Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 4655–4660

BIOORGANIC & MEDICINAL CHEMISTRY

Thio-Derived Disulfides as Potent Inhibitors of Botulinum Neurotoxin Type B: Implications for Zinc Interaction

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Received 1 April 2003; accepted 2 July 2003

Abstract—Botulinum neurotoxin type B causes the inhibition of acetylcholine release at the neuromuscular junction resulting in a flaccid paralysis designated botulism. This occurs through the cleavage of synaptobrevin, an intracellular critical component of neurotransmitter exocytosis, by the zinc-metallopeptidase activity of the smallest subunit of the toxin. Blocking the proteolytic activity may present an attractive approach to treat botulism as to date there is no efficient specific drug therapy avalaible. We have therefore recently described a series of β-amino-thiol derived pseudotripeptides able of inhibiting the toxin at low (10^{-8} M) concentration. In this study, binding characteristics of the protein's active site are explored through various structural modifications of the thiol functionality which was supposed to be a key structural constituent for effective zinc-ion chelation. Surprisingly, sulfanyl-derivatives such as symmetric disulfides were shown to be better inhibitors than their thiol-counterparts, the most potent compound displaying a K_i value of 3.4 nM.

Introduction

Botulinum neurotoxins are constituted of seven serotypes (A to G) that are among the most poisonous substances for humans known to date.[1] They cause botulism, a disease characterized by a flaccid paralysis, that can lead to death by respiratory arrest.[2] These toxins block the excitatory neurotransmission at the neuromuscular junction and at the autonomous nervous system level by cleaving one of the proteins involved in exocytosis.[3–7] They are composed of two chains linked by a disulfide bridge. The heavy chain (100 kDa) is involved in binding and translocation while the light chain (50 kDa) is endowed with the catalytic activity.[8,9] These light chains were demonstrated to be zinc-metallopeptidases.[10] To date, there is no specific drug therapy against botulism, whereas the use of serum is effective only in the first hour following the intoxication.[2] Moreover these toxins are potential biological weapons.[11] The design of compounds able of blocking their activity is an interesting target of therapeutical research. Botulinum neurotoxin type B (BoNT/B), which cleaves synaptobrevin, an integral protein of synaptic vesicles,[4] is the serotype responsible of the majority of cases of human botulism in Europe.[12]

Few selective and potent antagonists of this toxin have been described. Indeed, classical inhibitors of metalloproteases are ineffective on BoNT/B and small peptides derived from the substrate do not inhibit BoNT/B either.[4,13–17] Strong ligands of zinc are effective but have major adverse effects.[17–19] ICD 1578 (7-*N*-phenylcarbamoyl-amino-4-chloro-propyloxyisocoumarine), an inhibitor of the matrix metalloprotease elastase has only a slight inhibitory potency on BoNT/B[14,17] whereas inhibitors of serine protease, such as BABIM or keto-BABIM [respectively, bis(5-amidino-2-benzimidazolyl)-methane and bis(5-amidino-2-benzimidazolyl)oxo-methane] were proved to be more effective with inhibitory activities of 1.6 and 0.8 μM, respectively.[20] Buforin-1, a natural peptide, blocks the activity of BoNT/B in the micromolar range[21] and the thearubigin fraction of black tea was only shown to be efficient against the inhibiting activity of BoNT/B on the stimulated contractions of the mouse hemidiaphragm.[22]

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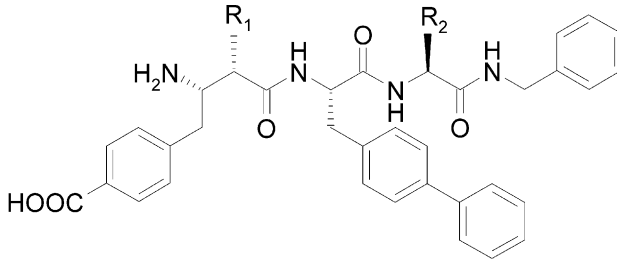
doi:10.1016/S0968-0896(03)00450-4

We have recently reported[23] potent and selective inhibitors of BoNT/B, the more potent being the pseudo-tripeptide compound **1** (Table 1). This compound displays an inhibitory activity of 20 nM on BoNT/B. Thiol-bearing inhibitors of zinc metalloproteases are generally proposed to interact with the Zn^{2+} ion through chelation with the thiolate whereas their disulfide derived counterparts are inactive.[24,25] In the case of matrix metallopeptidases, it has however been shown that the zinc present in the catalytic site must be chelated in a different mode as inhibitors are devoid of non-oxidized sulfur.[26] Sulfanyl-derivatives such as symmetric and dissymmetric disulfides and thio-ethers of some of the pseudo-tripeptides described previously were therefore synthesized as well as pseudo-tripeptide surrogates lacking a sulfur atom. All compounds were evaluated[27] for their ability to block the proteolytic activity of BoNT/B light chain (LC), which revealed some inhibitory potencies in the low nanomolar range.

Table 1. BoNT/B LC inhibitory potencies of compounds **1**–**12**

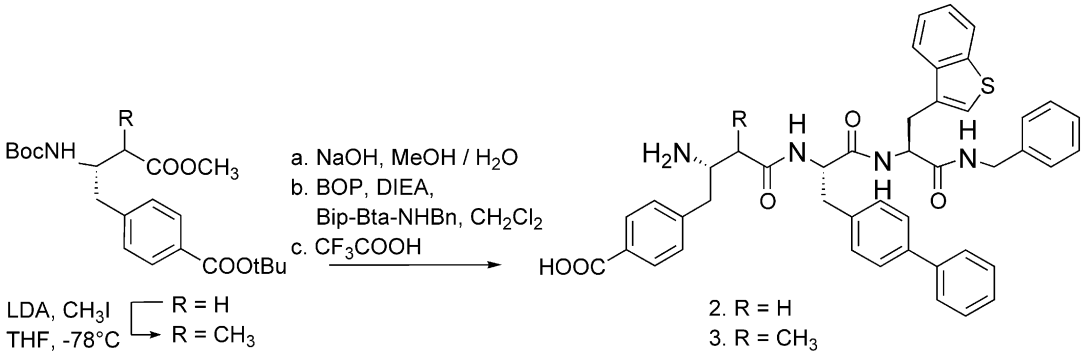


Compd	R_1[a]	R_2	$K_i \pm$SEM[b] (nM)
1	–SH	Benzo[*b*]thiophen-3-yl	20±2
2	–H	Benzo[*b*]thiophen-3-yl	193±10
3	$-CH_3$[c]	Benzo[*b*]thiophen-3-yl	697±157
4	$-S)_2$	Benzo[*b*]thiophen-3-yl	3.4±0.5
5	$-S)_2$	Phenyl	18±2
6	$-S)_2$	But-2-yl	23±2
7	$-S)_2$	4-Hydroxyphenyl	14±2
8	–SH	Phenyl	160±20
9	–SH	But-2-yl	540±41
10	–SH	4-Hydroxyphenyl	810±90
11	–S–S–Bn	Benzo[*b*]thiophen-3-yl	10.4±1.2
12	–S–(4-MeOBn)	Benzo[*b*]thiophen-3-yl	28±4

[a]Substituents indicated with $-S)_2$ represent the corresponding symmetric disulfide.
[b]K_i values represent the mean±SEM of three separate experiments each in triplicate.
[c]Undefined stereochemistry at the carbon atom connecting this substituent.

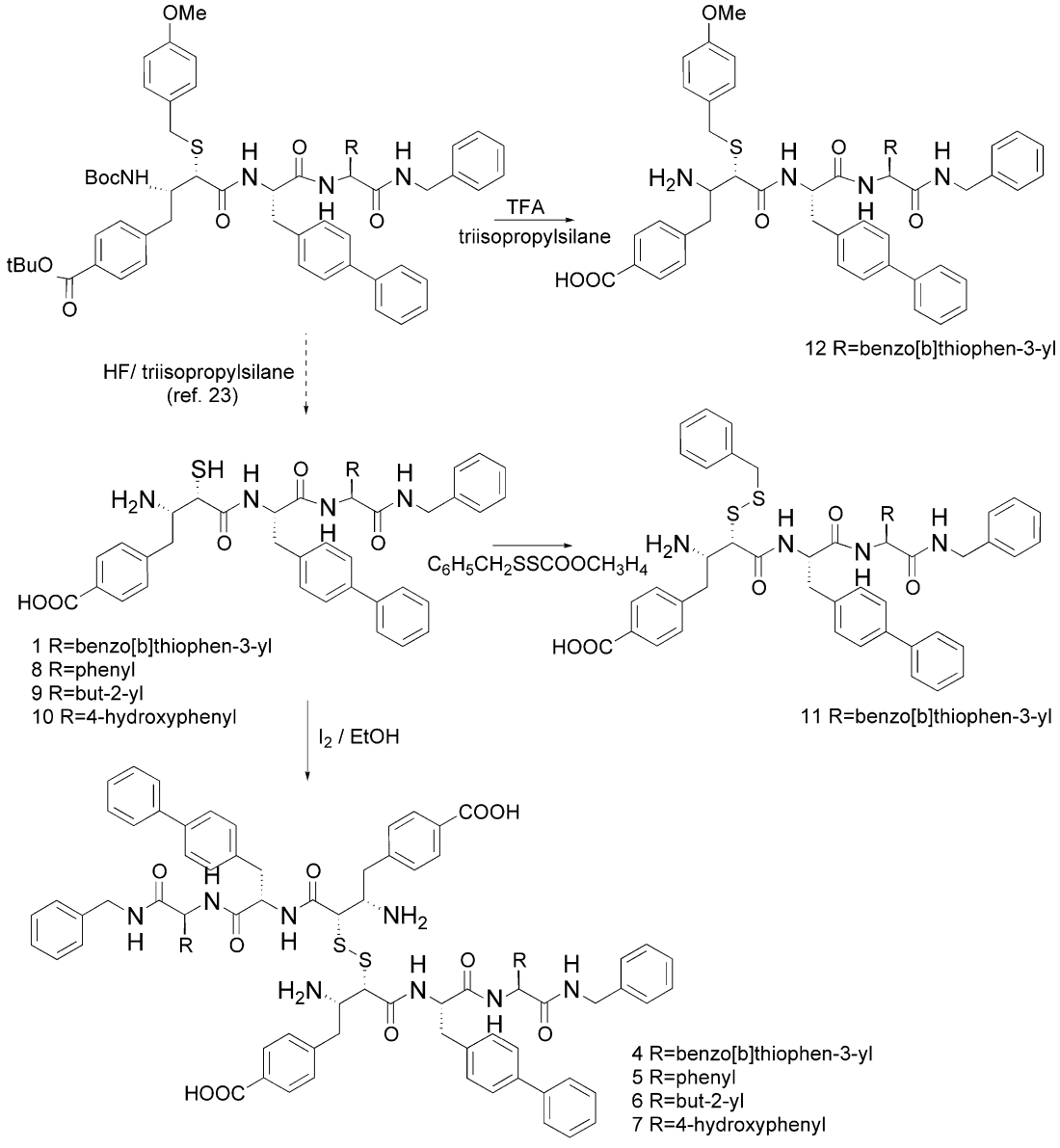
Results and Discussion

The values of the BoNT/B LC binding affinities of the newly synthesized compounds (Schemes 1 and 2) as well as some previously prepared analogues (**1**, **8**–**10**)[23] are listed in Table 1. Lead compound **1** showed high affinity for BoNT/B LC. The thiol functionality of compound **1** was either eliminated (**2**) or replaced with a methyl group (**3**) in order to demonstrate whether enzyme recognition might imply classical zinc chelation. The syntheses of compounds **2** and **3** are outlined in Scheme 1. 4-[(2*S*)-2-(1,1-Dimethylethyl)oxycarbonylamino-4-methoxy-4-oxo]benzoic acid-(1,1-dimethylethyl)ester was obtained as previously described[23] and was either directly hydrolyzed and subsequently coupled with the dipeptide Bip-Bta-NHBn[23] and deprotected to yield compound **2** or submitted to intermediate alkylation reaction conditions with methyliodide to yield compound **3**. Both compounds exhibit low inhibitory potencies as compared to **1** which is not withstanding the mode of metal complexation found for other metallopeptidases.[24,25] The thiol functionality of compound **1** was also derivatized into a symmetric disulfide, yielding compound **4** (see Scheme 2). Interestingly compound **4** displays a gain of affinity over an order of magnitude higher as compared to **1**. This was confirmed by synthesis of other symmetric disulfides **5**–**7** which were prepared from some previously described[23] thiol-bearing pseudo-tripeptides **8**–**10** (Scheme 2). As shown in Table 1 all disulfides are more potent inhibitors of BoNT/B LC than their corresponding sulfanyl counterparts. It can also be observed that the less potent the thiol is the greater the gain of activity for the corresponding disulfide (factor, respectively of 6, 9, 23 and 58 for compounds **4**–**7**). Zinc chelation does therefore not seem as essential for these large molecules which might perfectly complement several supplementary subsites of the protein binding pocket as compared to shorter sequences. Similarly, an acceptable inhibitory potency of BABIM showing weak amidinium–zinc ion chelation was explained through good enzyme subsite recognition.[28] A general sketch depicting the virtual pharmacophore is represented in Figure 1.



Scheme 1. Synthetic pathway of compounds **2** and **3**.

A short benzyl-derived dissymmetric disulfide (**11**) was prepared in order to reduce the number of putative interactions with additional subsites. Benzyl thiol was activated with *S*-chloro *O*-methyl thiocarbonate[29] and subsequently added to **1** as to enable the specific coupling to the thiol functionality yielding compound **11** (Scheme 2). As shown in Table 1, this compound, although very effective with a K_i of 10.4 nM, is a less potent inhibitor of BoNT/B LC as compared to **4** but slightly better in comparison to **1**. One can conclude that additional interactions of **4** with the binding pocket subsites as compared to **1** are involved in the gain of inhibitory potency but they do not seem to be the only contributors to this gain. The presence of the disulfide instead of the thiol might also be one essential factor. It can be proposed that the zinc is chelated either by the two pairs of electrons of the sulfur atoms or by only one pair of one sulfur atom. This last type of chelation exists for the copper ion, Cu^{2+} whereas bichelation of zinc by separated sulfur atoms was shown in the case of cryptants.[30] In order to attempt to elucidate the mode of chelation, a thio-ether (**12**) was prepared through retaining para-methoxy benzyl substitution by submitting the synthetic precursor[23] of **1** to selective deprotection conditions using trifluoro-acetic acid instead of hydrofluoric acid (Scheme 2). Compound **12** is nearly as potent as the corresponding thiol and only 8 times less potent as compared to the corresponding symmetric disulfide. Moreover it is only 3 times less effective than the dissymmetric disulfide **11** whose side chains are quite similar except for the methoxy in the *para* position of the benzyl group. The affinity of **12** suggests that the free pairs of electrons of the sulfur atom belonging to the thio-ether may be involved in zinc chelation as



Scheme 2. Synthesis of compounds **4**–**7**, **11** and **12** from previously described precursors.[23]

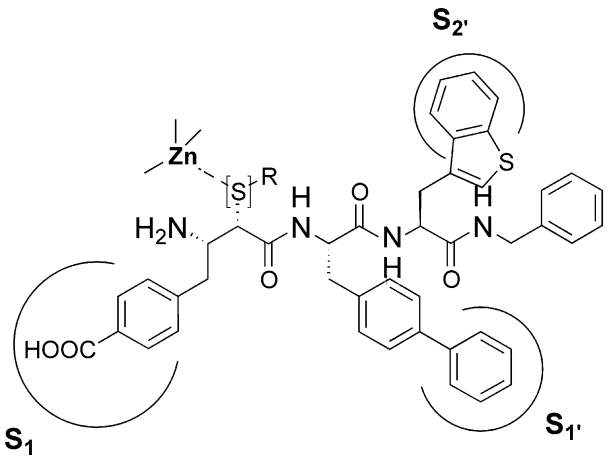


Figure 1. General structure (R = various substituents; $n = 0$, 1 or 2) and proposed mode for the interaction of potent inhibitors with BoNT/B LC. $S_{1'}$ $S_{1'}$ and $S_{2'}$ are schematic representations of subsites present in the BoNT/B LC active site which are supposed to complement the sequence surrounding the scissible bond (Glu^{76}-Phe^{77}) of the substrate synaptobrevin.

already described in the literature for copper Cu^{2+} ion[30,31] and zinc Zn^{2+}ion,[32] giving rise to weaker chelation, and thus affinity, in comparison with thiol **1**. A better complexation obviously enhances potency partially in the case of disulfides through either bi-chelation with two sulfurs or through the existence of an α-effect, reinforcing the nucleophilicity of one sulfur by the free pairs of the other one.

Conclusion

Although zinc chelation is strictly required for high inhibitory activity, the thiol functionality which generally provides an effective metal ion chelating group does not seem a prerequisite in our series of compounds for optimum BoNT/B metallopeptidase subunit recognition. Thus in disulfides the decrease of complexation with the metal as found in free thiol inhibitors could be compensated by multiplying interactions of the disulfide side chains with enzyme binding pocket subsites. These results suggest that BoNT/B recognition might be quite different from that of the other members of the zinc-metallopeptidase family as already assumed to explain the weak inhibitory potencies of classical zinc-metallopeptidase inhibitors on this toxin.[4,13–17] This is consistent with the mode of interaction of compounds such as BABIM studied by crystallography.[28] The insights provided by this study were taken into account for the further development of inhibitors with optimal adaptation to clefts and pockets of the enzyme active site and will be published shortly.

Experimental

Chemistry

^{1}H NMR spectra were measured on a Brucker AC 270MHz spectrometer using tetramethylsilane as internal standard. Electrospray mass spectra (MS-ES) were recorded on an Esquier-Brucker spectrometer. Flash column chromatography was performed using 40–63 μm silica gel. Reaction progress was determined by either TLC analysis or monitored using analytical reverse-phase HPLC (Shimadzu, LC10 AD-vp with a Class-VP5.03 software) using a Kromasil C_{18} column (100 Å, 5 μm, 250×4.6 mm, from Touzart-Matignon, France) with a mobile phase consisting of water containing 0.05% TFA (A), acetonitrile containing 0.05% TFA (B) or acetonitrile/water (9/1) containing 0.038% TFA (C). Preparative HPLC was performed on a Kromasil C_{18} column (100 Å, 5 μm, 250×20 mm). Reagents were obtained from commercial sources and are used without further purification.

4-[(2*S*,3*S*)-2-Amino-3-sulfanyl-4-[(1*S*)-2-[(1*S*)-1-(benzo[*b*]thiophen-3-ylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]amino]-4-oxobutyl]benzoic acid (2). 4-[(2*S*)-2-(1,1-Dimethylethyl) oxycarbonylamino-4-methoxy-4-oxo]benzoic acid-(1,1-dimethylethyl)ester was obtained as previously described[23] and treated overnight with NaOH (1.5 equiv) in a mixture of MeOH/H_2O at room temperature. The solvents were evaporated and H_2O (10 mL) was added. The pH was adjusted to 3 with an aqueous 10% citric acid solution and the product was extracted with EtOAc (3×20 mL). The combined organic layers were washed with water and brine, dried over Na_2SO4, filtered and evaporated to yield the corresponding acid as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.25 (s, 9H), 1.50 (s, 9H), 2.30 (m, 2H), 2.70 (m, 2H), 3.90 (m, 1H), 6.75 (d, 1H), 7.20 (d, 2H), 7.74 (d, 2H). The acid was subsequently coupled to dipeptide Bip-Bta-NHBn as previously reported.[23] The residual solid was purified by flash column chromatography with cHex/EtOAc (7/3) and treated for 4 h with trifluoroacetic acid. The solvent was evaporated and the product was freeze dried to yield compound **2** as a white solid. Yield 54%. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.3–3.3 (m, 8H), 3.48 (m, 1H), 4.2 (d, 2H), 4.55–4.75 (m, 2H), 7.0–7.45 (m, 19H), 7.7–7.95 (m, 6H), 8.4 (d, 1H), 8.5 (d, 1H), 8.55 (t, 1H). SM (ESI) $(M+H)^+$ m/z 739.3. HPLC (50% B): $R_t = 19.3$ min.

4-[(2*S*,3*S*)-2-Amino-3-(methylsulfanyl)-4-[(1*S*)-2-[(1*S*)-1-(benzo[b]thiophen-3-ylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]amino]-4-oxobutyl]benzoic acid (3). 4-[(2*S*)-2-(1,1-dimethylethyl)-oxycarbonylamino-4-methoxy-4-oxo]benzoic acid-(1,1-dimethylethyl)ester[23] was dissolved in anhydrous THF and treated at −78 °C with LDA which was freshly prepared from *n*BuLi (2 equiv) and diisopropylamine (2 equiv). MeI (1.1 equiv) was added and the mixture was allowed to warm to room temperature while stirring for 5 h. The solvent was then evaporated and EtOAc (10 mL) was added to the residue. The organic layer was washed with a 10% aqueous citric acid solution (3×10 mL), water, brine and the organic solution was dried over Na_2SO_4 and filtered. Evaporation of the solvent yielded a pale yellow oil which was chromatographed using cHex/EtOAc (9/1) as the eluting solvent. The resulting oil (yield 27%) was characterized by NMR: ^{1}H NMR ($CDCl_3$) δ 1.12 (d, 3H), 1.30 (s, 9H), 1.52 (s, 9H), 2.50 (m, 1H), 2.60–2.90 (m, 2H), 3.54 (s, 3H), 3.85 (m,

1H), 5.35 (d, 1H), 7.15 (d, 2H), 7.82 (d, 2H). The obtained intermediate was hydrolyzed and subsequently coupled with dipeptide Bip-Bta-NHBn followed by deprotection as above in the synthesis of compound **2** to give compound **3** as white solid. Yield 54%. ^{1}H NMR (DMSO-d_6 + TFA) δ 1.15–2.5 (m, 11H), 2.60–3.0 (m, 2H), 3.00–3.30 (m, 2H), 4.23 (d, 2H), 4.50 (m, 1H), 4.65 (m, 1H), 7.0-7.50 (m, 22H), 7.9 (m, 2H), 8.08 (bs, 1H), 8.45 (m, 2H). SM (ESI) $(M+H)^+$ m/z 753.3. HPLC (50% B): R_t = 19.7 min.

General procedure for the synthesis of compounds 4–7

Disulfides **4–7** were synthesized via dimerization of corresponding thiol monomers **1**, **8–10** which have been previously described.[23] Thiol compounds were dissolved in ethanol at a concentration of 1 mM. The mixture was stirred at room temperature and a solution of 0.25 M iodine in ethanol was added drop by drop until the solution became slightly yellow. After 15 min, the solvent was evaporated and the crude product was purified by preparative HPLC and freeze dried.

4,4′-Bis[disulfan-diyl[(2*S*,3*S*)-2-amino-3-[(1*S*)-2-[(1*S*)-1-(benzo[b]thiophen-3-ylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]carbamoyl]propane-1,3-diyl]dibenzoic acid (4). Yield 71%. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.8 (m, 3H), 3.1 (m, 2H), 3.25 (m, 1H), 3.8 (d, 1H), 3.9 (m, 1H), 4.2 (d, 2H), 4.75 (m, 2H), 7.0-7.50 (m, 20H), 7.80 (2d, 3H), 8.00 (m, 3H), 8.80 (m, 3H). SM (ESI) $(M+H)^+$ m/z 1541.0. HPLC (gradient 40–100% C in 20 min): R_t = 16.1 min.

4,4′-Bis[disulfan-diyl[(2*S*,3*S*)-2-amino-3-[(1*S*)-2-[(1*S*)-1-(phenylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]carbamoyl]propane-1,3-diyl]dibenzoic acid (5). Yield 58%. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.70 (m, 1H), 2.80 (m, 2H), 3.0 (m, 2H), 3.75 (d, 1H), 3.90 (m, 1H), 4.20 (d, 2H), 4.65 (m, 2H), 7.0-7.50 (m, 22H), 7.80 (d, 2H), 8.0 (m, 3H), 8.60 (t, 1H), 8.65 (d, 1H), 8.70 (d, 1H). SM (ESI) $(M+H)^+$ m/z 1429.8. HPLC (A/C: 30/70): R_t = 6.4 min.

4,4′-Bis[disulfan-diyl[(2*S*,3*S*)-2-amino-3-[(1*S*)-2-[(1*S*)-1-(but-2-ylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]carbamoyl]propane-1,3-diyl]dibenzoic acid (6). Yield 66%. ^{1}H NMR (DMSO-d_6 + TFA) δ 0.85 (m, 6H), 1.1 (m, 1H), 1.45 (m, 1H), 1.70 (m, 1H), 2.80–3.10 (m, 4H), 3.80 (d, 1H), 4.0 (m, 1H), 4.30 (m, 3H), 4.80 (m, 1H), 7.10–7.50 (m, 16H), 7.80 (d, 2H), 8.0 (m, 3H), 8.40 (d, 1H), 8.60 (t, 1H), 8.75 (d, 1H). SM (ESI) $(M+H)^+$ m/z = 1360.2. HPLC (gradient 40–100% C in 20 min): R_t = 13.6 min.

4,4′-Bis[disulfan-diyl[(2*S*,3*S*)-2-amino-3-[(1*S*)-2-[(1*S*)-1-(4-hydroxyphenylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]carbamoyl]propane-1,3-diyl]dibenzoic acid (7). Yield 60%. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.70 (m, 1H), 2.80 (m, 2H), 3.10 (m, 2H), 3.30 (m, 1H), 3.80 (d, 1H), 3.90 (m, 1H), 4.20 (d, 2H), 4.75 (m, 2H), 7.0–7.50 (m, 20H), 7.75 (2d, 3H), 8.0 (m, 3H), 8.60 (t, 1H), 8.70 (d, 2H). SM (ESI) $(M+H)^+$ m/z 1461.1. HPLC (gradient 40–100% C in 20 min): R_t = 9.5 min.

4-[(2*S*,3*S*)-2-Amino-3-(benzyldisulfanyl)-4-[(1*S*)-2-[(1*S*)-1-(benzo[b]thiophen-3-ylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]amino]-4-oxobutyl]benzoic acid (11). To a solution of **1** (0.01 mmol) and triethylamine (0.012 mmol) in 2 mL of chloroform was added benzylsulfanyl-*O*-methyl thiocarbonate[33] (0.011 mmol) at room temperature. The reaction was stirred at room temperature for 3 h. After evaporation in vacuo, the residue was purified by semi preparative HPLC and freeze dried.

Yield 44%. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.70 (m, 1H), 2.80 (m, 2H), 3.10 (m, 3H), 3.30 (m, 2H), 3.60 (d, 1H), 3.70 (m, 1H), 3.80 (q, 2H), 4.25 (d, 2H), 4.75 (m, 1H), 4.85 (m, 1H), 7.10–7.50 (m, 24H), 7.80 (m, 2H), 8.0 (m, 3H), 8.70 (t, 1H), 8.80 (d, 1H), 8.90 (d, 1H). HPLC (gradient 40–100% C in 20 min): R_t = 16.0 min.

4-[(2*S*,3*S*)-2-Amino-3-(4-methoxybenzylsulfanyl)-4-[(1*S*)-2-[(1*S*)-1-(benzo[b]thiophen-3-ylmethyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-ylmethyl)-2-oxoethyl]amino]-4-oxobutyl]benzoic acid (12). The synthetic precursor[23] of compound **1** (0.03 mmol) was stirred at 0 °C for 1 h with 1 mL of trifluoroacetic acid and 0.1 mL of triisopropylsilane. After evaporation, the residue was purified by semi-preparative HPLC and freeze-dried.

Yield 52%. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.65 (m, 1H), 2.85 (m, 2H), 3.10 (m, 2H), 3.30 (m, 2H), 3.40 (m, 2H), 3.60 (m, 4H), 4.25 (d, 2H), 4.75 (m, 2H), 6.60 (d, 2H), 6.80 (d, 2H), 7.10–7.45 (m, 19H), 7.80 (2d, 3H), 7.90 (m, 3H), 8.0 (d, 1H), 8.60 (d, 1H), 8.70 (m, 2H).

^{13}C NMR (DMSO-d_6) δ31.4, 35.5, 37.0, 37.8, 42.0, 47.0, 53.0, 54.0, 55.0, 114.0, 122.0, 123.0, 123.8, 124.0, 126.4, 126.9, 127.1, 128.3, 128.6, 128.8, 129.6, 129.6, 129.9, 130.1, 131.8, 136.8, 138.0, 138.5, 139.0, 139.4, 139.8, 140.6, 158.0, 167.0, 169.0, 171.0. SM (ESI) $(M+H)^+$ m/z 1005.2. HPLC (A/B: 15/85): R_t = 6.5 min.

Enzyme assay

The inhibitory potencies of all compounds were measured by using the light chain of BoNT/B as previously described[27] with some slight modifications. Briefly, BoNT/B LC (0.35 ng) was preincubated for 30 min at 37 °C in 90 μL of 20 mM Hepes, pH 7.4 with increasing concentrations (10^{-10}–10^{-5} M) of inhibitor. Compounds containing a free thiol group were tested in the presence of 0.1 mM DTT in order to avoid oxidation. All other compounds were tested in the absence of DTT. The fluorescent substrate Syb 60-94 [Pya^{74}-Nop^{77}] (K_m = 47 μM) was then incubated for 30 min and the reaction stopped by addition of 0.2 N HCl at 4 °C. Verification through HPLC analysis showed no reduction in situ of compounds containing disulfide bridges (data not shown). The percentage of degradation was measured directly in a 96-well plate. The IC_{50} values were determined from logarithmic dose–degradation curves and the values of the inhibitory constant (K_i) of compounds **1–12** were calculated according to the equation of Cheng and Prusoff and are expressed as the mean ± SEM of three separate experiments each in triplicate.

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