Partial Protection against Botulinum B Neurotoxin-Induced Blocking of Exocytosis by a Potent Inhibitor of Its Metallopeptidase Activity

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Clostridium botulinum *neurotoxins (BoNTs) cause botulism, which is characterized by a flaccid paralysis, through inhibition of acetylcholine release by peripheral cholinergic nerve terminals. This is due to the zinc metallopeptidase activity of the neurotoxin, cleaving one component (synaptobrevin for BoNT/B) of the exocytosis machinery. Yet, there are no specific agents able to control the peptidase-related effects of BoNT/B. We recently developed the first compounds to inhibit this enzymatic activity in the nanomolar range. Here we report that two of our best inhibitors prevent the BoNT/B-induced cleavage of native synaptobrevin on synaptic vesicles, and partially inhibit the suppression of [^{3}H]noradrenaline release from synaptosomes that is caused by BoNT/B. These results were obtained at micromolar concentrations, consistent with the measured inhibitory potency of these inhibitors on the native toxin. These compounds provide a new way to possibly prevent and/or to control the neurotoxin effects of botulinum.*

Introduction

The different strains of the anaerobic bacterium *Clostridium botulinum* produce seven immunologically distinct types (A–G) of neurotoxins, which are considered to be the most poisonous substances known for humans.[1] Each botulinum neurotoxin consists of a 50 kDa light chain (LC) linked by a disulfide bond to a 100 kDa heavy chain (HC).[2] The latter subunit binds with high selectivity to protein acceptors at the surface of neuron terminals and triggers internalization of the neurotoxin.[3] The light chain, which belongs to the group of zinc metallopeptidases, selectively cleaves one of the three proteins (synaptobrevin, syntaxin, SNAP-25) involved in the fusion of small vesicles to the external membrane of neurons.[4] Botulinum neurotoxin B (BoNT/B) selectively cleaves synaptobrevin (Sb),[5] a small protein embedded in the membrane of neurotransmitter-containing vesicles, preventing it from fusing with the two protein counterparts (syntaxin and SNAP-25) present in the cytosolic domain of the synaptic membrane.[6] This results in inhibition of neurotransmitter exocytosis and leads to botulism.[7] The clinical symptoms of botulism (blurred or double vision, difficulty swallowing and speaking, gastrointestinal problems, and, as botulism progresses, weakness or paralysis, starting with head muscles and progressing down the body, breathing difficulty) can easily be confused with different kinds of neuromuscular or brain diseases; this accounts for the necessity of a rapid and reliable diagnosis of the disease.[8]

BoNT/B is now currently used to induce locally a partial inhibition of the transmission of nerve impulse and thus to treat some skeletal muscle-related disorders and hyperfunctions of autonomic terminals, and to reduce wrinkles.[9] In spite of the relative safety of treatments with BoNTs, severe adverse reactions arising from local or sometimes large-scale diffusion of the toxin from the injection site have been reported.[10] Meanwhile, botulinum neurotoxins and, more precisely, the very stable BoNT/B, which is easily produced and purified, could be used in biological warfare.[11]

To date there is no potent and specific compound able to counteract the severe adverse effects of BoNT/B, and treatments consist of administering antibodies or symptomatic measures, such as intubation, when the botulism is in progress. Another possible way could be to inhibit the zinc metallopeptidase activity of the BoNT/B LC that is responsible for the neurotoxicity.[12]

Tetanus toxin and BoNT/B are two closely related toxins whose light chains cleave the same substrate at the same site.[4–5] In the case of the TeTx LC, several inhibitors have been designed, yet all these compounds have inhibitory potencies in the micromolar range.[13] In contrast, it has been possible to design highly potent inhibitors of the BoNT/B LC with nanomolar inhibitory potencies.[14–16] This could be due to differences in the mechanism of action of the two neurotoxins, as teta-

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nus toxin has been demonstrated to behave as an allosteric enzyme.[17]

In this study, we report the first demonstration that two highly potent BoNT/B inhibitors selected for their solubility in the buffer used for the given biochemical assay, counteract in vitro the adverse affect of this neurotoxin on the exocytosis process. These inhibitors of BoNT/B are able to protect native synaptobrevin present on synaptic vesicles from cleavage by BoNT/B and to prevent the blocking of neurotransmitter release from rat-brain synaptosomes induced by the neurotoxin. Several clinical applications could be expected from such selective BoNT/B inhibitors.

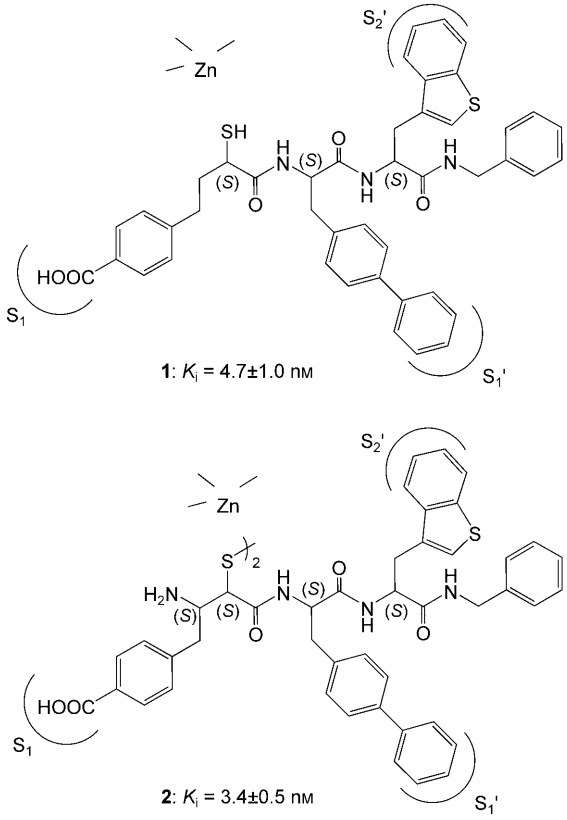
Results

Compounds 1 and 2 block BoNT/B LC-induced synaptobrevin cleavage

We have previously designed a new family of compounds inhibiting the metallopeptidase activity of the BoNT/B light chain in a biochemical in vitro assay using a synthetic fluorescent substrate.[18] The best compounds block this enzymatic activity at low nanomolar concentrations.[14–16] To evaluate the potency of these inhibitors under more physiological conditions, we have chosen the two most potent compounds **1** and **2**, the former with a zinc-coordinating thiol group in the free form and the latter with this group involved in a symmetric disulfide dimer-forming linkage (Scheme 1). The need for two inhibitors endowed with similar inhibitory potencies was caused by their different solubility in the medium used in the two biochemical experiments.

The secretion of neurotransmitters, such as acetylcholine, at the neuromuscular junction is achieved by the formation of a ternary fusion complex between synaptobrevin anchored in neurotransmitter-containing small vesicles and two proteins, SNAP 25 and syntaxin, embedded in the external membrane of the cell.[6] These proteins are the targets of botulinum neurotoxin light chains, which are released into the cytosol following reduction of the disulfide bond linking the small subunit to the heavy chain.[4] The cleavage of one component of the fusion complex, such as Sb by BoNT/B, interrupts neurotransmitter release. Inhibition of this cleavage can be studied by measuring the amounts of Sb protected from hydrolysis by BoNT/B LC.

At 15 μM, compound **2** reduces this degradation process by 62±3% (data not shown). As it was not possible to increase the concentration of **2** under the experimental conditions used, a dose-dependent study was performed with the most recently synthesized potent and more soluble inhibitor, **1**.[16] Compared to heat-inactivated BoNT/B LC taken as control (Figure 1, lane A) 4.4 nM of BoNT/B LC cleave Sb almost completely (Figure 1, lane E), with 14% of Sb remaining intact. In the presence of 100, 10, and 1 μM of **1** (Figure 1, lanes F, G, H), the hydrolysis of Sb by 4.4 nM BoNT/B LC is reduced dose-dependently by 100%, 40%, and 13%, respectively. This was calculated by a comparison of the immunoblots with those obtained with a mixture of the corresponding concentrations of



Scheme 1. Structures of the highly potent inhibitors of BoNT/B LC **1** and **2**. S_1, S_1' and S_2' are schematic representations of subsites present in the BoNT/B LC active site. The asymmetric carbons in the three molecules have the (*S*) configuration.

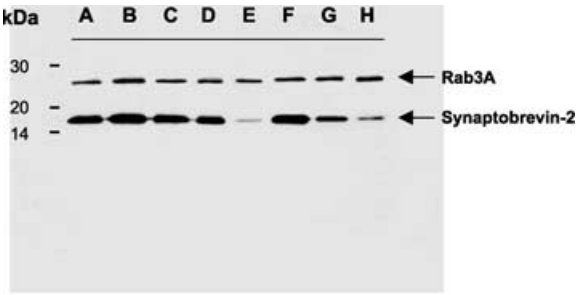


Figure 1. Analysis by immunoblot of the inhibitory effect of inhibitor **1** on the BoNT/B light chain-induced cleavage of synaptobrevin (Sb) present on synaptic vesicles. A) Sb and Rab3A from synaptic vesicles in the presence of heat-inactivated BoNT/B LC (control). B) Sb and Rab3A from synaptic vesicles in the presence of heat-inactivated BoNT/B LC and 100 μM of inhibitor (control with 100 μM inhibitor). C) Sb and Rab3A from synaptic vesicles in the presence of heat-inactivated BoNT/B LC and 10 μM of inhibitor (control with 10 μM inhibitor). D) Sb and Rab3A from synaptic vesicles BoNT/B LC in the presence of 1 μM of inhibitor (control with 1 μM inhibitor). E) Sb and Rab3A from synaptic vesicles incubated with 4.4 nM of BoNT/B LC (14% of intact Sb as compared to control column A). F) Sb and Rab3A from synaptic vesicles incubated with BoNT/B LC in the presence of 100 μM of inhibitor (100% of control in Sb). G) Sb and Rab3A from synaptic vesicles incubated with BoNT/B LC in the presence of 10 μM of inhibitor (54% of control in Sb) H) Sb and Rab3A from synaptic vesicles incubated with BoNT/B LC in the presence of 1 μM of inhibitor (25% of control in Sb). The percentages are calculated by using quantifications of Rab3A to normalize that of Sb, and the results of three different experiments are within ±5%.

1 and 4.4 nM of heat-inactivated BoNT/B LC (Figure 1, lanes B, C, D). No change in the levels of Rab3A, taken as internal protein standard noncleavable by BoNT/B, was observed. A IC_{50} of 18 ± 2 μM was evaluated for **1** from these experiments.

Reduction by inhibitor 2 of the BoNT/B-induced inhibition of [^{3}H]noradrenaline-evoked release from rat cortical synaptosomes

Given its inhibitory potency obtained with synaptobrevin (62% of BoNT/B LC at 15 μM), the ability of **2** to counteract the blocking of neurotransmitter release by the native BoNT/B two-chain protein was evaluated. In spite of its rather inferior solubility compared to **1**, compound **2** was selected because the presence of the disulfide bond enhances the bioavailability of the compound and thus its potency to cross cell–plasma membranes.[19] Although synaptosomes from rat cortex neurons have few neurotoxin acceptors at the surface,[20] they were chosen because, unlike peripheral-nerve preparation, biochemical experiments can be carried out with this preparation. [^{3}H]noradrenaline ([^{3}H]NA) was used instead of acetylcholine for technical reasons and was loaded into small vesicles of synaptosomes as described.[21] As shown in Figure 2, compound **2** alone is unable to modify the K^+-evoked release of [^{3}H]NA (compare lanes 1 and 2). As expected, preincubation for 120 min of the synaptosomes with BoNT/B (100 nM) led to a large reduction in the K^+-evoked release of [^{3}H]NA (compare lanes 1 and 3). Preincubated with the toxin under the same conditions, 15 μM of compound **2**, a concentration shown to be active in the previous experiment with synaptic vesicles, produced a 41.0 ± 2.8% reduction in the inhibitory effects of BoNT/B (compare lanes 3 and 4). This suggests that the designed inhibitors could have interesting protective effects against BoNT/B infection. The cell penetration of the inhibitor was also illustrated in another experiment, in which 15 μM of **2** was coincubated for 30 min with [^{3}H]NA-loaded synaptosomes (data not shown). After centrifugation, the pelleted synaptosomes were rapidly washed and then treated for 90 min with 100 nM BoNT/B. Even under these severe conditions, **2** was shown to reduce the inhibitory effect of BoNT/B on the evoked release of [^{3}H]NA by 26 ± 3% (means of three independent experiments).

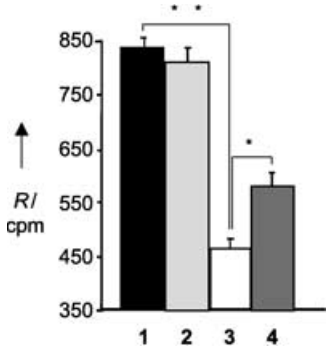


Figure 2. Reduction by **2** of the BoNT/B-induced inhibition of K^+-evoked release of [^{3}H]noradrenaline from rat cortical synaptosomes. *R* = radioactivity. 1) Evoked release of [^{3}H]NA in the presence of 100 nM of heat-inactivated BoNT/B. 2) Evoked release of [^{3}H]NA in the presence of 100 nM of heat-inactivated BoNT/B and compound **2** at 15 μM final concentration. 3) Inhibition of [^{3}H]NA-evoked release by 100 nM active BoNT/B. 4) Reduction by **2** at 15 μM final concentration of inhibition of [^{3}H]NA-evoked release induced by 100 nM BoNT/B. The results are the means ± SEM or four different experiments. ** $p < 0.01$; * $p < 0.05$ (Dunett's test).

Inhibitory properties of 2 on BoNT/B LC and native BoNT/B

The large differences (ca. a factor of 1000) observed in the inhibitory potency of **2** for BoNT/B LC in an enzymatic study[26] and its efficiency to counteract the inhibition of [^{3}H]NA release in a cellular assay performed with the native neurotoxin prompted us to determine its IC_{50} values towards BoNT/B LC and the whole-protein BoNT/B. This was done strictly under the same experimental conditions with Syb60–94 [Pya74-Nop77][35] as fluorescent substrate. Figure 3 shows that the IC_{50} values of **2** are 2 ± 0.3 nM and 16 ± 2 μM for BoNT/B LC and BoNT/B, respectively. These results account for the larger concentration required in the cellular assay and strongly suggest the occurrence of constraints to reaching the site of substrate hydrolysis for this type of molecules. Nevertheless, even at this micromolar concentration, they remain the most potent inhibitors of BoNT/B reported to date.

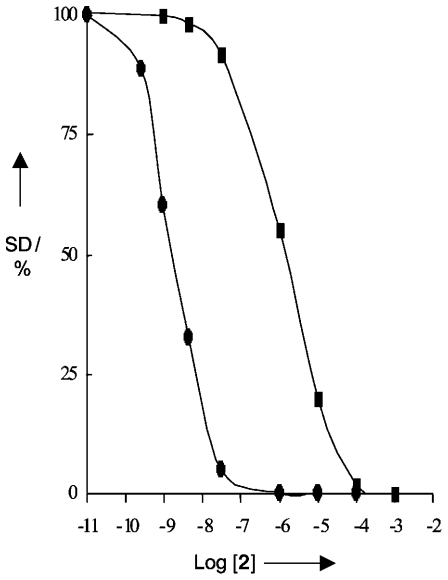


Figure 3. Determination of inhibitory potency (IC_{50} values) of **2** on BoNT/B LC (●) and native BoNT/B (■). BoNT/B LC (0.35 ng) or BoNT (5.25 ng) was preincubated for 30 min at 37 °C in 90 μL of 20 mM Hepes, pH 7.4 with increasing concentrations of **2** (from 10^{-10} to 10^{-4} M). The percentage of degradation of 18 μM Syb60–94 [Pya^{74}-Nop^{77}] incubated for 30 min after the previous step was calculated from the fluorescence increase as described.[34] SD = substrate degradation. The IC_{50} values of **2** are: 2 ± 0.3 nM for BoNT/B LC and 16 ± 2 μM for native BoNT.

Discussion

At this time, there is no specific compound available for an efficient protection or treatment of botulism. Immunotherapy

and drugs such as 4-aminopyridine, which are used to try to minimize the incapacitated peripheral motricity, are associated with significant side effects.[11,22] The goal of this study was to develop a new strategy aimed at inhibiting the neurotoxicity of BoNT/B. Several strategies could be theoretically used to block the action of the toxin. One way would be to prevent either the binding of the toxin to the cell by antibodies or the translocation of its light chain into the cytosol, but the mechanisms of the latter step remain unclear.[12] Moreover, there are a large number of isotypes for each botulinum neurotoxin; this limits the expected ubiquitous recognition and inhibition of cell penetration of botulinum toxins by using antibodies directed towards a single isotype.[37] Another approach could be to inhibit the zinc metallopeptidase activity of the toxin. Indeed, it has been clearly demonstrated that the light chain of BoNT/B released in the cytosol cleaves Sb, a protein essential for exocytosis.[5] Consistent with the relationship between this catalytic activity and the intrinsic toxicity of the toxin on exocytosis,[5] compounds with high inhibitory potencies were expected to prevent the BoNT/B blocking of neurotransmitter release. This approach was selected, and highly potent BoNT/B blockers have been designed. The best compounds of this series are the thiol **1** and the disulfide **2**,[15,16] which are the first reported compounds with nanomolar affinities for BoNT/B LC. Strikingly, in the case of the related tetanus toxin (TeTx), which cleaves Sb at the same peptide bond as BoNT/B, only micromolar inhibitors have been obtained, although a similar approach to that followed in this study was used.[13] This could be due to the allosteric-like mechanism of tetanus toxin,[17] which was not observed in the case of BoNT/B (data not shown).

Interestingly, compounds **1** and **2** have no affinity for TeTx or for the closely related neurotoxin BoNT/A, despite a high amino acid sequence alignment and a structural similarity with BoNT/B;[23,24] this indicates the presence of subtle differences in the active-site structure of all these neurotoxins. Thus, in BoNT/A,[23–24] an alanine residue could correspond to Lys242 in BoNT/B. The latter amino acid seems to be essential for the interaction of the selective inhibitors of BoNT/B with the enzyme active site, as shown by molecular modeling.[15]

The inhibitors designed in this study seem to have promising properties, at least as preventive agents, since they can reduce the blocking of exocytosis machinery induced by BoNT/B in complex bioassays at micromolar concentrations. The concentration of inhibitors required in these in vitro assays are more than 100 times higher than the IC_{50} values determined in biochemical experiments with the purified enzyme light chain.[25,26] This is consistent with the micromolar doses required to block the metallopeptidase activity of BoNT/B when the protein is used in its native form. The large differences in IC_{50} values between the LC and this subunit inside the whole protein is very likely due to structural constraints limiting the accessibility of the peptidase's catalytic site in native BoNT/B. This has been recently discussed in the light of crystallographic data on various clostridial toxins.[38–40]

Nevertheless, significant inhibition of the cleavage of Sb by BoNT/B LC was obtained in the 10–100 μM range by using rat brain synaptic vesicles. Moreover, in a more physiological assay, this inhibition was correlated with an effect on neurotransmitter exocytosis. The best compound prevents blocking of [^{3}H]noradrenaline release induced by BoNT/B by about 40%. It is important to observe that no compound was reported to display such in vitro activity in a "cell-mimicking" assay. Optimizations of these inhibitors to increase their bioavailability is now requested for in vivo assays on mice. Interestingly, both compounds remain unmodified after incubation with BoNT/B, as expected, but, more interestingly, also following 6 h incubation with human serum (data not shown).

Botulism is best prevented and its complications efficiently anticipated with adequate treatment at an early stage. This is expected to avoid the costly maintenance of patients in intensive care units until their affected nerve endings regenerate, a process that occurs over 3 to 5 weeks for BoNT/B and 4 to 7 weeks for BoNT/A.[27]

This paper reports the first biochemical and pharmacological assays of a family of molecules that might be used to control the local effects of botulinum B neurotoxin used in therapy. Nevertheless, with the aim of treating botulism, inhibitors must be administered during the whole period (from several weeks to months) over which the light chains of botulinum neurotoxins are effective intracellularly.[28,29] This would require enhancing the cell penetration of the systemically administered inhibitors, which could be achieved by the classical approaches used in medicinal chemistry as nicely shown with inhibitors of the HIV-1 protease.[30] These latter molecules, which have a size similar to that of the present inhibitors, are able to block the activity of huge levels of HIV-1 protease in contaminated cells. Finally unsophisticated approaches by using, for example, the isolated heavy chain of botulinum A or B neurotoxins to deliver the inhibitors into the cells could also be employed.[31] Peptides that target the neuromuscular junction and facilitate intrasynaptic penetration of the inhibitors could also be used.[32]

Experimental Section

Chemicals: Compound **1** belongs to a series of thiol inhibitors and compound **2** to its corresponding disulfide analogue (patent Fr 01.04895). Their synthesis has been described previously.[14–16] The entire BoNT/B protein and its derived light chain were obtained in pure forms as previously reported.[33] Synaptobrevin was synthesized by solid-phase synthesis and purified as described.[33]

Clostridial toxins: Native BoNT/B was prepared and purified in the laboratory of E. Johnson as reported.[31,33] BoNT/B light chain was purchased from Calbiochem (France)and its purity verified by SDS-PAGE. BoNT/E LC was from Sigma. Pure BoNT/A was a generous gift from Dr. Popoff (Pasteur Institute, Paris). The physiological metallopeptidases ACE, NEP, ECE were from our laboratory.[25,26]

Enzyme studies: The inhibitory potency of **2** was measured under the same experimental conditions by using the light chain of BoNT/B (from Calbiochem) or the native BoNT/B prepared as already described.[31,33] Due to its low solubility, the stock solution of **2** was made of DMF/H_2O, 10:90. In each assay, the amount of DMF was 2 M after dilution, a concentration unable to modify the metallopeptidase activity of BoNT, although this is the case in cellular assays. Due to the slower rate of degradation of the fluorescent

substrate Syb60–94[Pya^{74}-Nop^{77}][34] by the native toxin as compared to BoNT/B LC, a tenfold higher concentration of BoNT/B was used in the experiments. This led to a cleavage of about 3% of the substrate in 30 mn. BoNT/B LC (0.35 ng) or native BoNT/B (5.25 ng) was preincubated for 30 min at 37 °C in Hepes (90 μL, 20 mM, pH 7.4) with dithiothreitol (DTT, 0.1 mM; only in the case of BoNT/B LC) and increasing concentrations of **2**. Then the reaction was initiated by addition of the fluorescent substrate (10 μL, 18 μM).[34] Seven concentrations of **2** were used, and the fluorescence was measured by using a Cytofluor. With the BoNT/B LC, the determined IC_{50}, 2 ± 0.3 nM, was similar to that obtained previously,[16] while the IC_{50} of **2** for the native BoNT/B was 16 ± 2 μM.

Animals: Rats (Sprague Dawley) were used for the various experiments. They were treated and euthanized in accordance with the *NIH Guidelines for the Care and Use of Laboratory Animals* (1985) and in agreement with the local ethical committee.

Determination of the cleavage of VAMP/synaptobrevin present on synaptic vesicles: Synaptic vesicles prepared from rat brains were obtained as previously described.[35] Preincubation of BoNT/B LC (4.4 nM final conc) with compound **2** (1, 10, or 100 μM) or with compound **1** (15 μM) was performed for 30 min at 37 °C in buffer (20 mM HEPES, 1 mM DTT, 10 μM $ZnCl_2$, 0.1% BSA). Synaptic vesicles (0.6 mg mL^{-1} final conc) were then added, and incubation was continued for 1 h at 37 °C. Samples were subsequently denatured at 95 °C for 90 min in Laemli blue and analyzed by SDS-PAGE and immunoblotting to quantify intact synaptobrevin and intact Rab3A by using specific antibodies (anti-Sb and anti-Rab3A from Synaptic systems®). Evaluation of Sb and Rab3A quantities was achieved with a Vilber Lourmat apparatus connected to a VPN-120 videoprinter from Mitsubishi with Bio-Profil version 6.0 software.

Secretion assay from rat cortical synaptosomes: Synaptosomes were prepared as reported.[36] Briefly, cerebral cortices (5 rats) were homogenized in buffer (35 mL; 320 mM sucrose, 5 mM HEPES, pH 7.4) at 4 °C and separated in a centrifuge at 3500 *g* for 10 min at 4 °C. The supernatant was collected and separated in a centrifuge for 12 min at 14000 g at 4 °C. The pellet was suspended in sucrose (6.25 mL, 320 mM), and the fraction containing the synaptosomes was obtained by using a discontinuous Ficoll gradient that was spun for 40 min at 9000 *g* in a "swinging" rotor at 4 °C. The layer enriched in synaptosomes (9%) was removed, and buffer (12 mL; 132 mM NaCl, 4.8 mM KCl, 2.4 mM $MgSO_4$, 10 mM glucose, 20 mM HEPES, 2 mM $CaCl_2$, pH 7.4) was added. Finally, the Sb was obtained as a pellet after centrifugation at 14000 *g* for 12 min at 4 °C. It were suspended at 3 mg of protein per mL and at 25 °C in buffer (pH 7.4, 264 mM NaCl, 9.6 mM KCl, 4.8 mM $MgSO_4$, 20 mM glucose, 4 mM $CaCl_2$, 20 mM HEPES, 0.1% BSA, 0.35 μM pargyline, 1.2 mM ascorbic acid, 0.12 μM [^{3}H]noradrenaline ([^{3}H]NA)). This preparation was divided into four samples. The final active or heat-inactivated BoNT/B (100 nM), in the presence or not of compound **2** (15 μM final concentration), was preincubated in the absence of DTT for 30 min at 37 °C, then a suspension of [^{3}H]NA-loaded synaptosomes was added to each sample, and incubation was carried out for 90 min at 37 °C. The pellet was recovered and rapidly washed with the buffer lacking [^{3}H]NA. The secretory activity was then determined by stimulating the synaptosomes with K^+ (50 mM) in the presence of Ca^{2+} (2 mM). The [^{3}H]NA content of aliquots of the supernatant from each sample was determined by liquid scintillation counting.

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