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Small tripeptide surrogates with low nanomolar affinity as potent inhibitors of the botulinum neurotoxin B metallo-proteolytic activity

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Abstract—Botulinum neurotoxin type B is a high-weight (150 kDa) protein produced by the anaerobic bacillus *Clostridium botulinum*. This metallo-protease neurotoxin cleaves synaptobrevin, a protein, which is crucial to neurotransmission, resulting in the muscle paralysis, which characterizes botulism. Inhibition of the metallo-peptidase activity is a possible approach to obtain specific therapeutics to treat botulism. We have previously reported a successful attempt to block the proteolytic activity of this neurotoxin with new, selective amino-thiol inhibitors endowed with K_i values in the 15–20 nanomolar range. With the aim of increasing the affinity and bioavailability of this first series of inhibitors we have optimized the residue that fits the P_1 subsite of the enzyme by comparing a series of ligands that contain subtle but significant variants of the parent structure. In addition, this strategy provided a simplification of the synthesis of BoNT/B inhibitors by reducing the possible number of stereoisomers. As such we were able to enhance the inhibitory potency whilst reducing the size as compared to the initial privileged structure yielding the first pseudo-tripeptide inhibitors with K_i values in the low nanomolar range.

1. Introduction

Among the seven serotypes of botulinum neurotoxins (BoNT/A-G), neurotoxin type B, which is composed of a heavy chain of 100 kDa linked by a disulfide bridge to a light chain of 50 kDa specifically cleaves synaptobrevin, a membrane fusion protein, which is implicated in neuronal exocytosis.[1,2] Whereas the heavy chain is involved in neuronal cell adhesion, internalization and translocation, the light chain, which was shown to exhibit a zinc-dependent proteolytic activity is responsible for the hydrolysis of the Gln^{76}-Phe^{77} synaptobrevin peptide bond resulting in the blockade of excitatory neurotransmitter release causing the muscle paralysis, which is typical for botulism.[3–6]

The ability to selectively inhibit the enzymatic activity of the light chain, as for the lethal factor of *Bacillus antracis*,[7] has been postulated to present an attractive therapeutic approach for both the treatment following toxin exposure as well as to secure the use of botulinum toxin B as a therapeutical agent.[8] We therefore focussed our initial efforts on the design and synthesis of potent and selective inhibitors of the BoNT/B proteolytic activity. Such compounds were previously obtained by our group through screening available low molecular weight β-amino thiol derivatives, which were supposed to occupy the S_1 subsite and chelate the zinc atom.[9] The best β-amino thiol derivative with a K_i value of 11 μM was incorporated in extended peptidic structures in order to improve potency through putative interactions with S_1' and S_2' subsites of the BoNT/B light chain (LC) active site.[10,11] Amino acid residues were subsequently optimized to allow for the highest probability of enzymatic recognition at the S′ subsites only, yielding lead compound **1** with a K_i value of 20 nM (Fig. 1).[9]

In order to extend our study on these inhibitors we report here some structural modifications of the S_1 subsite molecular recognition group for optimal adaption to clefts and pockets of the toxin active site.[10,11] As such the functional groups at three different sites of the substituted β-amino thiol moiety were either one-by-one or simultaneously, eliminated or replaced. All compounds were evaluated[12] for their ability to block the proteolytic activity of the BoNT/B LC revealing molecules with inhibitory potencies in the low nanomolar range.

Keywords: Botulinum neurotoxin B; Metallo-peptidase inhibitors.
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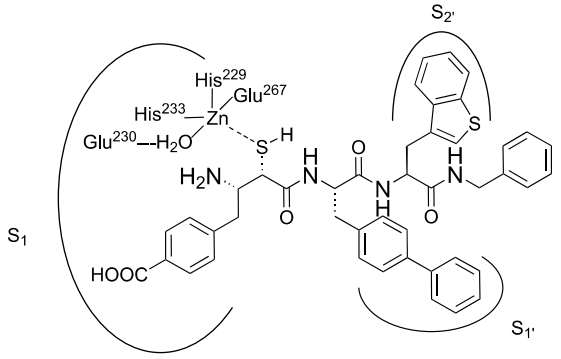


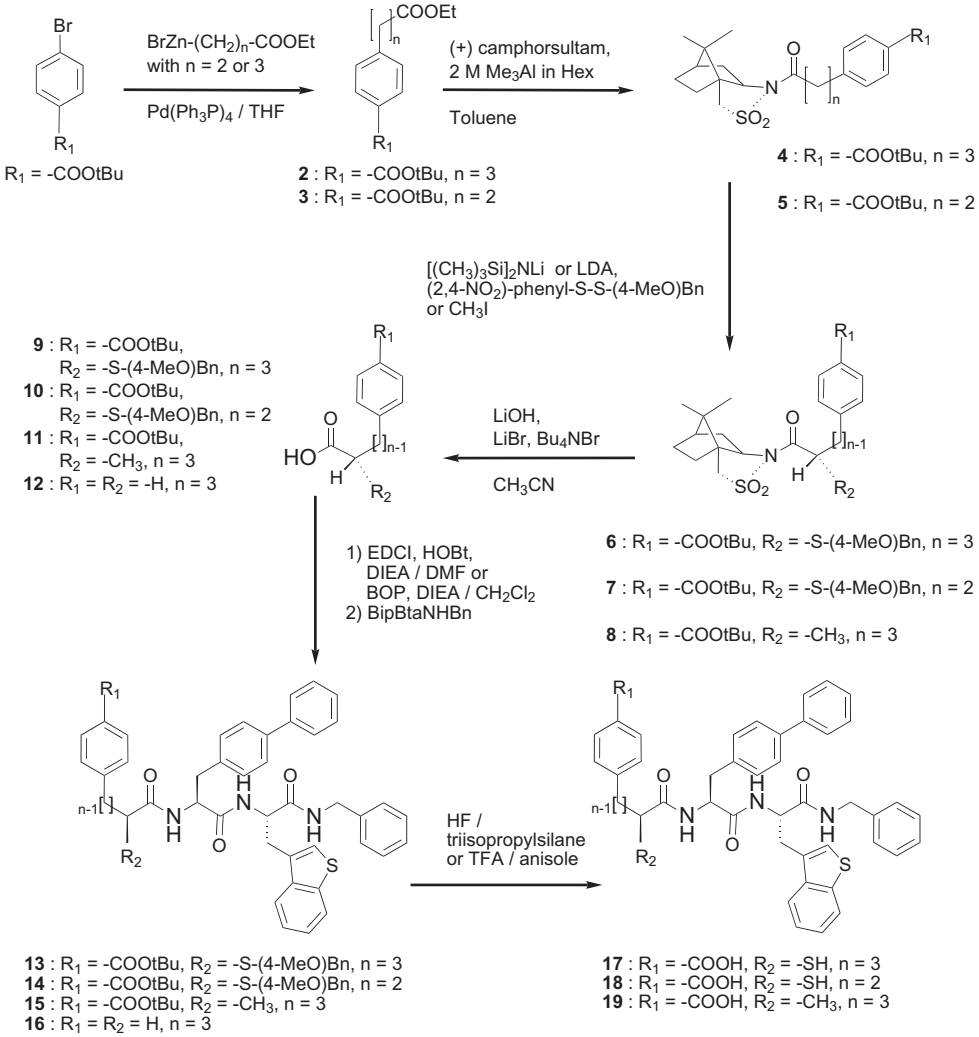
Figure 1. Proposed mode for the interaction of lead compound **1** with BoNT/B LC. S_1, S_1' and S_2' are schematic representations of subsites present in the BoNT/B LC active site, which are supposed to complement the sequence surrounding the scissible bond (Glu^{76}-Phe^{77}) of the substrate synaptobrevin. The numbering of amino acids around the zinc atom correspond to residues of BoNT/B LC.

2. Results and discussion

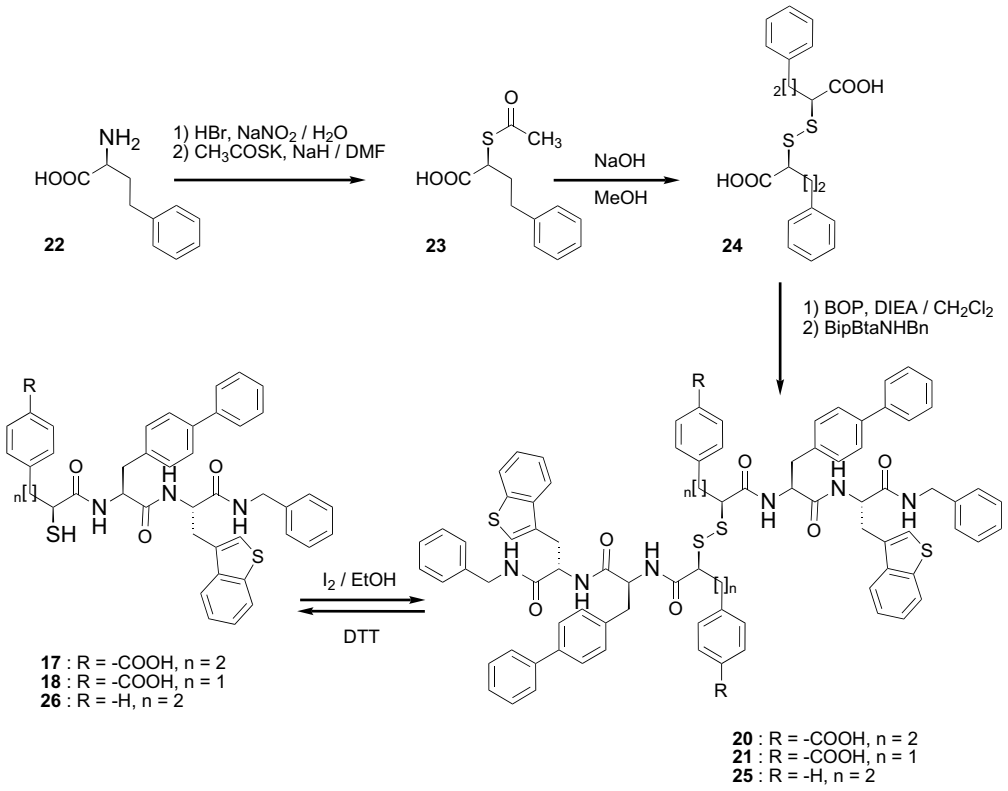
The values of the BoNT/B LC binding affinities of the newly synthesized compounds (Schemes 1 and 2) as well as lead compound **1** are listed in Table 1. As the β-amino thiol functionality, which presents an efficient metal-ion chelator is a key structural constituent of a wide variety of bioactive metallo-peptidase inhibitors we initially imagined that both the free primary amino- and the thiol-group could be of critical importance in our series of compounds.[14]

At least the presence of a sulfur atom was previously shown to be important for zinc chelation in this series of compounds[13] and we therefore eliminated the amino functionality of compound **1** with the aim to investigate the impact of this substituent on enzyme recognition. The synthesis of compound **17** and several structural analogues is outlined in Scheme 1.

The key step of the generally applicable synthetic pathway depicted in Scheme 1 consisted of either a stereospecific sulfenylation with 1-(4-methoxy-benzyl)disulfanyl-2,4-dinitrobenzene[15] or a stereospecific alkylation with methyliodide of the camphorsultam[16] containing precursors **4** and **5**. These compounds were appropriately prepared from commercially available 4-bromobenzoic acid *tert*-butyl ester through aromatic

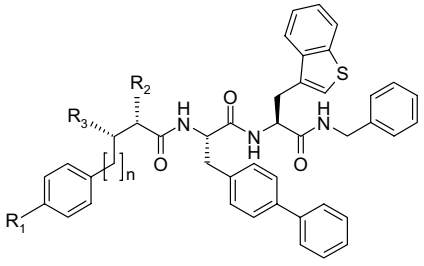


Scheme 1. Synthetic pathway of compounds **16**–**19**.



Scheme 2. Synthetic pathway of compounds **20**, **21**, **25** and **26**.

Table 1. BoNT/B LC inhibitory potencies of compounds **1**, **16**–**21**, **25** and **26**



Compound	R_1	R_2[a]	R_3	*n*	$K_i \pm$ SEM[b] (nM)
1	–COOH	–SH	$-NH_2$	1	20 ± 2
16	–H	–H	–H	1	871 ± 214
17	–COOH	–SH	–H	1	4.7 ± 1.0
18	–COOH	–SH	–H	0	3.3 ± 0.3
19	–COOH	$-CH_3$	–H	1	37 ± 3
20	–COOH	$-S)_2$	–H	1	2.3 ± 1.1
21	–COOH	$-S)_2$	–H	0	5.4 ± 0.8
25[c]	–H	$-S)_2$	–H	1	70 ± 14
26	–H	–SH	–H	1	76 ± 9

[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] This compound was tested as a mixture of three diastereomers in the ratio 16/23/11.

substitution with either 3-ethoxy-3-oxopropyl zinc bromide or 4-ethoxy-4-oxobutyl zinc bromide. Subsequent cleavage of the source of chirality, camphorsultam, in aprotic medium by a phase transfer catalysis method[17] afforded the intermediates **9**–**11** as pure *S*-enantiomers. Standard peptide coupling of acids **9**–**11** and commercially available 4-phenyl-butanoic acid (**12**) with the previously described[9] dipeptide Bip-Bta-NHBn yielded the corresponding pure *SSS*-diastereomers **13**–**16**. Final deprotection was subsequently conducted with either anhydrous hydrogen fluoride for the simultaneous removal of both the 4-methoxybenzyl group and the *tert*-butyl ester to give compounds **17** and **18** or by using trifluoroacetic acid in the case of just a *tert*-butyl ester (compound **19**).

As shown in Table 1, compound **17** displays a 4-fold better inhibitory potency as compared to **1**. This gain of activity indicates that metal complexation does imply the thiol group alone rather then the entire β-amino thiol moiety. As good affinity does not require the presence of a primary amino group we therefore discarded this functionality in studying any other structural modifications. Removal of the primary amino group does not only afford a gain in affinity but also implies a simplification of synthesis due to the elimination of one chiral center as compared to lead compound **1**.[9]

Compound **18** was prepared in order to examine the importance of the relative positioning of the benzoic acid moiety and the thiol group. This was achieved by prolonging the backbone with an additional methylene unit. No substantial gain, nor loss in activity was observed for compound **18** as compared to **17** indicating that some flexibility is permitted for this key residue,

which most probably interacts inside the large open cavity around the zinc binding domain as does the full length substrate synaptobrevin.[10]

The thiol functionality of compound **17** was replaced with a methyl group (compound **19**), which showed a 8-fold loss of activity, which is not withstanding earlier work[13] indicating the importance of the thiol functionality. Elimination of the carboxylic group on the other hand resulted in a 16-fold loss of affinity as displayed by compound **26**, which was, for synthetic convenience, obtained in only few steps but with poor optical activity by an alternative pathway as compared to Scheme 1. Indeed compound **26** was obtained and evaluated for its biological activity through reduction with dithiothrietol (DTT) of its corresponding disulfide analogue **25** as outlined in Scheme 2. Compound **25** was prepared by peptide coupling of the dipeptide Bip-Bta-NHBn[9] with the symmetrical acid **24**, which was readily available in our laboratory as it had been previously prepared from D-homophenylalanine **22**.

Although displaying a loss as compared to compound **17**, the affinities of both **19** and **26** remain still considerable considering the lack of one functional group in these short molecules as compared to previously reported analogues[9,13] and may suggest that other functionalities are optimum for enzyme recognition. Nevertheless, the need of at least one of these groups, which are expected to interact with the P_1 subsite, is illustrated by the simultaneous elimination of both the thiol group and the carboxylic acid moiety in compound **16**, which resulted in a drastic loss of affinity ($K_i = 871$ nM). Moreover it had been shown in a previous study[13] that the symmetric disulfide analogue of **1** displays a 6-fold better affinity than its parent compound (**1**) indicating that these larger double-sized molecules might complement several supplementary subsites of the protein binding pocket as compared to shorter sequences. Therefore the disulfide analogue **20** of compound **17** was prepared as outlined in Scheme 2 through oxidation with iodide. This compound did however not show any substantial gain in activity. This could be explained by the absence in **20** of the primary amino group, which could result in optimum enzyme subsite recognition for the shorter sequences.[13] This observation was confirmed by the synthesis and biological evaluation of another symmetric disulfide (**21**), which was obtained from the pseudo-tripeptide **18**. Similarly **25,** a less potent compound lacking the primary amino group showed a comparable affinity with its sulfanyl analogue **26**. Nevertheless one cannot eliminate the possibility that the mode of recognition of neurotoxin-associated metallo-peptidases could be completely different from those of the large number of enzymes belonging to this class of peptidases. This could also account for the somewhat striking SAR observed with our inhibitors.

3. Conclusion

Whereas a primary amino group was previously shown to be important in larger pseudo-hexapeptide sequences for good recognition of the metallo-peptidase subunit of botulinum neurotoxin B, it was demonstrated in this study that the elimination of the amine permitted a significant gain of affinity for the botulinum B active site. As such the best inhibitors yet reported have been obtained while maintaining small sized molecules with low molecular masses.

The different serotypes of the botulinum neurotoxins are all characterized by high substrate specificity and rather long substrate length requirements[18–22] as the proteolytic activity of the enzyme light chains is triggered by recognition and binding of two distinct regions of the substrate.[23] The design of small and potent inhibitors against botulinum neurotoxin catalytic domains in general is therefore difficult but not impossible as shown in the case of our new pseudo-tripeptides. These molecules are the most potent inhibitors of the botulinum neurotoxin B light chain reported to date and as such may be helpful not only in the search for pharmaceuticals able to counteract the botulinum neurotoxin B activity but also for the design and synthesis of inhibitors of other serotypes of botulinum neurotoxins.

4. Experimental

4.1. Chemistry

^{1}H NMR spectra were measured on a Bruker AC 270 MHz spectrometer using tetramethylsilane as internal standard. Electrospray mass spectra (MS-ES) were recorded on a 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% trifluoroacetic acid (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. General procedure for the synthesis of compounds 2 and 3

To a solution of tetrakis(triphenylphosphine)palladium (1.45 g, 1.25 mmol) and 4-bromo-benzoic acid *tert*-butyl ester (6.00 g, 23.34 mmol) in 50 mL dry tetrahydrofuran (THF) was added at room temperature under argon a solution of either 0.5 M 3-ethoxy-3-oxopropyl zinc bromide or 0.5 M 4-ethoxy-4-oxobutyl zinc bromide in THF (50 mL, 25 mmol). The mixture was stirred under argon for 48 h. The reaction was quenched at room temperature with 50 mL of an aqueous saturated solution of ammonium chloride. Diethyloxide (100 mL) was added and the organic layer was separated after extraction and washed with water and brine. After evaporation in vacuo

the residue was purified by column chromatography on silica gel with 25% of ethylacetate in cyclohexane to give the title compounds as colourless oils.

4.2.1. 4-(3-Ethoxycarbonyl-propyl)-benzoic acid *tert*-butyl ester (2). Yield 76% (5.22 g). TLC (cyclohexane/ethylacetate, 75/25): $R_f = 0.4$. ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H), 1.5 (s, 9H), 1.8 (m, 2H), 2.2 (t, 2H), 2.6 (t, 2H), 4.0 (q, 2H), 7.25 (d, 2H), 7.8 (d, 2H).

4.2.2. 4-(2-Ethoxycarbonyl-ethyl)-benzoic acid *tert*-butyl ester (3). Yield 55% (3.56 g). TLC (cyclohexane/ethylacetate, 85/15): $R_f = 0.32$. ^{1}H NMR (DMSO-d_6) δ 1.1 (t, 3H), 1.5 (s, 9H), 2.6 (t, 2H), 2.85 (t, 2H), 4.0 (q, 2H), 7.3 (d, 2H), 7.8 (d, 2H).

4.2.3. 4-[4-(10,10-Dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)-4-oxo-butyl]-benzoic acid *tert*-butyl ester (4). To a solution of (+)-camphorsultam (2.63 g, 12.2 mmol) in toluene (25 mL) at 0 °C was added 2 M trimethylaluminium in hexane (7.4 mL, 14.7 mmol). The mixture was stirred 15 min at room temperature and a solution of **2** (5.00 g, 17.12 mmol) in toluene (10 mL) was added dropwise. The mixture was stirred over night at 50 °C. The reaction was quenched at 0 °C with 15 mL of a 0.5 N aqueous solution of hydrochloric acid and then 30 mL of ethylacetate were added. The organic layer was separated after extraction and was washed with water and brine. After evaporation in vacuo the residue was purified by column chromatography on silica gel with 15% of ethylacetate in cyclohexane to give 3.53 g of compound **4** as a colourless oil, which crystallized on standing.

Yield 63%. TLC (cyclohexane/ethylacetate, 75/25): $R_f = 0.25$, mp = 125–127 °C. $[\alpha]_D^{22}$ 65.6 (*c* 1.3, CH_3CN). HPLC (A/B, 10/90): $t_R = 5.72$ min. ^{1}H NMR (DMSO-d_6) δ 0.85 (s, 3H), 1.0 (s, 3H), 1.2 (m, 1H), 1.35 (m, 1H), 1.5 (s, 9H), 1.8 (m, 7H), 2.6 (m, 4H), 3.7 (dd, 2H), 3.8 (d, 1H), 7.2 (d, 2H), 7.75 (d, 2H).

4.2.4. 4-[3-(10,10-Dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)-3-oxo-propyl]-benzoic acid *tert*-butyl ester (5). To a solution of (+)-camphorsultam (1.72 g, 8 mmol) in toluene (16 mL) at 0 °C was added 2 M trimethylaluminium in hexane (4 mL, 9.36 mmol). The mixture was stirred 15 min at room temperature and then a solution of **3** (1.85 g, 6.65 mmol) in toluene (7 mL) was added dropwise. The mixture was stirred over night at 50 °C and the reaction was treated as reported above for compound **4** to give 1.9 g of compound **5** as a colourless oil, which crystallized on standing.

Yield 64%. TLC (*n*-heptane/ethylacetate, 75/25): $R_f = 0.29$, mp = 132–134 °C. $[\alpha]_D^{22}$ 130.7 (*c* 1.13, CH_2Cl_2). HPLC (A/B, 10/90): $t_R = 5.37$ min. ^{1}H NMR (DMSO-d_6) δ 0.85 (s, 3H), 0.95 (s, 3H), 1.2 (m, 1H), 1.4 (m, 1H), 1.5 (s, 9H), 1.7 (m, 3H), 1.85 (m, 2H), 2.9 (m, 4H), 3.7 (dd, 2H), 3.75 (d, 1H), 7.2 (d, 2H), 7.75 (d, 2H).

4.2.5. 4-[(3*S*)-4-(10,10-Dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)-3-(4-methoxy-benzyl-sulfanyl)-4-oxo-butyl]-benzoic acid *tert*-butyl ester (6). To a solution of 1,1,1,3,3,3-hexamethyldisilazane (1.84 mL, 8.64 mmol) in dry THF (8 mL) at 0 °C under argon was added *n*-butyllithium (1.6 M in hexanes, 6 mL, 9.6 mmol). The mixture was stirred at 0 °C during 20 min. To a solution of **4** (1.0 g, 2.16 mmol) in dry THF (4 mL) at −78 °C under argon was added a volume of 7 mL of the previously prepared solution of lithium bis(trimethylsilyl)amide. The mixture was stirred at −78 °C during 30 min and 1-(4-methoxy-benzyldisulfanyl)-2,4-dinitrobenzene[15] (0.92 g, 2.59 mmol) was added. The reaction was subsequently stirred at −78 °C for 2 h and quenched at 0 °C with 10 mL of an aqueous saturated solution of ammonium chloride. The mixture was evaporated in vacuo and the crude oil was purified by semi-preparative HPLC yielding 0.54 g of the title compound as a colourless oil.

Yield = 41%. $[\alpha]_D^{22}$ −8.31 (*c* 1.7, CH_3CN). HPLC (A/B, 10/90): $t_R = 6.68$ min. ^{1}H NMR (DMSO-d_6) δ 0.90 (s, 3H), 1.1 (m, 5H), 1.40 (m, 2H), 1.5 (s, 9H), 1.7–2.2 (m, 9H), 2.6 (m, 2H), 3.65 (m, 4H), 3.8 (m, 4H), 6.8 (d, 2H), 7.15 (2d, 4H), 7.8 (d, 2H). MS (ESI) $(M+Na)^+$ $m/z = 636.3$, $(2M+Na)^+$ $m/z = 1249.4$.

4.2.6. 4-[(2*S*)-3-(10,10-Dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)-2-(4-methoxy-benzylsulfanyl)-3-oxo-propyl]-benzoic acid *tert*-butyl ester (7). To a solution of diisopropylamine (0.7 mL, 5 mmol) in dry THF (5 mL) at 0 °C under argon was added *n*-butyllithium (1.6 M in hexanes, 3.75 mL, 6.0 mmol). The mixture was stirred at 0 °C during 20 min. To a solution of **5** (0.25 g, 0.56 mmol) in dry THF (2 mL) at −78 °C under argon was added a volume of 2 mL of the previously prepared solution of lithium diisopropylamide. The mixture was stirred at −78 °C during 1 h and 1-(4-methoxy-benzyldisulfanyl)-2,4-dinitrobenzene[15] (0.24 g, 0.67 mmol) was added. The reaction was subsequently stirred at −78 °C for 90 min and quenched at 0 °C with 10 mL of an aqueous saturated solution of ammonium chloride. The mixture was evaporated in vacuo and the crude oil was purified by semi-preparative HPLC yielding 0.15 g of the title compound as a colourless oil.

Yield = 44%. $[\alpha]_D^{22}$ −43.2 (*c* 1.44, $CHCl_3$). HPLC (A/C, 10/90 during 12 min then A/C, 0/100 during 10 min): $t_R = 13.52$ min. ^{1}H NMR (DMSO-d_6) δ 0.95 (s, 6H), 1.1 (m, 4H), 1.5 (s, 9H), 1.7 (m, 5H), 2.9 (m, 1H), 3.2 (m, 1H), 3.8 (m, 7H), 6.8 (d, 2H), 7.15 (2d, 4H), 7.8 (d, 2H). MS (ESI) $(M+Na)^+$ $m/z = 622.3$, $(2M+Na)^+$ $m/z = 1221.3$.

4.2.7. 4-[(3*R*)-4-(10,10-Dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)-3-methyl-4-oxo-butyl]-benzoic acid *tert*-butyl ester (8). Compound **8** was prepared from compound **4** as described above using the procedure for compound **7** with methyliodide as the alkylating agent to yield 0.142 g of the title compound as a colourless oil.

Yield = 48%. TLC (cyclohexane/ethylacetate, 60/40): $R_f = 0.62$. ^{1}H NMR (DMSO-d_6) 0.85 (s, 3H), 0.95 (s, 3H), 1.1–1.4 (m, 6H), 1.5 (s, 9H), 1.6–2.0 (m, 8H), 2.5 (m, 2H), 3.0 (m, 1H), 3.7 (m, 2H), 7.2 (d, 2H), 7.8 (2d, 2H).

4.3. General procedure for synthesis of compounds 9–11

A solution of **6**, **7** or **8** (1 equiv), lithium hydroxide (4 equiv), lithium bromide (5 equiv), tetrabutylammonium bromide (0.4 equiv) in acetonitrile (10 mL/mmol of substrate) was stirred over night at room temperature. The reaction was quenched with 10 mL of a 0.1 N aqueous solution of hydrochloric acid and extracted with 20 mL of ethylacetate. The organic layer washed with water and brine. After evaporation in vacuo, the crude oil was purified by semi-preparative HPLC yielding the title compounds as colourless oils.

4.3.1. 4-[(3*S*)-3-Carboxy-3-(4-methoxy-benzylsulfanyl)-propyl]-benzoic acid *tert*-butyl ester (9). 0.190 g (90%). $[\alpha]_D^{22}$ −89.46 (*c* 0.90, CH_3CN). HPLC (A/B, 10/90): $t_R = 4.31$ min. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 9H), 1.7 (m, 1H), 1.95 (m, 1H), 2.6 (m, 2H), 3.0 (t, 1H), 3.7 (m, 5H), 3.75 (d, 2H), 6.80 (d, 2H), 7.1 (2d, 4H), 7.70 (d, 2H). MS (ESI) $(M+Na)^+$ $m/z = 439.1$.

4.3.2. 4-[(2*S*)-2-Carboxy-2-(4-methoxy-benzylsulfanyl)-ethyl]-benzoic acid *tert*-butyl ester (10). 0.088 g (73%). $[\alpha]_D^{22}$ −132.66 (*c* 0.88, $CHCl_3$). HPLC (A/C, 20/80): $t_R = 8.2$ min. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 9H), 2.85 (dd, 1H), 3.1 (dd, 1H), 3.4 (m, 1H), 3.7 (s, 3H), 3.75 (d, 2H), 6.80 (d, 2H), 7.15 (d, 2H), 7.25 (d, 2H), 7.75 (d, 2H). MS (ESI) $(M+Na)^+$ $m/z = 425.2$, $(2M+Na)^+$ $m/z = 827.1$.

4.3.3. 4-[(3*R*)-3-Carboxy-3-methyl-propyl]-benzoic acid *tert*-butyl ester (11). 0.060 g (79%). TLC (cyclohexane/ethylacetate, 60/40): $R_f = 0.40$. ^{1}H NMR (DMSO-d_6) δ 1.15 (d, 3H), 1.5 (s, 9H), 1.7 (m, 1H), 1.95 (m, 1H), 2.45 (m, 1H), 2.55 (m, 2H), 7.15 (d, 2H), 7.80 (d, 2H).

4.4. General procedure for the synthesis of compounds 13–16

To a solution of **9**, **10**, **11** or **12** (1 equiv) in either dimethylformamide (DMF) or dichloromethane (10 mL/mmol) was added successively 1.1 equiv of the dipeptide Bip-Bta-NHBn,[9] 1.1 equiv of coupling agent, either BOP (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium-hexafluorophosphate or EDCI (1-(3-dimethylamino-propyl)-3-ethylcarbodiimide) and 3 equiv of diisopropylethylamine (DIEA). The mixture was stirred for 3 h at room temperature. After removal of the solvent, the crude solid was successively washed with aqueous 10% $KHSO_4$ solution, water and diethylether. The obtained products were dried, characterized and used without further purification.

4.4.1. 4-[(3*S*)-3-[(1*S*)-1-((1*S*)-2-Benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl)-2-biphenyl-4-yl-ethyl-carbamoyl]-3-(4-methoxy-benzylsulfanyl)-propyl]-benzoic acid *tert*-butyl ester (13). 0.182 g (85%). HPLC (A/B, 10/90): $t_R = 7.07$ min. ^{1}H NMR (DMSO-d_6+TFA) δ 1.5 (s, 9H), 1.65 (m, 1H), 1.85 (m, 1H), 2.65 (m, 4H), 3.20 (m, 4H), 3.60 (s, 3H), 4.20 (m, 2H), 4.70 (m, 2H), 6.60 (d, 2H), 6.90 (d, 2H), 7.25 (m, 20H), 7.70 (d, 2H), 7.90 (2d, 2H), 8.25 (d, 1H), 8.45 (d, 1H), 8.55 (t, 1H). MS (ESI) $(M+Na)^+$ $m/z = 955.5$, $(2M+Na)^+$ $m/z = 1887.5$.

4.4.2. 4-[(2*S*)-2-[(1*S*)-1-((1*S*)-2-Benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl)-2-biphenyl-4-yl-ethyl-carbamoyl]-2-(4-methoxy-benzylsulfanyl)-ethyl]-benzoic acid *tert*-butyl ester (14). 0.170 g (93%). HPLC (A/C, 20/80 during 12 min then A/C, 0/100 during 15 min): $t_R = 20.0$ min. ^{1}H NMR (DMSO-d_6+TFA) δ 1.4 (s, 9H), 2.75 (m, 4H), 3.0–3.40 (m, 4H), 3.60 (m, 4H), 4.20 (d, 2H), 4.70 (m, 2H), 6.60 (d, 2H), 6.90 (d, 2H), 7.15 (m, 7H), 7.40 (m, 12H), 7.65 (d, 2H), 7.90 (d, 2H), 8.30 (d, 1H), 8.50 (m, 2H). MS (ESI) $(M+Na)^+$ $m/z = 940.5$, $(2M+Na)^+$ $m/z = 1857.4$.

4.4.3. 4-[(3*R*)-3-[(1*S*)-1-((1*S*)-2-Benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl)-2-biphenyl-4-yl-ethyl-carbamoyl]-3-methyl-propyl]-benzoic acid *tert*-butyl ester (15). 0.038 g (48%). ^{1}H NMR (DMSO-d_6+TFA) δ 1.3 (d, 3H), 1.45 (s, 1H), 1.65 (m, 1H), 2.15 (m, 1H), 2.6–3.3 (m, 6H), 4.15 (d, 2H), 4.60 (m, 2H), 6.1–7.5 (m, 17H), 7.65 (d, 2H), 7.80 (m, 2H), 8.0 (d, 1H), 8.2 (d, 1H), 8.45 (t, 1H).

4.4.4. *N*-{(2*S*)-2-[(1*S*)-1-((1*S*)-2-Benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl)-2-biphenyl-4-yl-ethyl}-4-phenyl-butanamide (16). 0.042 g (44%). HPLC (A/C, 30/70 during 12 min then A/C, 0/100 during 15 min): $t_R = 9.0$ min. ^{1}H NMR (DMSO-d_6+TFA) δ 1.6 (m, 2H), 2.0 (t, 2H), 2.32 (t, 2H), 2.70 (m, 1H), 2.95 (m, 1H), 3.10 (m, 1H), 3.25 (m, 1H), 4.20 (d, 2H), 4.55 (m, 1H), 4.65 (m, 1H), 7.0–7.5 (m, 18H), 7.90 (d, 2H), 8.05 (d, 1H), 8.30 (d, 1H), 8.45 (t, 1H). MS (ESI) $(M+H)^+$ $m/z = 680.86$.

4.5. General procedure for synthesis of compounds 17 and 18

The compounds **13** or **14** (0.1 mmol) were stirred at 0 °C for 1 h with 10 mL of anhydrous hydrogen fluoride (HF) and 0.15 mL of triisopropylsilane. After evaporation of HF, the residue was taken up with TFA and precipitated with a cold mixture of ether/*n*-hexane (1/1). After centrifugation, the precipitate was taken up with water and freeze dried. The product was finally purified by semi-preparative HPLC on Kromasil C_{18} column (100 Å, 5 μm, 250 × 20 mm). Analytical HPLC and RMN analysis confirmed the presence of one single diastereoisomer.

4.5.1. 4-[(3*S*)-3-[(1*S*)-1-((1*S*)-2-Benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl)-2-biphenyl-4-yl-ethyl-carbamoyl]-3-mercapto-propyl]-benzoic acid (17). 36 mg (40%). HPLC (gradient 50–100% C in 20 min): $t_R = 16.12$ min. ^{1}H NMR (DMSO-d_6+TFA) δ 1.75 (m, 1H), 1.90 (m, 1H), 2.60 (m, 2H), 2.80 (m, 1H), 2.95 (m, 1H), 3.10 (m, 1H), 3.25 (m, 2H), 4.15 (d, 2H), 4.55 (m, 1H), 4.70 (m, 1H), 6.90–7.50 (m, 20H), 7.80 (m, 4H), 8.20 (d, 1H), 8.30 (d, 1H), 8.40 (t, 1H). MS (ESI) $(M+H)^+$ $m/z = 756.2$.

4.5.2. 4-[(2*S*)-2-[(1*S*)-1-((1*S*)-2-Benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl)-2-biphenyl-4-yl-ethyl-carbamoyl]-2-mercapto-ethyl]-benzoic acid (18). 28 mg (38%). HPLC (A/C, 20/80): $t_R = 7.44$ min. ^{1}H NMR (DMSO-d_6+TFA) δ 2.75 (m, 2H), 2.90–3.30 (m, 4H), 3.70 (m, 1H), 4.20 (d, 2H), 4.55 (m, 1H), 4.65 (m, 1H), 7.00–7.40 (m, 17H), 7.50 (d, 2H), 7.75 (d, 2H), 7.90 (dd, 2H), 8.20 (d, 1H), 8.35 (d, 1H), 8.45 (t, 1H). MS (ESI) $(M+H)^+$ $m/z = 742.2$, $(2M+H)^+$ $m/z = 1483.0$.

4.5.3. 4-[(3*R*)-3-[(1*S*)-1-((1*S*)-2-Benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl)-2-biphenyl-4-yl-ethyl-carbamoyl]-3-methyl-propyl]-benzoic acid (19). Compound **19** was obtained following the above described procedure for compounds **17** and **18** but by using TFA/anisole instead of HF/triisopropylsilane to yield 0.029 g of the title compound as a white solid.

Yield = 90%. ^{1}H NMR (DMSO-d_6+TFA) δ 0.8 (d, 3H), 1.45 (m, 1H), 1.78 (m, 1H), 2.2 (m, 1H), 2.6-3.4 (m, 6H), 4.2 (d, 2H), 4.65 (m, 2H), 7.0–7.6 (m, 17H), 7.75 (d, 2H), 7.90 (d, 2H), 8.0 (d, 1H), 8.3 (d, 1H), 8.5 (t, 1H). MS (ESI) $(M+H)^+$ $m/z = 738.2$.

4.6. General procedure for the synthesis of compounds 20 and 21

Disulfides **20** and **21** were prepared via dimerization of corresponding thiol derivatives **17** and **18**, which 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.

4.6.1. 4,4′-Bis[disulfan-diyl-[(3*S*)-3-[[(1*S*)-2-benzo[*b*]thiophen-3-yl-methyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-yl-methyl)-2-oxoethyl]carbamoyl]-propane-1,3-diyl]-dibenzoic acid (20). HPLC (A/C = 25/75% for 10 min then gradient to 5/95% in 10 min): $t_R = 14.5$ min. ^{1}H NMR (DMSO-d_6+TFA) δ 1.75 (m, 2H), 1.90 (m, 2H), 2.60 (m, 4H), 2.80 (m, 2H), 2.95 (m, 2H), 3.10 (m, 2H), 3.25 (m, 4H), 4.15 (m, 4H), 4.55 (m, 2H), 4.70 (m, 2H), 6.90–7.50 (m, 40H), 7.80 (m, 8H), 8.20 (d, 2H), 8.35 (m, 4H). MS (ESI) $(M+H)^+$ $m/z = 1509.3$, $(M+Na)^+$ $m/z = 1532.4$.

4.6.2. 4,4′-Bis[disulfan-diyl-[(2*S*)-2-[[(1*S*)-2-benzo[*b*]thiophen-3-yl-methyl)-2-(benzylamino)-2-oxoethyl]amino]-1-(biphenyl-4-yl-methyl)-2-oxoethyl]carbamoyl]-ethane-1,2-diyl]-dibenzoic acid (21). HPLC (A/C, 20/80): $t_R = 17.3$ min. ^{1}H NMR (DMSO-d_6+TFA) δ 2.75 (m, 4H), 2.90–3.30 (m, 8H), 3.70 (m, 2H), 4.20 (m, 4H), 4.55 (m, 2H), 4.65 (m, 2H), 7.00–7.40 (m, 34H), 7.50 (d, 4H), 7.75 (d, 4H), 7.90 (m, 4H), 8.20 (d, 2H), 8.35 (m, 4H). MS (ESI) $(M+Na)^+$ $m/z = 1503.6$.

4.6.3. Bis[2,2′-disulfan-diyl-4,4′-diphenyl]-dibutanoic acid (24). To a solution of 10 mL of bromohydric acid in 10 mL of water was added 1.78 g (10 mmol) of D-homophenylalanine (**22**). The mixture was stirred for 10 min after which was added at 0 °C a solution of 4.5 g (65 mmol) sodium nitrite in 10 mL of water. Stirring was continued for 30 min then the aqueous solution was extracted with 3 × 30 mL of diethylether. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and evaporated to dryness to yield 1.29 g of a slightly coloured oil, which was used without further purification and characterization. The obtained oil was quantitatively added to a solution of 1.75 g (15 mmol) of potassium thioacetate in 10 mL of DMF in the presence of 1.0 g (42 mmol) of NaH. After 4 h of stirring at room temperature the solution was acidified and extracted with AcOEt and purified by chromatographic means to give 0.35 g of compound **23** (15% overall yield) as a yellow oil, which was characterized by HPLC and ^{1}H NMR: HPLC (A/B, 50/50): $t_R = 7.59$ min. ^{1}H NMR ($CDCl_3$) δ 2.0 (m, 1H), 2.2 (m, 1H), 2.34 (s, 3H), 2.65 (m, 2H), 4.15 (t, 1H), 7.05–7.28 (m, 5H). Coupling of **23** with a chiral amino acid and subsequent NMR analysis (data not shown) permitted to estimate the optical purity of **23** being 70%. To a solution of 55 mg (0.23 mmol) of **23** in 1 mL of MeOH was added at 0 °C 600 μL of 0.5 N NaOH. The solution was stirred for 3 h at room temperature after which the pH was adjusted with hydrochloric acid to permit extraction with AcOEt (3 × 30 mL). The combined organic layers were washed with brine, dried over sodium sulfate, filtered and evaporated to dryness to yield compound **24** as an oil, which crystallized on standing.

0.040 g (89%), mp 53 °C. ^{1}H NMR (DMSO-d_6+TFA) δ 1.8–2.1 (m, 4H), 2.55 (t, 4H), 3.4 (t, 2H), 7.1–7.3 (m, 10H). MS (ESI) $(M+Na)^+$ $m/z = 413.4$.

4.6.4. *N*,*N*′-Bis[(2*S*,2′*S*)-disulfanyl-diyl-[(2*S*)-[(1*S*)-2-benzo[*b*]thiophen-3-yl-1-benzylcarbamoyl-ethylcarbamoyl]-2-biphenyl-4-yl-ethyl]-4,4′-diphenyl]-dibutanamide (25). Compound **25** was obtained as a mixture of three diastereomers as evidenced by NMR and HPLC analysis following the procedure for the synthesis of compounds **13**–**16** but by using 1 equiv of acid **24**, 2.2 equiv of dipeptide Bip-Bta-NHBn,[9] 2.2 equiv of coupling agent BOP and 7 equiv of DIEA.

HPLC (gradient 60–100% B in 20 min): $t_R = 24.2$ min (32%), 24.5 min (46%), 28.2 min (22%). ^{1}H NMR (DMSO-d_6+TFA) δ 1.50–2.15 (m, 4H), 2.25–2.40 (m,

4H), 2.75–3.10 (m, 4H), 3.10–3.30 (m, 4H), 3.4 and 3.55 (t, 2H), 4.1–4.3 (m, 4H), 4.6–4.9 (m, 4H), 6.9–7.4 (m, 44H), 7.85 (m, 4H), 8.25–8.65 (m, 6H). MS (ESI) $(M+Na)^+$ $m/z = 1443.4$.

4.7. Enzyme assay

The inhibitory potencies of all compounds were measured by using the light chain of BoNT/B as previously described[12] 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} to 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}] ($K_m = 47$ M) 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) except for compound **25** when tested in the presence of 10 equiv of DTT added prior to incubation in order to evaluate the affinity of its thiol counterpart **26**. The percentage of degradation was measured directly in a 96 wells 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[24] and are expressed as the mean ± SEM of three separate experiments each in triplicate.

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