Development of Potent Inhibitors of Botulinum Neurotoxin Type B

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Received February 11, 2003

Botulinum neurotoxins are the most potent toxins known to date. They are zinc-metalloproteases able to cleave selectively an essential component of neurotransmitter exocytosis, causing the syndrome of botulism characterized by a flaccid paralysis. There is a great interest in designing antagonists of the action of these toxins. One way is to inhibit their catalytic activity. In this study, we report the design of such inhibitors directed toward BoNT/B. A study of the S_1 subsite specificity, using several β-amino thiols, has shown that this subsite prefers a *p*-carboxybenzyl moiety. The specificity of the S_1' and S_2' subsites was studied using two libraries of pseudotripeptides containing the S_1 synthon derived from the best β-amino thiol tested. Finally, a selection of various non natural amino acids for the recognition of the "prime" domain led to the most potent inhibitor of BoNT/B described to date with a K_i value of 20 nM.

Introduction

The seven serotypes of botulinum neurotoxins (BoNTs) designated A to G are 150 kDa-dichain proteins composed of a 100 kDa-heavy chain (HC) linked by a disulfide bridge to a 50 kDa-light chain (LC) and are produced by anaerobic bacteria, mainly *Clostridium botulinum*.[1] They are the most lethal substances known to mankind.[2] The toxins induce a flaccid paralysis, which can lead to death by respiratory arrest,[3] through inhibition of acetylcholine release at the neuromuscular junction.[4] The HC is responsible for cell-surface binding and internalization of the toxin whereas the LC posseses a zinc-metallopeptidase activity[4] and was shown to cleave one of the three proteins involved in exocytosis.[5,6] These toxins belong to the same family (clostridial neurotoxins) as tetanus toxin, which has a quite similar mode of action.[5,7] All these enzymes contain the consensus sequence HExxH found in thermolysin[8] and share a closely related spatial arrangement of the active site.[9,10]

The BoNTs cause food-borne, wound, and infant botulism, which is a rare but severe disease. Moreover they could be used as biological warfare agents because of their easiness of production and their very high toxicity.[3,11] On the other hand, for more than 10 years, there has been a medical use of botulinum neurotoxin, essentially BoNT/A and BoNT/B, for the treatment of various diseases such as hemifacial spasms, cervical dystonia and axilary hyperhydrosis.[12] The well-demonstrated efficacy of the neurotoxins toward these diseases has triggered a dramatic increase in their use to diminish facial lines and to reduce myofacial pain. However, severe drawbacks have been reported following local injection of botulinum toxin and even fatal issues.[13] An antitoxin exists, but its use during the first hours of infection results only in an improvement of the successful intensive care treatment and of a decrease in the time of hospital stay. Moreover, immunological drawbacks have been frequently reported following the use of antitoxin.[12]

Similarly to tetanus toxin, BoNT/B cleaves synaptobrevin, an integral membrane protein of synaptic vesicles, which was shown to be essential for the formation of a ternary complex with two other proteins of the plasma membrane (SNAP-25 and syntaxin). This fusion process triggers the synaptic release of neurotransmitters contained in the small vesicles.[14] BoNT/B cleaves the sequence of human synaptobrevin (Syb) between Glu 76 and Phe 77 which are located on the cytosolic part of Syb.[5] We have previously developed an efficient high-throughput assay for measuring the proteolytic activity of BoNT/B[15] and to determine the inhibitory potency of a great number of molecules derived from classical or combinatorial chemistry. Using this method, inhibitors of BoNT/B were rationally designed through a classical structure−activity relationship study. As tetanus toxin and BoNT/B cleave the same substrate at the same peptide bond, the strategy used to design BoNT/B inhibitors was initially based on that developed to obtain tetanus toxin inhibitors.[16]

In a first step, an exploration of the S_1 subsite of BoNT/B toxin by various β-amino thiols was performed. This subsite, which specifically recognizes the Glu 76 residue of its natural substrate, synaptobrevin,[10] is assumed to accommodate preferentially polar side chains, such as carboxylate, carboxamide, sulfonate, etc. In a second step, the optimized S_1 synthon was incorporated in structures able to fit the $S_1-S'_2$ subsites of BoNT/B. After determination by combinatorial chemistry of the best natural residues interacting with these subsites, non natural amino acids were used to improve the inhibitory potency. The best inhibitor of BoNT/B described to date ($K_i = 20$ nM) was obtained by this way.

Results

1. Chemistry. 1.1. The β-Amino Thiols. Among the various β-amino thiols used in this work, only three of them, compounds **4**, **8**, and **9**, have not been previously

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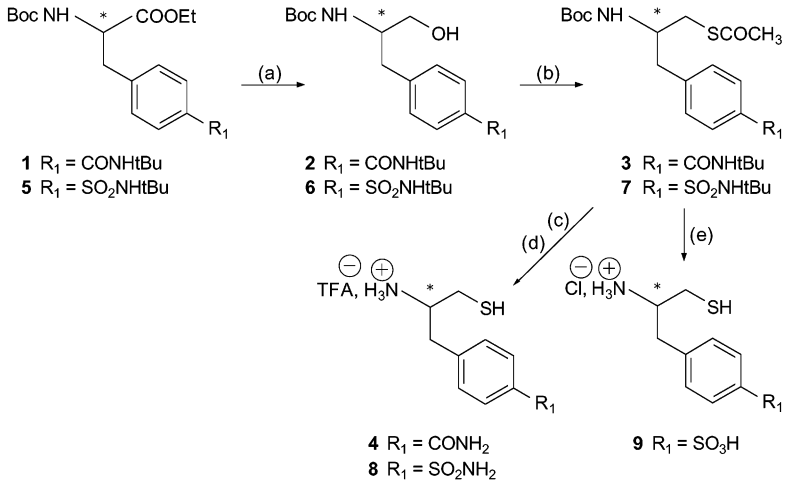
10.1021/jm0300680 CCC: $25.00
Published on Web 09/19/2003

Scheme 1. Synthesis of Inhibitors **4**, **8**, and **9**[a]



[a] (a) $NaBH_4$ LiCl; (b) DEAD, PPh_3, CH_3COSH, THF; (c) NaOH, MeOH, THF; (d) TFA, anisole, Δ; (e) 6 N HCl, Δ.

described.[17−21] They were prepared following reported methods[22] from their corresponding noncommercially available α-amino acids. The t-Boc-amino esters **1** and **5** were prepared using a solid−liquid-phase transfer catalytic method with the (benzylideneamino)acetic acid ethyl ester and either the 4-(chloromethyl)-(*tert*-butyl)-benzamide, or the 4-(bromomethyl)-phenyl(*tert*-butyl)-sulfonamide (prepared from the commercially available 4-(chloromethyl)benzoyl chloride and 4-(chlorosulfonyl)-benzoic acid, respectively), followed by acidic hydrolysis of the benzylidene moiety and protection of the free amine by a Boc group (data not shown).

The *β*-amino thiols **4**, **8**, and **9** were prepared by the following synthetic sequence: (i) reduction of the esters of **1** and **5** to afford the corresponding alcohols **2** and **6**; (ii) Mitsunobu reaction yielding the thioacetates **3** and **7**; (iii) a final deprotection step leading to the expected *β*-amino thiols **4**, **8**, and **9** (Scheme 1). These three compounds were obtained as racemic mixtures.

1.2. Synthesis of the Synthon 16. The chosen method was an Oppolzer synthesis[23] using the 2-(benzydrylideneamino)-1-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)ethanone previously described.[24] Its stereospecific alkylation with 4-(bromomethyl)benzoic acid *tert*-butyl ester in the presence of butyllithium as base afforded **10** which was then deprotected by acidic hydrolysis in **11** and reprotected by a Boc function to give **12** (Scheme 2). The source of chirality, camphorsultam, is then cleaved in aprotic medium by a phase-transfer catalysis method,[24] leading to the pure *S* enantiomer **13**. The next steps were (i) the Arndt−Eistert homologation of **13** leading to the *β*-amino ester **14** with retention of configuration; (ii) the stereospecific sulfenylation of **14** with 1-(4-methoxybenzyl)disulfanyl-2,4-dinitrobenzene;[25] and (iii) the hydrolysis of the ester with bis(tributyltin) oxide which avoids racemization. In these conditions, the synthon **16** is obtained as pure 2*S*,3*S* diastereoisomer (Scheme 2).

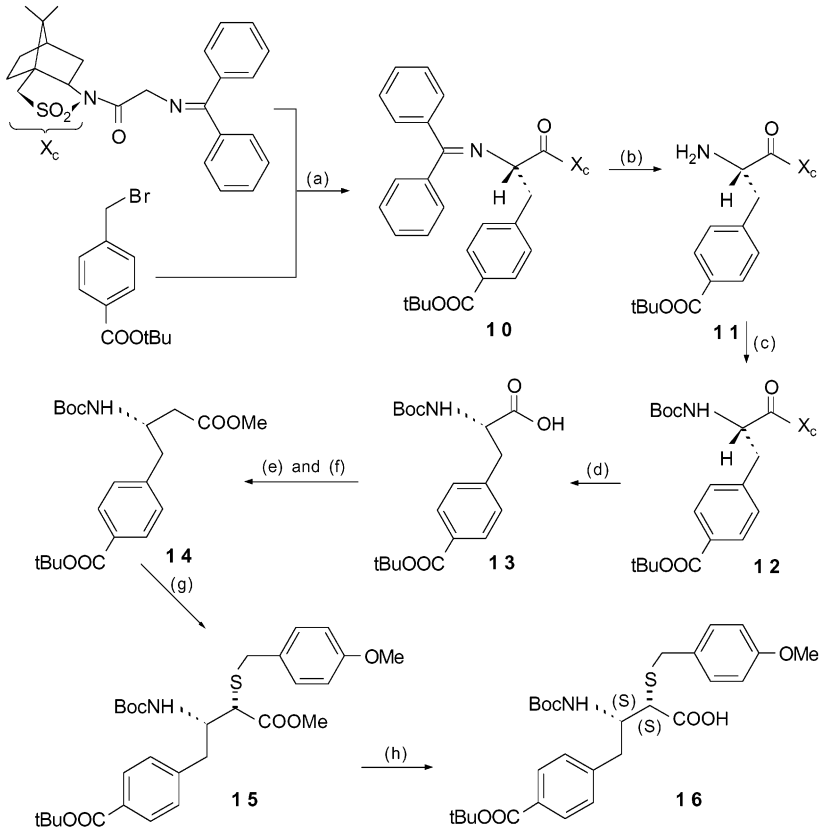
1.3. Combinatorial Synthesis of Two Libraries of Pseudotripeptides. Two pseudotripeptide libraries, one to study the S_1' subsite of BoNT/B LC and the other one to investigate its S_2' subsite, were generated by the Divide/Couple/Recombine method using natural α-amino acids.[16] The peptides were obtained by solid-phase synthesis on a chlorotrityl resin. For the first library (Figure 1A), an equimolar mixture of 18 natural amino acids as P_2' residues was introduced on the resin and coupled successively with 18 amino acids (all natural amino acids except proline and cysteine) corresponding to the P_1' residue and finally coupled with the protected synthon **16**. The second library (Figure 1B) was achieved by a parallel synthesis of 10 pseudotripeptides containing the same P_1 synthon, a Trp residue as P_1' group, and one of the 10 selected amino acids for the S_2' subsite recognition.

All the coupling steps were performed using (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (Bop) and minimum amounts of diisopropylethylamine to preserve the stereochemistry. The final deprotection necessitated anhydrous hydrogen fluoride owing to the use of 4-methoxybenzyl group as thiol protecting moiety.

2. Inhibitory Potencies of the Various Inhibitors. The various *β*-amino thiols were tested in vitro on BoNT/B metallopeptidase activity, as described previously.[15] From the results reported in Table 1, it appears that (i) the decreasing order of BoNT/B LC recognition for the various side chains in P_1 position is phenyl > cyclohexyl > alkyl; (ii) the inhibitory potency is increased when the benzyl (or cyclohexyl) moiety bears a substituent in position 4; and (iii) this substituent is preferentially a carboxylate group. Although the study was not exhaustive, it can be noticed that the best *β*-amino thiol of this series, (compound **24**, *p*-carboxyphenylalanine thiol) exhibits a rather good affinity (K_i = 11 μM) for BoNT/B.

From the data reported in Figure 1A, it can be observed that aromatic residues were preferred in the P_1' position. The tryptophan led to a slightly better inhibition than a phenylalanine or a tyrosine residue (30−35% inhibition at 1 μM), while the less active mixtures contained charged (Glu, Lys, Arg, etc.) or small (Ser, Gly) amino acids. In the second series of inhibitors (Figure 1B), a Trp residue was introduced in P_1' position, and among the 10 residues tested as P_2' components, aromatic amino acids and Asn are preferred with 35−40% of inhibitory potency at 1 μM. These results clearly indicate a large preference of the S_1'−S_2' domain of the peptidase for aromatic residues. Consequently, to tentatively improve the inhibitory potency of these

Scheme 2. Synthesis of Synthon **26**[a]



[a] (a) nBuLi 2.5 M/hexane, THF, HMPA; (b) citric acid 10%, THF; (c) NEt_3, $(Boc)_2O$, DMF; (d) LiOH, LiBr, nBu_4NBr, acetonitrile; (e) iBuOCOCl, NMM, THF; (f) diazomethane followed by silver benzoate, NEt_3, methanol; (g) nBuLi 1.6 M/hexane, HMDS, THF then HMPA, 1-(4-methoxybenzyldisulfanyl)-2,4-dinitrobenzene; (h) bis(tributyltin) oxide, acetonitrile.

pseudotripeptides, various compounds were synthesized in large quantities by liquid-phase method, with a benzylamide at the C-terminus.[16]

Compound **26** issued from the library, with the dipeptide Trp-Phe-benzylamide for $S_1'-S_2'$ domain recognition, inhibits BoNT/B with a K_i value of 3.5×10^{-7} M. The replacement of Trp by either the 1-naphthylmethyl (compound **27**), the NMe-Trp (**28**), or the biphenyl methyl moiety (**29**) induces a slight increase in the inhibitory potency of the related inhibitors (Table 2).

The effect of the stereochemistry of residues in P'_1 and P'_2 positions on the metallopeptidase activity of BoNT/B was also studied (compounds **29**−**31**), and it was shown that the *S*,*S* isomer yielded a slightly better BoNT/B inhibitor. The rather weak stereochemical dependence of BoNT/B LC for optimal inhibition is similar to that observed with TeTx.[16]

In the second part of the study, the S'_2 recognition of BoNT/B LC was optimized. With this aim, three different amino acids were used: an aromatic amino acid with a hydroxyl group, tyrosine (compound **33**), an amino acid with a nonaromatic but hydrophobic side-chain, isoleucine (compound **34**). As compared to **29** which contains a phenylalanine in P_2', **33** and **34** are slightly less efficient consistent with the results of the library (Figure 1B). Then, a non natural aromatic amino acid with a bulky side-chain corresponding to benzothienylalanine was introduced in P_2' position, leading to **35**. As shown in Table 2, an improvement in inhibitory potency was obtained, suggesting the presence of a large S'_2 subsite in BoNT/B LC. To our knowledge, **35** is the best inhibitor of BoNT/B reported so far with a K_i value of 20 nM.

3. Molecular Modeling. All the compounds described in this work have been synthesized before the publication of the crystal structure of BoNT/B[26] and a substrate-sequence of synaptobrevin bound to the BoNT/B LC.[10] The results of these two studies were used to investigate the possible mode of interaction of **35** with BoNT/B LC**.** Figure 2 shows a view of the enzyme−inhibitor complex. The biphenyl group seems to fit the hydrophobic and deep pocket corresponding to the S'_1 subsite of BoNT/B LC and interacts with several hydrophobic residues of the protein. The carboxylate group, of the P_1 residue, faces a highly hydrophilic area and was found at distances compatible with hydrogen bonding or ionic interactions with the amino acids Lys 242 and Arg 183 of the metallopeptidase. The P_1 benzyl moiety of **35** participates to the correct orientation of the *p*-carboxylate group. The benzothienyl heterocycle seems to be stacked on the aromatic residue Tyr 53. This could explain its favorable influence on BoNT/B LC inhibition. Regarding the amino group, no evidence for specific interaction with the enzyme was observed. The result of this docking experiment are consistent with those of the structure−activity studies.

The inhibitory potency of compound **35** was tested with BoNT/A LC [27] and with various physiological enzymes such as angiotensin-converting enzyme (ACE),[28] endothelin-converting enzyme (ECE),[29] and aminopeptidases A and N (APA or APN).[30] In all cases, the

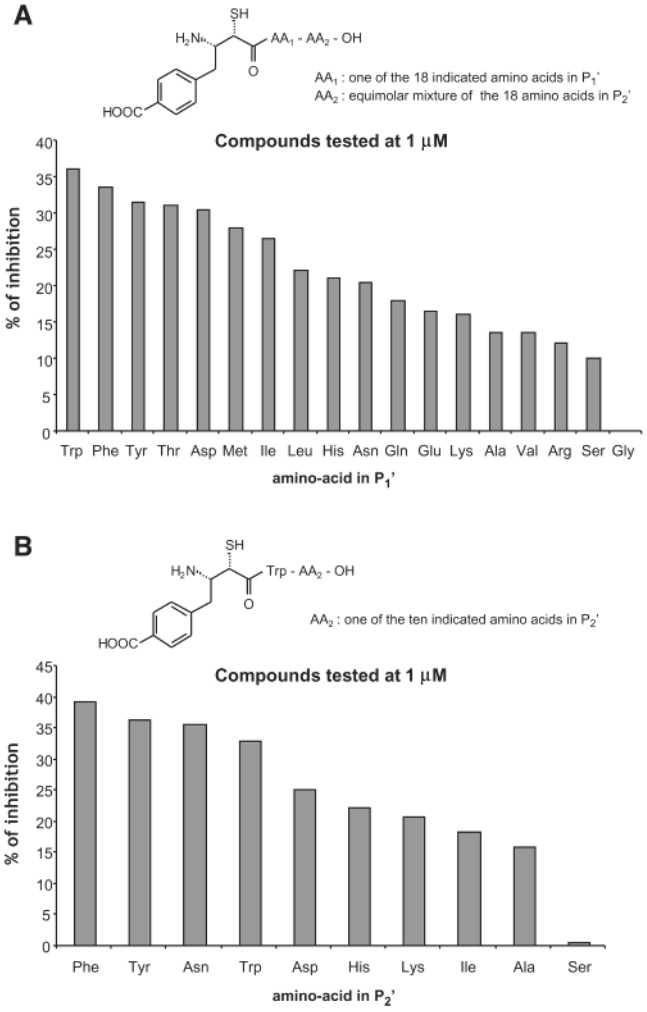


Figure 1. Histograms representing the percentage of inhibition obtained with mixtures of pseudotripeptides derived from the β-amino thiol **16** on botulinum B neurotoxin light chain endoprotease activity. The general formula of these mixtures of compounds are given above the histograms. (A) Each mixture differs by an amino acid putatively interacting in S_1' which is represented in the abscissa as a three-letter code. A stoechiometric amount of each amino acid is present in P_2'. These mixtures were tested at 1 μM on BoNT/B LC. (B) In each case, a tryptophan is present in P_1' position. Each mixture differs by the residue present in S_2' which corresponds to an amino acid indicated as a three-letter code in the abscissa. These mixtures were tested at 1 μM on BoNT/B LC.

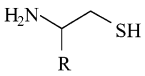
inhibitory potency (IC_{50}) was found at least 2 orders of magnitude higher as for BoNT/B LC (data not shown). Thus, **35** appears as a selective BoNT/B inhibitor.

Discussion

Inhibitors of botulinum neurotoxins could be devoted to the protection against infection by the toxins or to alleviate adverse effects when the toxins are used in therapy. The first role would be preventive in the case where a contamination is suspected, too late to allow the use of serum but soon enough so that the inhibitors could prevent the intracellular effect of the toxin. The second one would be to block the evolution of the disease by protecting the intracellular target proteins which are not already cleaved by the toxin. Thus the duration and the severity of the disease could be reduced.

In this work, our main objective was to investigate the nature of the best residues interacting with the

Table 1. Inhibitory Potencies of Various β-Amino Thiols on BoNT/B



compounds	R	K_i (BoNT/B)[a]
17	$(CH_2)_3CH_3$	425 μM
18	$(CH_2)_2CO_2H$	250 μM
19	CH_2-cyclohexyl	160 μM
20	CH_2-(3-COOH)-cyclohexyl	>1 mM
21	CH_2-(4-COOH)-cyclohexyl	21 μM
22	CH_2-Ph	100 μM
23	CH_2-(3-COOH)-Ph	250 μM
24	CH_2-(4-COOH)-Ph	11 μM
4	CH_2-(4-$CONH_2$)-Ph	60 μM
8	CH_2-(4-SO_2NH_2)-Ph	65 μM
9	CH_2-(4-SO_3H)-Ph	22 μM
25	CH_2-(4-CH_2COOH)-Ph	97 μM

[a] The K_i values are the mean ± SEM of three independent experiments performed in triplicate.

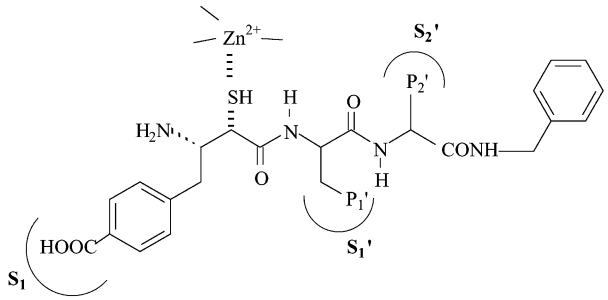
subsites of the active site of BoNT/B. Indeed this neurotoxin is easily produced and purified and is very stable, accounting mainly for its possible use as a weapon.[11] Moreover BoNT/B is now currently used for the treatment of various neuromuscular diseases[12] and for the reduction of wrinkles.[31] In addition, BoNT/B remains efficient in patients who have developed antibodies against BoNT/A.[32] Nevertheless, the use of BoNT/B is sometimes associated with more or less severe side effects.[13]

At this time, there is no potent inhibitor of BoNT/B. Zinc chelating agents are effective blockers but are devoid of selectivity and have important adverse effects and thus cannot be used in therapy.[33−35] Small peptides derived from the substrate of BoNT/B are neither substrates nor inhibitors.[5,34] Classical inhibitors of zinc metalloproteases (ACE, NEP, etc.) were shown to be inactive.[5,36−38] Other inhibitors have also been tested: 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin (elastase inhibitor) with an inhibitory potency of 27.6 μM,[35,36] phosphonates and phenylmethylsulfonyl fluoride (serine protease inhibitor) which were not really effective,[35,39] BABIM (bis(5-amidino-2-benzimidazolyl)-methane) or keto-BABIM with inhibitory potencies of 1.6 and 0.8 μM,[40] and buforin-1 with an inhibitory potency of 1 μM were the first synthetic substances with a relatively good affinity for BoNT/B.[41] However, their potency was too low for a possible clinical use. Consequently, there was a great interest in designing highly potent and selective inhibitors of BoNT/B.

With this aim, pseudo-tripeptides were synthesized since such molecules have been shown to be quite effective for the inhibition of TeTx,[16] a protease very similar to BoNT/B, cleaving the same substrate (synaptobrevin) at the same position. This was achieved step by step by characterizing the best residues for S_1, S'_1, and S'_2 recognition.

In the case of BoNT/B, the benzyl side chain in P_1 position requires a *p*-carboxylate group for optimal interaction whereas a *m*-sulfonamide group seems to be preferred in tetanus toxin.[18] The importance of the carboxylate could be related to interactions with constituting amino acids present in the S_1 subsite as suggested by molecular modeling. The inhibitors' potencies are modulated by the position of the charged

Table 2. Inhibitory Potency of Molecules **26**−**35** on BoNT/B LC



no.	P_1'	absolute configuration Cα(P_1')	P_2'	absolute configuration Cα(P_2')	K_i (BoNT/B),[a] M
26	indolyl	*S*	benzyl	*S*	$(3.5 \pm 0.7) \times 10^{-7}$
27	1-naphthyl	*S*	benzyl	*S*	$(2.2 \pm 0.4) \times 10^{-7}$
28	N-Me-indolyl	*S*	benzyl	*S*	$(1.7 \pm 0.3) \times 10^{-7}$
29	biphenyl	*S*	benzyl	*S*	$(1.6 \pm 0.2) \times 10^{-7}$
30	biphenyl	*R*	benzyl	*S*	$(5.4 \pm 0.3) \times 10^{-7}$
31	biphenyl	*S*	benzyl	*R*	$(5.8 \pm 0.4) \times 10^{-7}$
32	biphenyl	*S*	imidazolylmethyl	*S*	$(5.0 \pm 0.9) \times 10^{-7}$
33	biphenyl	*S*	4-OH-benzyl	*S*	$(8.1 \pm 0.9) \times 10^{-7}$
34	biphenyl	*S*	1-methylpropyl	*S*	$(5.4 \pm 0.4) \times 10^{-7}$
35	biphenyl	*S*	benzothienylmethyl		$(2.0 \pm 0.2) \times 10^{-8}$

[a] The K_i values are the mean ± SEM of three independent experiments performed in triplicate.

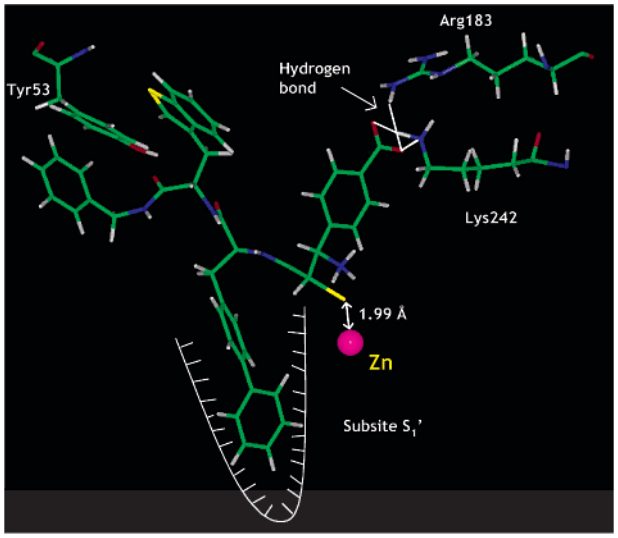


Figure 2. Molecular modeling of the best inhibitor **35**. Schematic representation of the putative interactions with BoNT/B.

carboxyl group and the length of the side chain bearing this group (compare compounds **23**, **24**, **25**). This seems to be in relation to an optimal interaction with amino acids present in the S_1 subsite.

In a second step, the interaction with the S'_1 subsite was studied using a library of inhibitors obtained by combinatorial chemistry with natural amino acids. This showed that the best residue is a hydrophobic aromatic residue, which is consistent with the cleavage site of synaptobrevin, the substrate of BoNT/B, where the P'_1 amino acid is a phenylalanine. The nature of this residue was shown to be essential for a good interaction with BoNT/B LC[38,42] and for optimal recognition of almost all zinc metallopeptidases.[43]

An optimization of the S'_1 interaction was achieved using non natural amino acids, leading to bulky aromatic residues as preferred ligands. This could be explained by the deep and hydrophobic S'_1 subsite as evidenced by crystallographic studies[10,26] and molecular modeling (this study). It seems that this subsite is so deep that side chains longer than biphenyl could possibly improve the enzyme recognition.

Cleavage of synaptobrevin analogues by BoNT/B has suggested that interaction with the S'_2 subsite would be less essential as that with the S'_1.[42] This was not the case with inhibitors, illustrating the absence of optimization of metallopeptidase recognition by substrate as already observed with another zinc peptidase, NEP.[9] Thus, the best inhibitor of BoNT/B LC was obtained in our series with a large bicyclic heteroaromatic residue (compound **35**) while a glutamate was found at this P'_2 level in the substrate.[5] This suggests that the S'_2 interactions with synaptobrevin are not optimal, the selectivity being related to the recognition of the long cleft of BoNT/B LC by the side chains of the constituent amino acids of the substrate.[10] This could explain why specifically changing the glutamate does not really modify the kinetics of cleavage. In the case of small inhibitors such as those reported here, there is only a small number of possible interactions, and their optimization is essential to improve the inhibitory potency (Table 2). This is clearly demonstrated with the benzothienyl group. This molecule is the best inhibitor ever described for BoNT/B with an inhibitory potency of 20 nM (patent Fr. 01.04.895).[44] This is probably due to the various favorable interactions (ionic, hydrophobic, aromatic ring stackings) evidenced in the enzyme active site by molecular modeling. Nevertheless, the precise mechanism of BoNT/B LC inhibition remains to be investigated in detail since several modes of enzyme-recognition have been identified in crystal structures of the complex between BoNT/B and the less potent inhibitor BABIM.[45]

Because the inhibitory potency is already in the high nanomolar range, we could expect a rapid optimization of this type of compound. This promising approach could

allow the design of molecules for in vivo use. Thus, docking experiments have shown that the amino group seems to have no role in enzyme recognition. Therefore, removal of this hydrophilic group could lead to compounds with similar inhibitory potencies in vitro with probably a better in vivo bioavailability.

Experimental Procedures

Molecular Modeling. Molecular mechanics calculations were performed using InsightII package (Accelrys Inc.) and AMBER force field. A distance-dependent dielectric screening constant of 4*r* was used. The protein was built using as a starting point the PDB crystal structure of BoNT/B (PDB entry 1EPN).[26] The inhibitor was first docked into the enzyme by performing a sequence of six molecular dynamics runs. Each run was done at 300 K during 10000 steps of 1 fs. A distance constraint was imposed between the S atom of the thiol group and the metal ion of the enzyme. This distance constraint was sequentially reduced from 12 Å with the inhibitor outside the protein active site for the first dynamics run to 2.3 Å for the sixth one. For these runs, the protein backbone was held frozen but its side chains were relaxed and the inhibitor was completely relaxed. A conformational search of the best inhibitor position was then performed using the AFFINITY module of InsightII with a distance between the metal ion and the S atom of the thiol group constrained to 2.3 Å, assuming that the thiol acts as zinc chelating group. Among 50, 17 structures fulfilling these criteria and low energy were found by the program. One of these structures was chosen for its very low nonbinding energy between the enzyme and the inhibitor in comparison to the others. This structure also seemed acceptable by geometrical criteria, considering experimental values of K_i for different inhibitors. Finally, 50 steps of dynamics were performed on this structure. Each was done at 300 K during 2000 steps of 1 fs. For these final runs, no distance constraint was imposed between the enzyme and the inhibitor. The resulting structure was submitted to steepest-descent and conjugate-gradient minimization and stored. Analysis of these structures showed a large family of low energy overlapping structures. The minimum-energy structure was used as a model for this study.

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-Brüker 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) (Touzart-Matignon, France), with a mobile phase consisting of water containing 0.05% TFA (A) and acetonitrile/water 9/1 containing 0.038% TFA (B). Reagents were obtained from commercial sources and are used without further purification.

General Procedure for the Synthesis of Compounds 2 and 6. A mixture composed of the appropriate carboxylic acid in dimethoxyethane (1 mL/mmol) was stirred at −15 °C under argon. *N*-Methylmorpholine (1 equiv) and isobutylchloroformate (1 equiv) were then added successively and the reaction was stirred during 2 min. at −15 °C. After filtration, a solution of sodium borohydride (1.5 equiv) in water (0.7 mL/mmol) was added, and the reaction was immediately hydrolyzed with water (26 mL/mmol). The product was extracted two times with ethyl acetate, and the combined organic layers were washed with HCl (1 N) and brine. After evaporation in vacuo, the title compounds were obtained as colorless oils which were used without further purification.

[1-(4-*tert*-Butylcarbamoylbenzyl)-2-hydroxyethyl]carbamic acid *tert*-butyl ester (2): 1.25 g (71.5%). TLC (dichloromethane:methanol, 90:10): $R_f = 0.43$. ^{1}H NMR (DMSO-d_6) δ 1.25 (s, 9H), 1.3 (s, 9H), 2.55 (dd, 2H), 2.8 (dd, 2H), 3.55 (m, 1H), 4.7 (t, 1H), 6.6 (d, 1H), 7.2 (d, 2H), 7.6 (s, 1H), 7.7 (d, 2H).

[2-(4-*tert*-Butylsulfamoylphenyl)-1-hydroxymethylethyl]carbamic acid *tert*-butyl ester (6): 0.64 g (95%). TLC (dichloromethane:methanol, 90:10): $R_f = 0.83$. ^{1}H NMR (DMSO-d_6) δ 1.05 (s, 9H), 1.25 (s, 9H), 2.45 (m, 4H), 3.55 (m, 1H), 4.75 (t, 1H), 6.6 (d, 1H), 7.3 (d, 2H), 7.35 (s, 1H), 7.65 (d, 2H).

General Procedure for the Synthesis of Compounds 3 and 7. To a solution of triphenylphosphine (2 equiv) in anhydrous tetrahydrofuran (2 mL/mmol), stirred at 0°C, was added diisopropylazodicarboxylate (2 equiv), and the mixture was stirred at this temperature during 30 min. A yellow solid was formed at this step. Then a mixture composed of thiolacetic acid (2 equiv) and the alcohol **2** or **6** (1 equiv) in anhydrous tetrahydrofuran (2 mL/mmol of alcohol) was added at 0 °C. The reaction was stirred at room temperature during 72 h. After evaporation in vacuo, the residue was dissolved in ethyl acetate and the organic layer washed once with water, a solution of hydrogenocarbonate, water, and brine. After evaporation in vacuo, the residue was purified by column chromatography on silica gel with 30% of ethyl acetate in cyclohexane to give the compounds as a colorless oil.

4-[1-(Acetylsulfanyl)-2-(Boc-amino)propyl]-(*N*-*tert*-butyl)benzamide (3): 1.01 g (69%). TLC (cyclohexane:ethyl acetate, 70:30): $R_f = 0.31$. ^{1}H NMR (DMSO-d_6) δ 1.25 (s, 9H), 1.3 (s, 9H), 2.25 (s, 3H), 2.8 (dd, 2H), 3.0 (dd, 2H), 3.7 (m, 1H), 6.8 (d, 2H), 7.2 (d, 2H), 7.6 (s, 1H), 7.7 (d, 2H).

4-[1-(Acetylsulfanyl)-2-(Boc-amino)propyl]phenyl-(*N*-*tert*-butyl)sulfonamide (7): 0.5 g (68%). TLC (cyclohexane: ethyl acetate, 70:30): $R_f = 0.26$. ^{1}H NMR (DMSO-d_6) δ 1.05 (s, 9H), 1.25 (s, 9H), 2.25 (s, 3H), 2.75 (dd, 2H), 2.75 et 2.95 (dd, 2H), 3.65 (m, 1H), 6.85 (d, 1H), 7.25 (d, 2H), 7.35 (s, 1H), 7.65 (d, 2H).

4-(2-Amino-3-sulfanylpropyl)benzamide (4). To a solution of **3** (1.0 g, 2.46 mmol) in a mixture of methanol (5 mL) and tetrahydrofuran (5 mL) at 0°C under argon was added a 1 M sodium hydroxide solution (2.6 mL, 2.6 mmol). The mixture was stirred at room temperature during 90 min. The organic solvent was evaporated in vacuo, and after acidification with a 1 N HCl solution, the solid was filtered and dried to yield 0.805 g (90%), which was used without purification.

A solution of this compound (795 mg, 2.18 mmol) in trifluoroacetic acid (6.7 mL, 84 mmol) and anisole (3.6 mL) was heated at 60 °C during one week. After drying, the product was precipitated with diethyl ether and purified by HPLC (C18 Kromasil, 100 Å, 5 μm). After freeze-drying, compound **4** was obtained as a white powder: 0.13 g, (30%). ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6 (m, 2H), 2.9 (d, 2H), 3.5 (m, 1H), 7.2 (d, 2H), 7.65 (d, 2H), 7.9 (m, 3H). MS (ESI) $(M + H)^+$ $m/z = 210.9$.

4-(2-Amino-3-sulfanylpropyl)benzenesulfonamide (8). To a solution of a compound **7** (0.25 g, 0.56 mmol) in a mixture of methanol and tetrahydrofuran (1/1, 2 mL) at 0 °C under argon was added NaOH (0.6 mL, 0.59 mmol). The mixture was stirred at room temperature during 90 min. After acidification with HCl (1 N), the product was filtered and dried to yield a white powder, 0.157 g (70%).

A solution of this crude product in trifluoroacetic acid (1 mL) and anisole (0.5 mL) was stirred overnight at room temperature. After drying, the product was precipitated with diethyl ether. Compound **8** was obtained as a white powder: 0.08 g (94%). ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6−3.1 (m, 4H), 3.65 (m, 1H), 7.4 (d, 2H), 7.75 (d, 2H), 8.05 (m, 3H). MS (ESI) $(M - Cl)^+$ $m/z = 247$.

4-(2-Amino-3-sulfanylpropyl)benzenesulfonic Acid (9). A solution of **7** (0.25 g, 0.56 mmol) in HCl (6 N) (2 mL) was heated at 120 °C overnight. After drying, the product was purified on cation-exchange resin DOWEX 50W×8. After freeze-drying, the product was obtained as yellow powder: 0.06 g (40%). ^{1}H NMR (DMSO-d_6 + TFA) δ 2.75 (m, 2H), 2.85 (m, 2H), 3.65 (m, 1H), 7.4 (d, 2H), 7.75 (d, 2H), 8.05 (m, 3H). MS (ESI) $(M - Cl)^+$ $m/z = 247.0$

4-[2-(Benzhydrylideneamino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)-3-oxopropyl]benzoic Acid *tert*-Butyl Ester (10). A solution of 2-(benzhydrylideneamino)-1-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-aza-tricyclo[5.2.1.0^{1,5}]dec-4-yl)ethanone (17 g, 38.9 mmol) in

200 mL of anhydrous tetrahydrofuran was stirred at −78 °C under argon. ^{n}BuLi (2.5 M in hexane, 16.8 mL, 42.5 mmol) was added dropwise, and the mixture was stirred 15 min at this temperature. A solution of 4-bromomethylbenzoic acid *tert*-butyl ester (12.7 g, 46.68 mmol) in 30 mL of hexamethylphosphoramide and 100 mL of anhydrous tetrahydrofuran was added dropwise at −78 °C. The mixture was allowed to warm to room temperature under stirring during 3 h. The reaction was stopped with a mixture of tetrahydrofuran and acetic acid (4/1, 15 mL) at 0 °C. After removal of the solvent, the residue was dissolved in 300 mL of ether. The organic layer was washed once with 10% NH_4Cl solution, water, and brine. After drying with Na_2SO_4 the solvent was removed. The oily residue was triturated in a mixture of cyclohexane/ ethyl acetate 95/5 to give a white solid: 17.6 g (72%). mp = 174 °C. $[\alpha]^{22}_D$ = −605.1 (c = 0.787 in MeOH). ^{1}H NMR (DMSO-d_6) δ 0.45 (s, 3H), 0.75 (s, 3H), 1.45 (s, 9H), 1.50−1.85 (m, 6H), 2.35 (m, 2H), 2.86 (m, 1H), 3.40−3.75 (m, 3H), 4.80 (t, 1H), 6.85−7.0 (m, 4H), 7.30−7.50 (m, 8H), 7.50 (d, 2H).

4-[2-Amino)-3-(10,10-dimethyl-3,3-dioxo-3λ^6-thia-4-azatricyclo[5.2.1.0^{1,5}]dec-4-yl)-3-oxopropyl]benzoic Acid *tert*-Butyl Ester (11). To a solution of **10** (16.1 g, 25.8 mmol) in 270 mL of tetrahydrofuran was added 272 mL of a 10% citric acid solution. The mixture was stirred at room temperature during 2 h. After removal of the solvent, the aqueous layer was washed twice with ether and the pH adjusted to 7−8 with saturated aqueous $NaHCO_3$. The product was extracted with dichloromethane. The organic layer washed with brine, dried over Na_2SO_4, and concentrated to give the product as a colorless oil: 9.3 g (78%). ^{1}H NMR (DMSO-d_6) δ 0.75 (s, 3H), 0.85 (s, 3H), 1.50 (s, 9H), 1.60−2.10 (m, 6H), 2.35 (m, 2H), 2.70 (m, 1H), 2.95 (m, 1H), 3.50−3.80 (m, 3H), 4.0 (t, 1H), 7.30 (d, 2H), 7.75 (d, 2H).

4-(2-*tert*-Butoxycarbonylamino-2-carboxyethyl)benzoic Acid *tert*-Butyl Ester (13). To a solution of **11** (8.7 g, 18.7 mmol) and triethylamine (5.26 mL, 37.42 mmol) in 90 mL of dimethylformamide at 0 °C was added di-*tert*-butyl dicarbonate (4.5 g, 20.58 mmol). The mixture was stirred during 48 h at room temperature. After removal of the solvent, the crude mixture was diluted with 250 mL of ethyl acetate, washed with 10% $KHSO_4$, water, and brine, dried over Na_2SO_4, and concentrated. The resulting crude oil **12** (8.5 g, 75%) was used without purification (TLC (cyclohexane:ethyl acetate: dichloromethane, 7:1.5:1.5): R_f = 0.21).

A solution of **12** (8.4 g, 15.03 mmol), LiOH (1.44 g, 60.12 mmol), LiBr (6.51 g, 75.15 mmol), and ^{n}Bu$_4$NBr (1.93 g, 6.01 mmol) in 150 mL of acetonitrile was stirred overnight at room temperature. After removal of the solvent, 150 mL of water was added and the aqueous layer washed twice with ether. The pH was adjusted at 2−3 with 10% $KHSO_4$, and the product was extracted with dichloromethane. The organic layer washed with brine, dried over Na_2SO_4, and concentrated. The resulting crude mixture was purified by silica gel column chromatography (cyclohexane:ethyl acetate:acetic acid, 7.5:2.5:0.25) to give the product as a colorless oil: 6.2 g (71%). $[\alpha]^{22}_D$ = 100.4 (c = 1.068 in MeOH). TLC: (cyclohexane:ethyl acetate:acetic acid, 5:5:0.5) R_f = 0.75. ^{1}H NMR (DMSO-d_6) δ 1.25 (s, 9H), 1.50 (s, 9H), 2.80 (m, 1H), 3.0 (m, 1H), 4.05 (t, 1H), 7.00 (d, 1H), 7.30 (d, 2H), 7.75 (d, 2H).

4-(2-*tert*-Butoxycarbonylamino-3-methoxycarbonylpropyl)benzoic Acid *tert*-Butyl Ester (14). Isobutyl chloroformate (0.99 mL, 9 mmol) was added to a solution of **13** (3.29 g, 9 mmol) and *N*-methylmorpholine (0.99 mL, 9 mmol) in 27 mL of tetrahydrofuran at −20 °C. The mixture was stirred 15 min and filtered. At 0 °C a solution of diazomethane (80 mmol) in 100 mL of ether was added to the filtrate. After 2 h at room temperature and removal of the solvent, 170 mL of methanol, silver benzoate (144 mg, 0.72 mmol), and triethylamine (3.79 mL, 27 mmol) were successively added. The mixture was stirred 15 min at 0 °C and 30 min at room temperature. The dark suspension was filtered on Celite 545. After removal of the methanol, the crude mixture was diluted with ether (250 mL), washed with water and brine, dried over Na_2SO_4, and concentrated. The resulting crude yellow oil was purified by silica gel column chromatography (cyclohexane: ethyl acetate, 6:4) to give the product as a yellow solid: 2.36 g (67%). mp = 71 °C. $[\alpha]^{22}_D$ = −82.8 (c = 1.03 in $CHCl_3$). HPLC (A:B, 20:80): t_R = 5.50 min. ^{1}H NMR (DMSO-d_6) δ 1.25 (s, 9H), 1.50 (s, 9H), 2.35 (d, 2H), 2.71 (d, 2H), 3.45 (s, 3H), 3.90 (m, 1H), 6.80 (d, 1H), 7.25 (d, 2H), 7.75 (d, 2H).

4-[2-*tert*-Butoxycarbonylamino-3-(4-methoxybenzylsulfanyl)-3-methoxycarbonylpropyl]benzoic Acid *tert*-Butyl Ester (15). To a solution of freshly distilled hexamethyldisilazane (3.46 mL, 16.4 mmol) in 40 mL of dry tetrahydrofuran at 0 °C under argon was added ^{n}BuLi (1.6 M in hexane, 10.24 mL, 16.4 mmol). The mixture was stirred 15 min at 0 °C and cooled to −70 °C. A solution of **14** (2.3 g, 5.85 mmol) in 23 mL of dry tetrahydrofuran was added dropwise. After 2 h under argon at −70 °C, 1-(4-methoxybenzyldisulfanyl)-2,4-dinitrobenzene (2.89 g, 8.19 mmol) and hexamethylphosphoramide (2 mL, 8.19 mmol) were added, and the mixture was stirred 90 min at −70 °C. The reaction was quenched with 10% NH_4Cl, diluted with 150 mL of ethyl acetate. The organic layer washed with water and brine, dried over Na_2SO_4, and concentrated. The crude mixture was purified by silica gel column chromatography (cyclohexane:dichloromethane: ethyl acetate, 7:1.5:1.5) to give the product as pale yellow solid: 1.55 g (47%). mp = 79 °C. HPLC (A:B, 20:80): t_R = 10.05 min. ^{1}H NMR (DMSO-d_6) δ 1.15 (s, 9H), 1.50 (s, 9H), 2.35 (d, 2H), 3.0 (m, 1H), 3.60 (s, 3H), 3.70 (s, 3H), 3.8−4.0 (m, 3H), 6.80 (m, 3H), 7.25 (m, 4H), 7.70 (m, 2H).

4-[2-*tert*-Butoxycarbonylamino-3-carboxy-3-(4-methoxybenzylsulfanyl)propyl]benzoic Acid *tert*-Butyl Ester (16). To a solution of bis(tributyltin) oxide (8.2 mL, 16.1 mmol) in 32 mL of acetonitrile was added a solution of **15** (1.5 g, 2.75 mmol) in 5.5 mL of acetonitrile. The mixture was heated under reflux. After 5 days, the mixture was cooled to 0 °C and 20 mL of aqueous 2 N HCl was added. After stirring 3 h at room temperature, the reaction was diluted in 100 mL of ethyl acetate, washed with water and brine, dried over Na_2SO_4, and concentrated to give a pale yellow oil. Silica gel chromatography (cyclohexane:ethyl acetate:acetic acid, 8:2:0.5) gave the product as a yellow solid: 1.15 g (79%). mp = 76 °C. HPLC (A:B, 20:80): t_R = 6.1 min. $[\alpha]^{22}_D$ = −489.2 (c = 0.732 in MeOH). ^{1}H NMR (DMSO-d_6) δ 1.2 (s, 9H), 1.45 (s, 9H), 2.35 (d, 2H), 3.0 (m, 1H), 3.75 (s, 3H), 3.8−4.0 (m, 3H), 6.80 (m, 3H), 7.25 (m, 4H), 7.70 (m, 2H).

General Procedure for the Synthesis of Deprotected Dipeptides. Synthesis of Combinatorial Libraries of Thiol-Containing Inhibitors. A pseudotripeptide library, corresponding to 18 mixtures of thiol-containing peptides of formula (synthon **26**)-AA′$_{1x}$-AA′$_{2x}$, where AA′$_{1x}$ and AA′$_{2x}$ represent the 18 different natural amino acids (Cys and Pro omitted), was obtained by solid-phase peptide synthesis on 2-chlorotrityl chloride resin, by using a previously described protocol.[16] The Divide/Couple/Recombine method was used to synthesize 18 sublibraries. A first amino acid mixture is prepared by combining the 18 different amino acids linked to the 2-chlorotrityl resin. This homogenized mixture is then split into 18 identical samples to which a known amino acid is coupled. This gives rise to 18 different mixtures, each of them containing synthon **26** in each sample, allowing the synthesis of 18 mixtures containing 18 couples of diastereomers. The 18 fractions of peptidyl resin were then cleaved from the resin by using a dichloromethane/trifluoroethanol mixture (8/2). The deprotection of the TFA-labile protecting groups was achieved by using trifluoroacetic acid, and the final deprotection step used to hydrolyze the benzylcarbamate group and the 4-methoxybenzyl group was performed with anhydrous hydrogen fluoride. The freeze-dried sublibraries were soluble in water at 1 mM. Electrospray mass spectra and quantitative amino acid analysis showed in each case a correct mass distribution and a good stoechiometry in the S_2' position, respectively (data not shown).

A second library corresponding to the parallel synthesis of pseudotripeptides of formula (synthon **26**)-Trp-AA$_2$ was achieved on 2-chlorotrityl chloride resin with the same protocol described above.

To a solution of Boc-amino acid (5 mmol) in dichloromethane (3 mL/mmol) was added successively benzylamine (6 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (Bop) (6 mmol), and diisopropylethylamine (17.5 mmol). The reaction was stirred 3 h at room temperature. After evaporation to dryness, the oily residue was diluted with ethyl acetate and washed with aqueous 1 M $KHSO_4$, 1 M $NaHCO_3$, water, and brine. The organic phase was concentrated to give the product as an oil which was used in the next step without purification.

To a solution of the Boc-benzylamide amino acid (2.5 mmol) in dichloromethane (5 mL/mmol) at 0 °C was added trifluoroacetic acid (TFA) (50 mmol). The mixture was stirred 2 h at 0 °C and 1 h at room temperature. After evaporation to dryness, the product was obtained as a white solid after trituration of the oily residue with ether/hexane (1/1). The product was used in the next step without purification.

The deprotected dipeptide was obtained after coupling with Boc-amino acid using Bop and deprotected with TFA. The dipeptides are used without purification in the next step.

General Procedure for the Synthesis of Pseudotripeptides 26−35. To a solution of (2*S*,3*S*)-3-amino-3-(3-*tert*-butylsulfamoylphenyl)-2-(4-methoxybenzylsulfanyl)propionic acid[23] or synthon **16** (0.24 mmol) in dimethylformamide (6 mL/mmol) were added the selected dipeptide (0.29 mmol), Bop (0.29 mmol), and diisopropylethylamine (0.84 mmol). The mixture was stirred 3 h at room temperature. After removal of the solvent, the crude solid was washed with aqueous 10% $KHSO_4$ solution and water, dried, and used without purification.

The residue (0.12 mmol) was stirred at 0 °C for 1 h with 10 mL of anhydrous hydrogen fluoride (HF), 0.6 mL of anisole, and 0.6 mL of dimethyl sulfide. 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 semipreparative 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-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*S*)-2-phenylethylcarbamoyl)-(2*S*)-2-(1*H*-indol-3-yl)ethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (26): 22 mg (27%). HPLC (55% B): t_R = 5.2 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6−3.15 (m, 6H), 3.6 (m, 2H), 4.15−4.25 (m, 2H), 4.6 (m, 2H), 6.8−7.25 (m, 16H), 7.55 (d, 1H), 7.85 (d, 2H), 7.9 (m, 3H), 8.5 (m, 3H), 10.7 (s, 1H). MS (ESI) (M + H)$^+$ *m*/*z* = 678.4.

4-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*S*)-2-phenylethylcarbamoyl)-(2*S*)-2-naphthalen-1-yl-ethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (27): 15 mg (18%). HPLC (60% B): t_R = 5.7 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.7−3.7 (m, 8H), 4.1−4.8 (m, 4H), 4.6 (m, 2H), 6.8−8.8 (m, 27H). MS (ESI) (M + H)$^+$ *m*/*z* = 689.5.

4-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*S*)-2-phenylethylcarbamoyl)-(2*S*)-2-(1-methyl-1*H*-indol-3-yl)ethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (28): 18 mg (22%). HPLC (50% B): t_R = 7.4 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6−3.15 (m, 6H), 3.55 (m, 5H), 4.15−4.25 (m, 2H), 4.6 (m, 2H), 6.8−7.25 (m, 16H), 7.55 (d, 1H), 7.85 (d, 2H), 7.9 (m, 3H), 8.5 (m, 3H). MS (ESI) (M + H)$^+$ *m*/*z* = 692.6.

4-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*S*)-2-phenylethylcarbamoyl)-(2*S*)-2-biphenyl-4-yl-ethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (29): 19 mg (22%). HPLC (60% B): t_R = 5.5 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6−3.1 (m, 6H), 3.6 (m, 2H), 4.2 (d, 2H), 4.6 (m, 2H), 7.0−7.4 (m, 21H), 7.8 (d, 2H), 7.9 (m, 3H), 8.55 (m, 3H). MS (ESI) (M + H)$^+$ *m*/*z* = 715.9.

4-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*S*)-2-phenylethylcarbamoyl)-(2*R*)-2-biphenyl-4-yl-ethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (30): 26 mg (31%). HPLC (gradient 40−100% B in 20 min): t_R = 12.6 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.85−3.45 (m, 6H), 3.55 (m, 1H), 3.7 (m, 1H), 4.25−4.35 (2dd, 2H),4.75 (m,1H), 4.85 (m, 1H), 7.1−7.5 (m, 21H), 7.75 (d, 2H), 7.95 (m, 3H), 8.45 (d, 1H), 8.65 (m, 2H). MS (ESI) (M + H)$^+$ *m*/*z* = 715.6.

4-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*R*)-2-phenyl-ethylcarbamoyl)-(2*S*)-2-biphenyl-4-yl-ethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (31): 32 mg (32%). HPLC (gradient 40−100% B in 20 min): t_R = 13.2 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.7−3.2 (m, 6H), 3.55 (m, 1H), 3.65 (m, 1H), 4.15−4.25 (2dd, 2H),4.65 (m, 2H), 7.05−7.5 (m, 21H), 7.8 (d, 2H), 7.95 (m, 3H), 8.45 (d, 1H), 8.65 (m, 2H). MS (ESI) (M + H)$^+$ *m*/*z* = 715.5.

4-(2*S*)-2-Amino-3-{1-[1-benzylcarbamoyl-(2*S*)-2-(3*H*-imidazol-4-yl)-ethylcarbamoyl]-(2*S*)-2-biphenyl-4-ylethylcarbamoyl}-3(*S*)-sulfanylpropyl)benzoic acid (32): 21 mg (25%). HPLC (35% B): t_R = 6.2 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6−3.15 (m, 6H), 3.35−3.45 (dd, 1H), 3.7 (m, 1H), 4.1 (2dd, 2H), 4.65 (m, 2H), 6.7 (d, 2H), 7.05−7.55 (m, 18H), 7.8 (d, 2H), 8.0 (d, 1H), 8.45 (t, 1H), 8.65 (d, 1H), 8.55 (m, 1H), 8.8 (s, 1H). MS (ESI) (M + H)$^+$ *m*/*z* = 705.3.

4-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*S*)-2-(4-hydroxyphenyl)ethylcarbamoyl)-(2*S*)-2-biphenyl-4-ylethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (33): 13 mg (14%). HPLC (gradient 40−100% B in 20 min): t_R = 11.1 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6−3.2 (m, 6H), 3.65 (m, 2H), 4.2 (d, 2H), 4.55−4.65 (m, 2H), 6.55 (d, 2H), 6.9−7.45 (m, 18H), 7.8 (d, 2H), 7.9 (m, 3H), 8.55 (m, 3H). MS (ESI) (M + H)$^+$ *m*/*z* = 731.1.

4-{(2*S*)-2-Amino-3-[1-(1-benzylcarbamoyl-(2*S*)-2-methylbutylcarbamoyl)-(2*S*)-2-biphenyl-4-ylethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (34): 28 mg (26%). HPLC (gradient 40−100% B in 20 min): t_R = 11.0 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 0.75 (m, 6H), 1.1−1.45 (m, 2H), 1.75 (m, 1H), 2.75−3.05 (m, 6H), 3.65 (m, 2H), 4.2 (m, 3H), 4.7 (m, 1H), 7.1−7.55 (m, 16H), 7.8 (d, 2H), 7.95 (m, 3H), 8.3 (d, 1H), 8.55 (t, 1H), 8.6 (d, 1H). MS (ESI) (M + H)$^+$ *m*/*z* = 681.1.

4-{(2*S*)-2-Amino-3-[1-(2*S*)-2-benzo[*b*]thiophen-3-yl-1-benzylcarbamoylethylcarbamoyl)-(2*S*)-2-biphenyl-4-yl-ethylcarbamoyl]-3(*S*)-sulfanylpropyl)}benzoic acid (35): 27 mg (30%). HPLC (gradient 55−100% B in 15 min): t_R = 8.7 min. ^{1}H NMR (DMSO-d_6 + TFA) δ 2.6−2.33 (m, 6H), 3.6 (m, 2H), 4.2 (d, 2H), 4.65−4.75 (m, 2H), 6.9−7.45 (m, 19H), 7.7−7.85 (m, 4H), 7.9 (m, 3H), 8.6 (m, 3H). MS (ESI) (M + H)$^+$ *m*/*z* = 769.6.

Acknowledgment. The authors gratefully acknowledge C. Dupuis for her assistance in drafting this manuscript and Dr. A. Blommaert for helpful comments and stylistic revision.

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JM0300680