) was non-toxic to mice [22].

Therefore, based on the possibility of repurposing a biologically relevant chemotype, **1** was chosen as a candidate for synthetic modification to provide derivatives with increased BoNT/A LC inhibitory potencies. Specifically, based on the strategy used to increase the inhibitory potency of lead **I** (Fig. 1) [18], it was hypothesized that systematically tethering terminal 4,7-ACQ motifs to **1** with short methylene chains would also provide significantly more potent derivatives.

## 2. Chemistry

Initially, we attempted to substitute  $-(\text{CH}_2)_n-4,7\text{-ACQ}$  components directly onto the terminal amidines of **1**. However, as



**Scheme 1.** Reagents and conditions. i) 4-CN- $\text{PhB}(\text{OH})_2$ ,  $\text{Pd}(\text{OAc})_2$ , DME,  $\text{Na}_2\text{CO}_3(\text{aq})$ , MW,  $80\ ^\circ\text{C}$ , 2 h; ii) DIBAH, PhMe, 1 h,  $0\ ^\circ\text{C}$ , Ar; iii) (step 1)  $\text{NH}_2-(\text{CH}_2)_n-4,7\text{-ACQ}$ ,  $\text{NaBH}_4$ , AcOH,  $\text{MeOH}/\text{CH}_2\text{Cl}_2$  and (step 2) TFA; iv) (step 1) LHMDS, THF, r.t. and (step 2) HCl/EtOH.

**Table 1**  
BoNT/A LC inhibition constants for lead **1** and derivatives **4a–d**.

Inhibitor	$K_i$ ( $\mu\text{M}$ )
<b>1</b>	$10.88 \pm 0.90\ \mu\text{M}$
<b>4a</b>	$0.882 \pm 0.11\ \mu\text{M}$
<b>4b</b>	$0.302 \pm 0.03\ \mu\text{M}$
<b>4c</b>	$0.535 \pm 0.60\ \mu\text{M}$
<b>4d</b>	$0.889 \pm 0.11\ \mu\text{M}$

previously encountered when attempting the same amidine substitution with a different BoNT/A LC inhibitor chemotype [18], prohibitive synthesis and/or degradation during purification were encountered. To circumvent these obstacles, an alternative cationic motif, with the terminal amidines of **1** replaced with secondary amines, provided an efficient synthetic route for the generation of a congeneric series to further test our hypothesis for increasing the inhibitory potency of chemotype **1**.

The synthesis of **1** and derivatives **4a–d** are outlined in Scheme 1. Key intermediate di-nitrile **2** was obtained in 94% yield by coupling 4-cyanophenylboronic acid and 2,5-dibromothiophene under Suzuki conditions. The synthesis of **1**, to ensure purity (as it was initially obtained from a chemical repository (*vide supra*)), was achieved in two-steps by reacting **2** with LHMDS at room temperature, followed by isolation as an HCl salt (97% yield). Di-aldehyde **3** was prepared in 77% yield via the DIBAH-mediated reduction of **2** at  $0\ ^\circ\text{C}$ . Following, simultaneous reductive amination of **3** and coupling with  $\text{NH}_2-(\text{CH}_2)_n-4,7\text{-ACQ}$  motifs provided targets **4a–d**, which were isolated as TFA salts (yields ranged from 24 to 49%).

## 3. BoNT/A LC inhibition

Table 1 provides the  $K_i$  values for inhibitors **1** and **4a–d** when examined *in vitro* employing a well documented HPLC-based assay for BoNT/A LC inhibition [23–29]. The potencies of the derivatives, in support of our hypothesis, provide further evidence that tethering  $-(\text{CH}_2)_n-4,7\text{-ACQ}$  components onto the termini of di-cationic lead BoNT/A LC inhibitors (*i.e.*, such as **I** (Fig. 1)) and **1** (Scheme 1 and Table 1) can significantly improve inhibitory potency. Interestingly, as was encountered when tethering 4,7-ACQ components onto the terminal amides of inhibitor chemotype **I** [18] (Fig. 1), trimethylene linkers also afforded the most significant increase in the inhibitory potency of **1**. Specifically, tethering  $-(\text{CH}_2)_3-4,7\text{-ACQ}$  groups onto the secondary amino termini of the 2,5-bis(4-methylaminophenyl) thiophene scaffold (**4b**, Scheme 1) increased inhibitory potency by approximately (approx.) 36-fold versus **1** (Table 1).

Moreover, as was also previously observed for derivatives of **I** (Fig. 1) [18], decreasing or increasing the methylene tether length above or below that of trimethylenes also resulted in decreased inhibitor **1** derivative potencies. For example, when the tethers were shortened to dimethylenes (**4a**, Scheme 1), inhibitory potency decreased by approx. 3-fold versus **4b** (Table 1). Likewise, when tether lengths were increased to tetramethylenes (**4c**, Scheme 1), inhibitory potency also decreased, but less dramatically than observed for **4a** (Scheme 1) (i.e., an approx. 1.8-fold decrease in potency versus **4b**) (Table 1). Finally, derivative **4d** (Scheme 1), which possesses pentamethylene tethers, was approx. 3-fold less potent than **4b** (Table 1), and with respect to inhibitory potency, is equivalent to **4a** (Table 1). Nevertheless, it is noteworthy that regardless of the tether length, **4a–d** are all significantly more potent than lead **1** (Table 1).

The data from this study, and the previous study for developing more potent derivatives of inhibitor **I** (Fig. 1) [18], provide further evidence that using trimethylene tethers to link 4,7-ACQ moieties onto the termini of di-cationic BoNT/A LC inhibitors consistently results in derivatives with significantly improved potencies versus dimethylene, tetramethylene, or pentamethylene tethers (Table 1). However, unlike the substitution pattern used to generate more potent derivatives of **I** (Fig. 1), the results from this research indicate that  $-(\text{CH}_2)_n-4,7\text{-ACQ}$  components may be tethered directly onto the inhibitor's two cationic substituents to achieve increased potency. For example, **II** (Fig. 1) is approx. 15-fold more potent than **I** (Fig. 1), while **4b** is approx. 36-fold more potent than **1** (Table 1) – even though the  $K_i$  values of the two leads (i.e., **I** (Fig. 1) and **1** (Table 1)) indicate that their inhibitory potencies are approx. equivalent. Additionally, the  $K_i$  values for **4a–d** indicate that lower  $pK_a$  cationic amines may be used to translate  $\mu\text{M}$  range, di-amidine substituted leads (such as **1** (Scheme 1)) into nM-range derivatives via an efficient synthetic route (Scheme 1).

#### 4. Conclusions

In summary, we report that tethering  $-(\text{CH}_2)_n-4,7\text{-ACQ}$  motifs onto the modified cationic components of lead BoNT/A LC inhibitor 2,5-bis(4-amidinophenyl)thiophene (i.e., **1**) significantly increases derivative potencies. The data from this study corroborates and further validates a previous report in which a similar strategy was used to translate a bis[3-amide-5-(imidazolino)phenyl]terephthalamide-based BoNT/A LC lead inhibitor into submicromolar-range derivatives [18]. However, in this study, we show that tethering  $-(\text{CH}_2)_n-4,7\text{-ACQ}$  components directly onto the lead inhibitor chemotype's terminal di-cationic components provides a more pronounced increase in potency. The implications of this research: such modifications may be made to a variety of our lead, di-cationic BoNT/A LC inhibitors [10–15,18,30] to significantly increase derivative potencies. Finally, results from this study preface future syntheses to optimize the elemental composition and rigidity of the tethered components, i.e., beyond that of  $-(\text{CH}_2)_n-4,7\text{-ACQ}$  motifs.

#### 5. Experimental section

##### 5.1. Chemistry

Melting points were determined using a Boetius PMHK apparatus (Carl Zeiss, Germany) and were not corrected. IR spectra were recorded on a Perkin–Elmer spectrophotometer FTIR 1725X.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Varian Gemini-200 spectrometer (at 200 and 50 MHz, respectively) and a Bruker Ultrashield Advance III

spectrometer (at 500 and 125 MHz, respectively) employing indicated solvents (*vide infra*) using TMS as the internal standard. Chemical shifts are expressed in ppm ( $\delta$ ) values and coupling constants ( $J$ ) in Hz. ESI–MS (HRMS) spectra of the synthesized compounds were acquired on a Agilent Technologies 1200 Series instrument equipped with a Zorbax Eclipse Plus C18 ( $100 \times 2.1$  mm i.d.  $1.8 \mu\text{m}$ ) column and DAD detector (190–450 nm) in combination with a 6210 Time-of-Flight LC/MS instrument in positive ion mode. The samples were dissolved in pure  $\text{H}_2\text{O}$  (HPLC grade). The selected values were as follows: capillary voltage = 4 kV, gas temperature =  $350^\circ\text{C}$ , drying gas =  $12 \text{ L min}^{-1}$ , nebulizer pressure = 45 psig, and fragmentator voltage = 70 V. Lobar LichroPrep Si 60 ( $40\text{--}63 \mu\text{m}$ ) or LichroPrep RP-18 columns (Merck, Germany), coupled to a Waters RI 401 detector, were used for preparative column chromatography. Microanalyses were performed using a Vario ELP III (Elementary, Germany). Thin-layer chromatography was performed on pre-coated Merck silica gel 60 F254 and Merck RP-18 F254 plates. Reactions carried out employing microwave (MW) conditions were performed using a Biotage Initiator Eight Robot with an automatic sampler (USA and Sweden). Compounds were analyzed for purity (HPLC) using a Waters 1525 HPLC dual pump system equipped with an Alltech, Select™ degasser system, and dual  $\lambda$  2487 UV–VIS detector. HPLC analyses were performed using two distinct methods:

**Method A:** Octadecylsilica was used as the stationary phase (Symmetry C18 analytical column, 4.6–150 mm, 5  $\mu\text{m}$ , series no. 021336278136 37 (Waters Corporation, Milford, Massachusetts 01757-3696, USA). Compounds were dissolved in 5% formic acid in methanol. Final concentrations (concs.) were 0.1–0.5 mg/ml, and injection volumes were 10  $\mu\text{L}$ . Eluent was prepared from the following solvents: 0.2% formic acid in water and methanol. Gradient method runs from 10% to 90% MeOH over 15 min and back to 90% aqueous over 5 min, followed by a 5 min 10% MeOH isocratic period, were performed. The analyses were performed at the UV max of the compounds (330 nm) to maximize selectivity. For data processing, Empower software (Waters Corporation, Milford, Massachusetts 01757-3696 U.S.A) was used. All compounds were >95% pure.

**Method B:** Octadecylsilica was used as the stationary phase (Nucleosil C18 analytical column, 4–150 mm, 5  $\mu\text{m}$ , series no. 72923.40 (Macherey-Nagel GmbH & Co. KG, Düren, Germany)). Compounds were dissolved in 5% formic acid in methanol. Final concs. were 0.1–0.5 mg/ml, and the injection volume was 10  $\mu\text{L}$ . Eluent was prepared from 0.5% formic acid in water and methanol. Gradient method runs from 10% to 90% MeOH over 15 min and back to 90% aqueous over 5 min, followed by a 5 min 10% MeOH isocratic period, were performed. The analyses were performed at the UV max of the compounds (330 nm) to maximize selectivity. For data processing, Empower software was used. All compounds were >95% pure.

##### 5.1.1. 4,4'-Thiene-2,5-diylidibenzenecarboximidamide dihydrochloride (**1**)

Di-nitrile **2** (*vide infra*) (2.24 g, 7.82 mmol) was suspended in freshly distilled THF (40 ml) and treated with a solution of lithium bis(trimethylsilyl)amide (LHMDS, 5.24 g, 31.32 mmol) in freshly distilled THF (40 ml). The reaction was stirred for 48 h at r.t., and the mixture was clear and orange in color. The reaction mixture was cooled to  $0^\circ\text{C}$ , followed by the addition of HCl-saturated ethanol (100 ml). The mixture was stirred overnight, diluted with ether, and the resulting solid was collected by filtration (3.21 g). The crude product was purified by neutralization with 1 N NaOH, and collected by filtration. The di-amidine product (2.50 g, 7.81 mmol) was saturated with 36.7% HCl/EtOH (200 mL). The mixture was stirred for 12 h, diluted with ether, and the resulting solid was collected by filtration. Final product **1** was a yellow amorphous powder. The yield was 2.99 g (97%, mp =  $385\text{--}398^\circ\text{C}$ ) [21]. IR (KBr): 3254m, 3074m, 1668s, 1652m, 1605s, 1556w, 1533w, 1517w, 1485m, 1451w, 1415w,

1385w, 1286m, 844w, 832w, 800w, 771w, 747w, 704w  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (200 MHz,  $d_6$ -DMSO): 9.473 (s,  $2\text{NH}_2=\text{C}$ ), 9.250 (s,  $2\text{NH}_2-\text{C}$ ), 7.99–7.93 (m, 8H-Ar), 7.882 (s, H-C(3) and H-C(4)).  $^{13}\text{C}$  NMR (50 MHz,  $\text{CD}_3\text{OD}$ ): 164.82, 142.48, 138.04, 129.16, 127.67, 126.80, 125.39. Anal. Calcd. for  $\text{C}_{18}\text{H}_{18}\text{Cl}_2\text{N}_4\text{S} \times 0.3\text{H}_2\text{O}$ : C, 54.14; H, 4.71; N, 14.03; S, 8.03. Found: C, 54.23; H, 4.82; N, 13.35; S, 8.00.

#### 5.1.2. 4,4'-Thiene-2,5-diyl dibenzonitrile (**2**)

A reaction vessel containing a solution of  $\text{Pd}(\text{OAc})_2$  (20 mg, 0.09 mmol) and  $\text{PPh}_3$  (94.5 mg, 0.36 mmol) in DME (9 mL) was stirred for 10 min under Ar atmosphere. Following, 2,5-dibromothiophene (0.1 mL, 0.89 mmol) and 2 M  $\text{Na}_2\text{CO}_3$  (1.6 mL, 3.2 mmol) were added. After 5 min, 4-cyanophenylboronic acid (290 mg, 1.97 mmol) was added, and the mixture was stirred for 2 h at 80 °C in a MW reactor. The solvent was then removed under reduced pressure, and the reaction mixture was suspended in dichloromethane (DCM), transferred to a separation funnel, and washed well with saturated  $\text{Na}_2\text{CO}_3$  solution ( $2 \times 25$  mL) containing 1 mL  $\text{NH}_3$ . The organic layer was collected, dried with anhydrous  $\text{Na}_2\text{SO}_4$ , and the solvent was filtered. Subsequently, the solvent was removed under reduced pressure and intermediate **2** was isolated using column chromatography (dry-flash,  $\text{SiO}_2$ , eluent DCM), and collected as a pale yellow amorphous powder. The yield was 240 mg (94%, mp = 282–284 °C) [21]. IR (KBr): 2925w, 2359w, 1535m, 1506m, 1380w, 1207s, 1150s, 1035s, 825s, 781m, 732m, 702m  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (200 MHz,  $\text{CD}_3\text{OD}$ ): 7.80–7.60 (m, 8H-Ar), 7.50–7.45 (s, H-C(3) and H-C(4)).  $^{13}\text{C}$  NMR (50 MHz,  $\text{CD}_3\text{OD}$ ): 143.31, 137.90, 132.88, 126.36, 125.98, 118.64, 111.19. HPLC purity: method A, RT 15.397 min, area 99.03%; method B: RT 14.656 min, area 96.58%.

#### 5.1.3. 4,4'-Thiene-2,5-diyl dibenzaldehyde (**3**)

Di-nitrile **2** (400 mg, 1.50 mmol) was dissolved in dry toluene (50 mL) under Ar atmosphere, and DIBAH (9 mL, 1 M in toluene) was added drop-wise. After stirring at 0 °C for 1 h, the reaction was quenched with 5%  $\text{H}_2\text{SO}_4$  (12 mL), and stirring was continued for 1 h at r.t. The reaction mixture was transferred into a separation funnel as an EA emulsion. The organic layer was separated, and the aqueous layer was extracted with DCM ( $8 \times 50$  mL). The combined organic layers were washed with brine ( $2 \times 10$  mL) and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . After filtration, the solvent was removed under reduced pressure. Resulting intermediate **3**, a pale yellow amorphous powder, was sufficiently pure and was used in the next reaction step. The yield was 315 mg (77%, mp = 148–152 °C) [31]. IR (ATR): 3349w, 3198w, 3069m, 2923m, 2854m, 2764w, 1694s, 1599s, 1568m, 1498w, 1451w, 1423w, 1397w, 1346w, 1310w, 1280m, 1216m, 1167m, 1116w, 838m, 796m, 688w.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ ): 10.01 (s,  $2\text{H}-\text{C}=\text{O}$ ), 7.96 (s, 8H-Ar), 7.90–7.80 (m, H-C(3) and H-C(4)).  $^{13}\text{C}$  NMR (125 MHz,  $\text{DMSO}-d_6$ ): 192.27, 142.98, 138.57, 135.24, 130.46, 127.65, 125.73. HRMS:  $m/z$  293.06242 corresponding to molecular formula  $\text{C}_{18}\text{H}_{12}\text{O}_2\text{SH}^+$  (error in ppm: –2.25).

#### 5.1.4. 4,4'-[Thiene-2,5-diylbis(4,1-phenylenemethyleneammonioethane-2,1-diylimino)]bis(7-chloroquinolinium) tetra(trifluoroacetate) (**4a**)

A mixture of *N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (340 mg, 1.53 mmol), di-aldehyde **3** (150 mg, 0.51 mmol), and anhydrous AcOH (72  $\mu\text{L}$ , 1.2 mmol) in  $\text{MeOH}/\text{CH}_2\text{Cl}_2$  (2/1, v/v, 30 mL) was stirred at r.t. for 14 h, followed by the addition of  $\text{NaBH}_4$  (240 mg, 6.34 mmol) in 40 mg portions over 2 h. After stirring for an additional 6 h at r.t., the solvent was removed under reduced pressure, and the product was isolated using column chromatography (dry-flash,  $\text{SiO}_2$ , gradient  $\text{EtOAc}/\text{MeOH}/\text{NH}_3$  36/1  $\rightarrow$  36/3). Fractions containing the product were combined, and the crude product was dissolved in  $\text{MeOH}/\text{CH}_2\text{Cl}_2$  (1/1, v/v, 15 mL), followed by the addition of TFA (0.6 mL). The solvent and excess of TFA were removed

under reduced pressure, and the product was crystallized as a salt from MeOH, and dried at 40 °C in a vacuum oven. Final product **4a** was a yellow amorphous powder. The yield was 200 mg (34%, mp 242–244 °C). IR (ATR): 3268w, 3030w, 2822w, 1678s, 1608s, 1569m, 1455m, 1426w, 1381w, 1246w, 1197s, 1132m, 838w, 802m, 721w  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (200 MHz,  $\text{DMSO}-d_6$ ): 9.40 (bs, 2H-C(8')), 8.67 (d,  $J = 6.8$ , 2H(2')), 8.48 (d,  $J = 9.0$ , 2H(5')), 8.02 (d,  $J = 2.2$ , H-C(3) and H-C(4)), 7.90–7.50 (m, 2H(6'), 8H-Ar), 6.99 (d,  $J = 6.8$ , 2H-C(3')), 4.30 (s,  $\text{CH}_2$ -Ar), 3.94 (bs, 4H-C(9')), 3.35 (bs, 4H-C(10')).  $^{13}\text{C}$  NMR (50 MHz,  $\text{DMSO}-d_6$ ): 159.26, 158.63, 158.01, 157.37, 155.07, 143.28, 141.75, 138.36, 137.60, 133.53, 130.86, 130.31, 126.45, 125.29, 124.90, 119.62, 118.99, 115.29, 113.67, 98.36, 49.21, 43.82. HRMS:  $m/z$  703.2172 corresponding to molecular formula  $\text{C}_{40}\text{H}_{36}\text{Cl}_2\text{N}_6\text{SH}^+$  (error in ppm: –1.54). HPLC purity: method A, RT 6.912, area 98.95%; method B, RT 5.856, area 98.79%. Anal. Calcd. for  $\text{C}_{40}\text{H}_{36}\text{Cl}_2\text{N}_6\text{S} \times 4$  TFA: C, 49.71%; H, 3.48%; N, 7.25%; S, 2.76%. Found: C, 49.56%; H, 3.63%; N, 7.03%; S, 2.52%.

#### 5.1.5. 4,4'-[Thiene-2,5-diylbis(4,1-phenylenemethyleneammonioisopropane-3,1-diylimino)]bis(7-chloroquinolinium) tetra(trifluoroacetate) (**4b**)

Compound **4b** was synthesized according to the procedure used to generate **4a** using di-aldehyde **3** (75 mg, 0.26 mmol) and *N*-(7-chloroquinolin-4-yl)propane-1,3-diamine (144 mg, 0.61 mmol), but with a modification to the column chromatography solvent mixture. Specifically, crude product was purified by column chromatography (dry-flash,  $\text{SiO}_2$  gradient  $\text{EtOAc}/\text{MeOH}/\text{NH}_3$  (36/1  $\rightarrow$  36/3)), and the product was crystallized from MeOH as a TFA salt. Final product **4b** was a yellow amorphous powder. The yield was 150 mg (49%, mp = 165–168 °C). IR (ATR): 3612w, 3414w, 3273m, 3070m, 3076m, 2966m, 2816m, 2478w, 2366w, 2331w, 1674s, 1638w, 1613s, 1553m, 1456m, 1422w, 1368w, 1244w, 1199s, 1128s, 900w, 835m, 799m, 721m  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ): 8.38–8.35 (m, 2H-C(2') and 2H-C(8')), 7.85–7.82 (m, 2H-C(5')), 7.66 (d,  $J = 8.25$ , 2H-C(7) and 2H-C(11)), 7.61 (dd,  $J = 2.02$ ,  $J = 9.07$ , 2H-C(6')), 7.50 (d,  $J = 8.30$ , 2H-C(8) and 2H-C(10)), 7.38 (s, H-C(3) and H-C(4)), 6.87 (d,  $J = 7.20$ , 2H-C(3')), 4.25 (s,  $2\text{CH}_2$ -Ar), 3.70 (t,  $J = 6.85$ , 2H-C(9')), 3.22 (t,  $J = 7.82$ , 2H-C(11')), 2.24–2.18 (m, 2H-C(10')).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ): 157.52, 143.94, 143.75, 141.01, 139.81, 136.38, 131.66, 131.25, 128.72, 126.87, 126.16, 125.82, 120.23, 119.16, 116.82, 99.65, 51.73, 45.42, 41.56, 25.54. HRMS:  $m/z$  731.24576 corresponding to molecular formula  $\text{C}_{42}\text{H}_{40}\text{Cl}_2\text{N}_6\text{SH}^+$  (error in ppm: –3.74). HPLC purity: method A, RT 7.191 min, area 97.64%; method B: RT 6.113 min, area 99.08%. Anal. Calcd. for:  $\text{C}_{42}\text{H}_{40}\text{Cl}_2\text{N}_6\text{S} \times 4$  TFA: C, 50.56%; H, 3.73%; N, 7.07%; S, 2.70%. Found: C, 50.40%; H, 3.85%; N, 6.92%; S, 2.57%.

#### 5.1.6. 4,4'-[Thiene-2,5-diylbis(4,1-phenylenemethyleneammonioisobutane-4,1-diylimino)]bis(7-chloroquinolinium) tetra(trifluoroacetate) (**4c**)

Compound **4c** was synthesized according to the procedure used to obtain **4b** using di-aldehyde **3** (70 mg, 0.23 mmol) and *N*-(7-chloroquinolin-4-yl)butane-1,4-diamine (180 mg, 0.72 mmol), but with an additional purification step. Specifically, the reaction mixture was initially sub-purified using column chromatography (dry-flash,  $\text{SiO}_2$  gradient  $\text{EtOAc}/\text{MeOH}/\text{NH}_3$  36/1  $\rightarrow$  36/3), and solvent was removed under reduced pressure. Following, the semi-crude product was dissolved in  $\text{MeOH}/\text{CH}_2\text{Cl}_2$  (1/1, v/v) with TFA added (0.5 mL). After evaporation to dryness, the remaining solid was purified using a second column chromatography procedure: LOBAR, RP-18 column, eluent  $\text{MeOH}/0.1\%$  TFA. Final product **4c** was obtained as a yellow amorphous powder. The yield was 70 mg (24%, mp = 154–158 °C). IR (ATR): 3388w, 3247m, 3034m, 2956m, 2824m, 2366w, 1680s, 1614s, 1561m, 1454m, 1424m, 1365w, 1202s, 1133s, 1016w, 901w, 834m, 800m, 767w, 718m  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR

(200 MHz, CD<sub>3</sub>OD): 8.40–8.35 (m, 2H-C(2') and 2H-C(8')), 7.86 (d,  $J = 1.5$ , 2H-C(5')), 7.73 (d,  $J = 8.5$ , 2H-C(7) and 2H-C(11)), 7.66 (dd,  $J = 2.0$ ,  $J = 9.0$ , 2 × H-C(6')), 7.53 (d,  $J = 8.5$ , 2H-C(8) and 2H-C(10)), 7.44 (s, H-C(3) and H-C(4)), 6.88 (d,  $J = 7.0$ , 2H-C(3')), 4.24 (s, 2CH<sub>2</sub>-Ar), 3.64 (bs, 2H-C(9')), 3.15 (bs, 2H-C(12')), 1.92–1.85 (m, 2H-C(10'), 2H-C(11')). <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD): 162.65, 157.82, 144.33, 143.97, 141.17, 140.18, 136.72, 131.95, 128.85, 127.19, 126.50, 126.10, 120.48, 117.09, 99.91, 52.07, 48.17, 44.27, 26.28, 24.79. HRMS:  $m/z$  759.27980 corresponding to molecular formula C<sub>44</sub>H<sub>44</sub>Cl<sub>2</sub>N<sub>6</sub>SH<sup>+</sup> (error in ppm: −1.21). Anal. Calcd. for C<sub>44</sub>H<sub>44</sub>Cl<sub>2</sub>N<sub>6</sub>S × 4 TFA × 2H<sub>2</sub>O: C, 49.89%; H, 4.19%; N, 6.71%; S, 2.56%. Found: C, 49.74%; H, 4.25%; N, 6.50%; S, 2.33%.

#### 5.1.7. 4,4'-[Thiene-2,5-diylbis(4,1-phenylenemethyleammonio-pentane-5,1-diylimino)]bis(7-chloroquinolinium) tetra(trifluoroacetate) (**4d**)

Compound **4d** was synthesized according to the procedure used to obtain **4c** using di-aldehyde **3** (70 mg, 0.23 mmol) and *N*-(7-chloroquinolin-4-yl)pentane-1,5-diamine (190 mg, 0.72 mmol). Note: the additional purification step used to obtain **4c** (*vide supra*) was also used. Final product **4d** was obtained as a yellow amorphous powder. The yield was 70 mg (24%, mp = 146–149 °C). IR (ATR): 3273w, 3033m, 2939m, 2825m, 2360w, 1670s, 1613s, 1454m, 1365w, 1198s, 1128s, 901w, 832w, 798m cm<sup>−1</sup>. <sup>1</sup>H NMR (200 MHz, CD<sub>3</sub>OD): 8.42–8.28 (m, 2H-C(2') and 2H-C(8')), 7.88–7.78 (m, 2H-C(5')), 7.72 (d,  $J = 8.0$ , 2H-C(7) and 2H-C(11)), 7.64 (dd,  $J = 1.8$ ,  $J = 9.2$ , 2H-C(6')), 7.53 (d,  $J = 8.0$ , 2H-C(8) and 2H-C(10)), 7.43 (s, H-C(3) and H-C(4)), 6.83 (d,  $J = 6.8$ , 2H-C(3')), 4.22 (s, 2CH<sub>2</sub>-Ar), 3.57 (t,  $J = 6.8$ , 4H-C(9')), 3.15–3.00 (m, 4H-C(13')), 1.90–1.70 (m, 4H-C(10'), 4H-C(12')), 1.53–1.45 (m, 4H-C(11')). <sup>13</sup>C NMR: 157.52, 144.12, 143.68, 140.91, 140.00, 136.45, 131.86, 128.62, 127.02, 126.36, 125.98, 120.30, 116.84, 99.69, 51.84, 44.50, 28.44, 26.73, 24.86. HRMS:  $m/z$  787.31125 corresponding to molecular formula C<sub>46</sub>H<sub>48</sub>Cl<sub>2</sub>N<sub>6</sub>SH<sup>+</sup> (error in ppm: 0.20). HPLC purity: method A: RT 8.071 min, area 97.10%; method B: RT 6.918 min, area 97.59%. Anal. Calcd. for: C<sub>46</sub>H<sub>48</sub>Cl<sub>2</sub>N<sub>6</sub>S × 4 TFA: C, 52.14%; H, 4.21%; N, 6.76%; S, 2.58%. Found: C, 51.98%; H, 4.36%; N, 6.52%; S, 2.42%.

#### 5.2. Biology (HPLC-based assay to calculate BoNT/A LC inhibition)

A previously described HPLC *in vitro* assay was employed to determine inhibitor-mediated BoNT/A LC inhibition kinetics [23–29], with slight modifications [23]. Briefly, the assay utilized an N-terminal acetylated, C-terminal aminated, synthetic peptide identical in sequence to residues 187–203 of SNAP-25. Compounds with intrinsic fluorescence quenching capability do not interfere with the activity measurements of the assay because substrate hydrolysis is determined by HPLC separation of the cleaved product and the intact substrate, followed by measurement of the peak areas. All HPLC separations were conducted on a Shimadzu Prominence Ultra Fast Liquid Chromatography XR system using a Hypersil Gold Javelin c18 guard column (Thermo Fisher Scientific, Waltham, MA, USA) and a Hypersil Gold c18 reverse-phase analytical column (50 × 2.1 mm, 1.9 μm) (Thermo Fisher Scientific, Waltham, MA, USA). Peak areas were measured with LC solution automated integration software (Shimadzu Corporation, Kyoto, Japan) and used to calculate enzyme reaction rates. Assay mixtures consisted of 40 mM HEPES–0.05% Tween (pH 7.3), recombinant BoNT/A LC (MetaBiologics, Madison, WI, USA), peptide substrate, 0.5 mg/ml Bovine Serum Albumin, 1 mM Dithiothreitol, 50 μM excess zinc, and 20 μM inhibitor. The assays were run at 37 °C, quenched via the addition of TFA, and analyzed by reverse-phase HPLC.

For  $K_i$  value determination, plots of reaction velocity versus substrate conc., at multiple inhibitor concs., were analyzed by

global kinetic analysis. Data were fit to a competitive inhibition model and analyzed by nonlinear regression analysis using the program GraphPad Prism, version 5.01 (GraphPad Software, San Diego, CA, USA).

#### Author contributions

Sina Bavari: group leader, biological testing, sponsor of J.C.B., L.M.G. and J.E.N.

James C. Burnett: compound design and inhibitory potency interpretation.

Vuk Filipovic: compound synthesis and purity analysis.

Laura M. Gomba: biological testing.

Rick Gussio: group leader, compound design and inhibitory potency interpretation, sponsor of J.C.B.

Jon E. Nuss: biological testing.

Jgor Opsenica: compound synthesis and purity analysis.

Dejan Opsenica: compound synthesis and purity analysis.

Bogdan A. Solaja: group leader, compound synthesis and purity analysis, sponsor of V.F., I.G., and D.O.

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