β-Amino-thiols Inhibit the Zinc Metallopeptidase Activity of Tetanus Toxin Light Chain

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Tetanus neurotoxin is a 150-kDa protein produced by *Clostridium tetani*, which causes the lethal spastic paralytic syndromes of tetanus by blocking inhibitory neurotransmitter release at central synapses. The toxin light chain (50 kDa) has a zinc endopeptidase activity specific for synaptobrevin, an essential component of the neuroexocytosis apparatus. Previous unsuccessful attempts to block the proteolytic activity of this neurotoxin with well-known inhibitors of other zinc proteases led us to study the design of specific inhibitors as a possible drug therapy to prevent the progressive evolution of tetanus following infection. Starting from the synaptobrevin sequence at the level of the cleavage site by tetanus neurotoxin (Gln^{76}-Phe^{77}), a thiol analogue of glutamine demonstrated inhibitory activities in the millimolar range. A structure−activity relationship performed with this compound led us to determine the requirement for the correct positioning of the thiol group, the primary amino group, and a carboxamide or sulfonamide group on the side chain. This resulted in the design of a β-amino-(4-sulfamoylphenyl)glycine-thiol, the first significantly efficient inhibitor of tetanus neurotoxin with a K_i value of 35 ± 5 μM.

Introduction

Tetanus neurotoxin (TeNt) is a 150-kDa protein produced by the anaerobic bacillus *Clostridium tetani* which blocks the release of inhibitory neurotransmitter in central synapses leading to a lethal spastic paralysis.[1] TeNt is a member of the clostridial neurotoxin family, including seven botulinum neurotoxins responsible for the flaccid paralysis of botulism.[2] TeNt is composed of a heavy chain of 100 kDa linked by a disulfide bridge to a light chain of 50 kDa. The heavy chain ensures the binding, internalization, and retrograde axonal transport of the light chain into the neuronal cytosol.[3] The light chain possesses proteolytic activity and cleaves specifically VAMP/synaptobrevin,[4] an integral membrane protein of small synaptic vesicles,[5−7] which plays a critical role in the neuroexocytosis apparatus.[8−10] TeNt belongs to the M_{27} family of Zn^{2+}-metallopeptidases[11] which contains the HEXXH consensus sequence, found in the majority of zinc endopeptidases, where His^{233} and His^{237} are involved in zinc chelation and Glu^{234} in the catalytic process.[12−15] Abolition of any enzymatic activity by double mutations of Glu^{270}-Glu^{271} suggests that one of these glutamates, highly conserved among all the members of this family, could be the third zinc ligand.[15] A possible role of Tyr^{243} as an additional ligand,[16] like in the family of metzincins,[17] has also been proposed. The proteolytic activity of tetanus neurotoxin has been shown to be directed toward synaptobrevin only at its Gln^{76}-Phe^{77} peptide bond.[4] Such a narrow specificity, not common for metalloproteases, has recently been explained by an allosteric-like mechanism for TeNt. Indeed, the binding of both an acidic (S 27−55) and a basic (S 82−93) domain of synaptobrevin to tetanus toxin "exosites" is required to induce the conformational change switching on its proteolytic activity[18,19] (Figure 1A).

At the present time, there is no effective drug therapy to prevent the progressive evolution of tetanus or botulism following intoxication or infection. For these reasons, we identified the inhibition of the proteolytic activity of tetanus neurotoxin as a possible strategy for treatment following toxin exposure. Potent and selective inhibitors against TeNt proteolytic activity have yet to be obtained. Strong chelating agents specific for divalent metallic cation like EDTA or 1,10-*o*-phenanthroline[20−22] give a weak protection against this toxin at millimolar concentrations. Potent blockers of zinc peptidases such as captopril, thiorphan, and phosphoramidon have no inhibitory activity in vitro[21,23] or ex vivo.[22] Moreover, various synaptobrevin-derived peptides spanning the sequence surrounding the scissible bond failed to antagonize tetanus neurotoxin proteolytic activity even when tested at concentrations up to 1 mM.[4,21]

With the aim of designing the first selective inhibitors of TeNt, we began this study using the only available clue concerning the preference of the catalytic site: the synaptobrevin sequence at the cleavage site (QAGASQ/FETSA) (Figure 1B). Starting with synaptobrevin-derived peptides containing a thiol group as a zinc ligand, a significant inhibition in the 250 μM range was obtained. An extended structure−activity relationship analysis on this compound revealed the requirement and the position of a primary amino group, the nature of the zinc chelating group, and the nature and the length of the amino-thiol side chain. Finally, a β-amino-phenylglycine-thiol substituted in the meta position by a sulfonamide group was synthesized and shown to inhibit TeNt with a K_i value of 35 μM. This molecule

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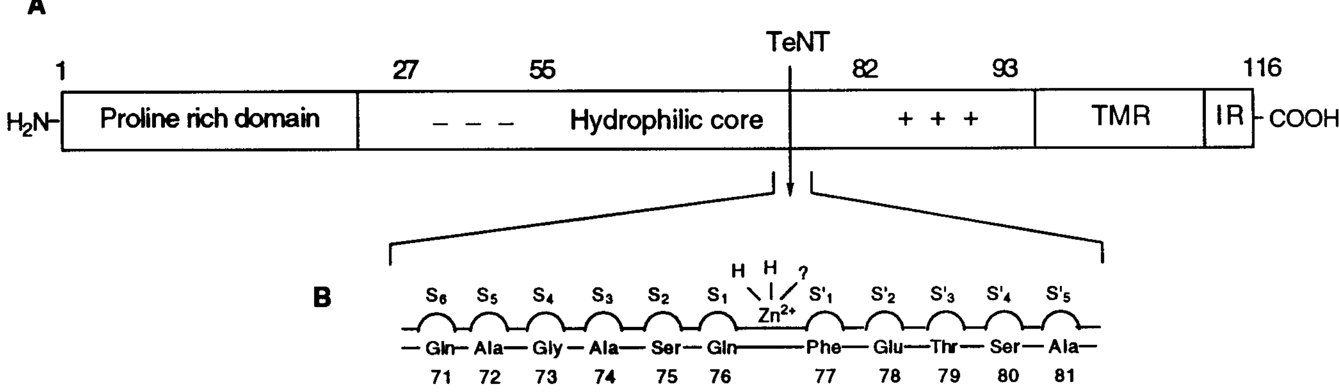


Figure 1. (A) Schematic representation of VAMP/synaptobrevin II. TMR indicates the transmembrane region and IR, the intravesicular region. The cleavage site Gln[76]-Phe[77] is indicated by the arrow. Domains 27−55 and 82−93 represent respectively the acidic and basic clusters whose binding to tetanus neurotoxin is required to switch on its proteolytic activity. (B) Human synaptobrevin II sequence surrounding the scissible bond. The side chains of the different synaptobrevin residues are putatively interacting with subsites of the catalytic domain of TeNt designated S_1−S_6 at the left of the cleavage bond and S'_1−S'_5 at the right, respectively.

is the first described relatively potent inhibitor of tetanus neurotoxin.

Results

Chemistry. The various thiol-containing pseudopeptides **1**−**5** spanning the putative S'_1−S'_5 subsites of TeNt active site (Table 1A) were synthesized by coupling the different tetrapeptides with the racemic (2*SR*)-3-(acetylsulfanyl)-2-benzylpropanoic acid as described in the Experimental Section.

The thiol derivatives **6**−**12** (Table 1B) and **14** (Table 2) encompassing the putative subsites defined as S_1−S_5 of TeNt active domain were obtained as disulfides by coupling various protected amino acids or peptides with the β-glutamine-thiol synthon **13** as described in the Experimental Section. The synthesis of **13** (Scheme 1) was achieved by using the commercially available Boc-LGln(Trt)-OH. After reduction of the α-carboxylate,[24] a nucleophilic substitution of the hydroxy group by potassium thioacetate (via the Mitsunobu reaction)[25] gave a thioester group which was deprotected in alkaline conditions. A selective deprotection of the Boc group led to the synthon **13**, whereas β-amino-glutamine-thiol **12** was formed by cleavage of both the Boc and trityl groups with trifluoroacetic acid.

Scheme 1. Synthesis of the Glutamine Derivatives[a]

[a] (a) NMM, ^{i}BuOCOCl, $NaBH_4$; (b) PPh_3, (^{i}PrN=)$_2$, CH_3COSH; (c) NaOH, I_2; (d) TFA; (e) $BzlONH_2$, BOP, DIEA; (f) H_2, Pd/C; (g) $(C_2H_5)_2O{\cdot}BF_3$, AcOH; (h) NMM, ^{i}BuOCOCl, CH_2N_2; (i) Ag_2O, Na_2CO_3, $Na_2S_2O_3$, H_2O.

The different compounds described in Table 2 represent derivatives of glutamine or glutamate bearing various zinc-coordinating entities (SH, COOH, CONHOH, or PO_3H_2). Compounds **12**−**19** were synthesized following the protocol shown in Scheme 1. Compounds **37**−**39** were prepared as previously described.[26−28] The hydroxamate derivative **15** (Table 2) was synthesized from Boc-LGln(Trt)-OH by coupling benzyloxyamine and subsequent deprotection. Compounds **17** and **18** were obtained by the same route as **12** and **15** (Scheme 1) using the intermediate compound **16**, which has been obtained previously by Arndt−Eistert homologation of Boc-LGln(Trt)-OH.[29]

Compounds **40**−**53** (Table 3), **54**, and **55** (Table 4) were prepared as previously described.[26,27,30,31]

Scheme 2 depicts the synthesis of compound **24** (Table 4), the sulfonamide analogue of the β-glutamine-thiol derivative. The disulfide group of the N- and C-protected DL-homocysteine **20** was oxidized by chlorine

Scheme 2. Synthesis of the Sulfonamide Derivative[a]

[a] (a) BzlOCOCl, NaOH; (b) CH_2N_2; (c) Cl_2, MeOH, CCl_4; (d) ^{t}BuNH$_2$; (e) $NaBH_4$; (f) PPh_3, (^{i}PrN=)$_2$, CH_3COSH; (g) NaOH; (h) TFA; (i) HF.

to give the sulfonyl chloride,[32] which was subsequently treated with *tert*-butylamine to form the *tert*-butylsulfonamide **21**. The carboxylic ester of **21** was therefore transformed into the corresponding thiol derivative **24** via the alcohol **22** substituted by potassium thioacetate to give **23** and fully deprotected by saponification and cleavage with trifluoroacetic acid and anhydrous hydrogen fluoride in succession.

Scheme 3 shows the pathway used for the preparation of the *m*-carbamoyl analogues of β-phenylglycine-thiol. Starting from 3-cyanobenzaldehyde, the Bucherer–Berg reaction[33] gave the α-amino acid, which was protected to give **25**. The α-carboxylic ester was reduced to the corresponding alcohol, whereas the aryl ester, after saponification, was transformed to the carboxamide derivatives **27a**,**b**. The replacement of the hydroxy group by potassium thioacetate led to the thiol inhibitors **29a**,**b** as described above.

Scheme 3. Synthesis of the β-Amino-(4-carbamoylphenyl)glycine Derivatives[a]

[a] (a) $SOCl_2$, EtOH; (b) $(Boc)_2O$, NEt_3; (c) $NaBH_4$; (d) NaOH; (e) NMM, $^iBuOCOCl$, NH_3; (e′) NMM, $^iBuOCOCl$, $(CH_3)_2NH_2$; (f) CH_3SO_2Cl, NEt_3; (g) CH_3COSK; (h) I_2; (i) HCl.

Scheme 4. Synthesis of the β-Amino-(4-sulfamoylphenyl)glycine Derivative[a]

[a] (a) $BzlNH_2$; (b) NMM, $^iBuOCOCl$, $NaBH_4$; (c) DMSO, $(COCl)_2$, NEt_3; (d) $(Ph)_2CHNH_2$, KCN, AcOH; (e) HCl, MeOH; (f) Amberlite 120, MeOH; (g) $NaBH_4$, LiCl; (h) PPh_3, $(^iPrN{=})_2$, CH_3COSH; (i) NaOH; (j) HF.

For the synthesis of the (3-sulfamoylphenyl)glycine inhibitor **36** (Scheme 4), the commercially available 3-(chlorosulfonyl)benzoic acid was transformed into the benzylsulfonamide analogue; subsequent reduction of carboxylate led to the corresponding benzaldehyde **31**. A modified Strecker reaction allowed compound **32** to be obtained. This compound, treated successively with HCl-saturated methanol and Amberlite IR-120 in refluxing methanol, yielded the desired amino ester **33**. The subsequent steps of the synthesis, similar to those described in Scheme 2, gave the corresponding β-aminothiol **36**.

Inhibitory Properties. Enzymatic studies were performed using the fluorescent synaptobrevin derivative [Pya[88]]S 39-88 as substrate, according to the protocol described by Solheihac et al.[34] The various pseudopeptides **1**–**11** (Table 1A,B), derived from synaptobrevin sequences at the cleavage site, did not inhibit or gave weak inhibition of TeNt activity at 1 mM, whereas the β-amino-glutamine-thiol **12**, which is hypothesized to interact with the S_1 subsite, completely inhibited the peptidase action of TeNt at this concentration.

Table 1. Exploration of the Catalytic Active Site of TeNt Light Chain with Various Thiol-Containing Pseudopeptides Putatively Mimicking Synaptobrevin S'_1–S'_5 Binding Sites (A) and S_1–S_6 Binding Sites (B)

A

n°	sequence (S_1 – Zn^{2+} – S'_1 S'_2 S'_3 S'_4 S'_5): —Gln—Phe—Glu—Thr—Ser—Ala—	inhib at 10^{-3} M (%)
1	HS–Phe*–Glu–Thr–Ser–Ala-OH	0
2	HS–Phe*–Ala–Thr–Ser–Ala-OH	0
3	HS–Phe*–Phe–Thr–Ser–Ala-OH	0
4	HS–Phe*–Gln–Thr–Ser–Ala-OH	0
5	HS–Phe*–OH	0

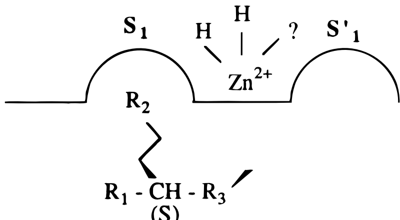
B

n°	sequence (S_6 S_5 S_4 S_3 S_2 S_1 – Zn^{2+} – S'_1): —Gln—Ala—Gly—Ala—Ser—Gln—Phe—	inhib at 10^{-3} M (%)
6	Ac-Gln-Ala-Gly-Ala-Ser-Gln(S)-SH	0
7	H-Phe-Gln(S)-SH	30
8	H-Glu-Gln(S)-SH	25
9	H-Val-Gln(S)-SH	26
10	H-Lys-Gln(S)-SH	16
11	H-Ser-Gln(S)-SH	18
12	H-Gln(S)-SH	100

HS–Phe*– = HS–CH_2–CH*(CH_2Ph)–CO–

–Gln(S)–SH = –NH–CH(S)($CH_2CH_2CONH_2$)–CH_2–SH

Table 2. Comparison of Inhibitory Potencies of Various Glutamine and Glutamate Analogues Containing, as a Zinc Ligand, a Thiol, Hydroxamate, Carboxylate, or Phosphonate Group: Importance of the Amine and the Chelation with Zinc



no.	R_1	R_2	R_3	inhib at 10^{-3} M (%)
	H_2N	$CONH_2$	COOH	0
19	H_2N	$CONH_2$	CH_2COOH	70
15	H_2N	$CONH_2$	CONHOH	0
18	H_2N	$CONH_2$	$CH_2CONHOH$	0
17	H_2N	$CONH_2$	CH_2CH_2SH	0
12	H_2N	$CONH_2$	CH_2SH	100
14	AcNH	$CONH_2$	CH_2SH	12
37	H_2N	COOH	CH_2SH	65
38	(Me)HN	COOH	CH_2SH	0
39	H_2N	COOH	PO_3H_2	0

On the basis of this result, various derivatives of glutamine bearing different functional groups capable of chelating the zinc atom of TeNt L-chain were tested. As reported in Table 2, the change of the position of the sulfanyl group in **17** strongly decreased its inhibitory effect as compared to **12**. The β- and γ-hydroxamate analogues **18** and **15** did not inhibit TeNt activity at 1 mM. Interestingly, a 70% inhibition was observed with 1 mM β-amino-homoglutamine **19**, while 1 mM L-glutamine was inactive. The phosphonate **39**[28] was a poor inhibitor. Thus, the sulfanyl group at the β-position represented the best zinc ligand to obtain significant inhibitory effect toward TeNt.

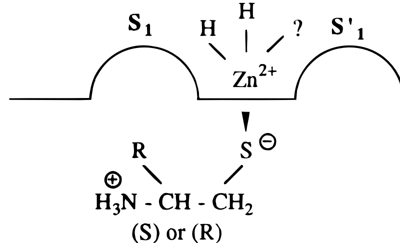
The need for a free primary amino group was investigated by introducing either an acetyl (**14**) or a methyl (**38**) group on the amino group of the β-amino-glutamine-thiol **12** or the β-amino-glutamate-thiol **37**, respectively. An almost complete abolition of any inhibitory effect was obtained with these modified compounds (Table 2).

Finally, various β-amino-thiols differing in their side chain structure were studied in order to explore the S_1 subsite specificity of TeNt (Tables 3 and 4). As shown in Table 3, the length of the aliphatic side chains does not seem to be very important, with only a slight preference for an ethylene linker (compare **12** to **40**). The charged or polar groups such as $-CO_2^-$, $-SO_3^-$, $-NH_3^+$, or $-CONH_2$ are more favorable than $-PO_3^{2-}$ for TeNt inhibition, and the chirality of the α-carbon is unimportant (compare **37** to **41** and **42** to **43**). The cyclic or aromatic side chains are poorly recognized (compound **48**) except when they are substituted at a meta position by a charged group (compound **53**). In Table 4, the K_i values of selected inhibitors are reported, and these indicate that a sulfonamide substituent is preferred in both linear ($K_i \sim 100\ \mu$M) and aromatic ($K_i \sim 35\ \mu$M) side chains.

Discussion

The sequence of synaptobrevin at the C-terminus of the scissible bond (FETSA), putatively interacting with

Table 3. Exploration of the S_1 Subsite of TeNt Light Chain with β-Amino-thiols Having Various Aromatic and Aliphatic Side Chains



no.	config	side chain	inhib at 10^{-3} M (%)
12	(*S*)	R = -$(CH_2)_2$-$CONH_2$	100
40	(*S*)	R = -$(CH_2)_3$-$CONH_2$	75
37	(*S*)	R = -$(CH_2)_2$-COOH	65
41	(*R*)	R = -$(CH_2)_2$-COOH	73
42	(*S*)	R = -$(CH_2)_3$-COOH	92
43	(*R*)	R = -$(CH_2)_3$-COOH	69
44	(*S*)(*R*)	R = -$(CH_2)_4$-COOH	70
45	(*S*)	R = -$(CH_2)_4$-NH_3^+	90
46	(*S*)(*R*)	R = -$(CH_2)_2$-SONa	100
47	(*S*)(*R*)	R = -$(CH_2)_2$-PO_3H_2	46
48	(*S*)(*R*)	R = -CH_2-Ph	0
49	(*S*)(*R*)	R = -CH_2-(4-COOH)Ph	30
50	(*S*)(*R*)	R = -CH_2-(4-COOH)cHex	62
51	(*S*)(*R*)	R = -CH_2-(3-COOH)cHex	47
52	(*S*)(*R*)	R = -(4-COOH)Ph	0
53	(*S*)(*R*)	R = -(3-COOH)Ph	100

S'_1–S'_5 subsites of TeNt active site, was modified by substituting the amino-terminal group by a sulfanyl-methylene group, which is known to be a highly potent zinc ligand. For the five analogues described in Table 1A, no activity was detected even at a millimolar concentration of inhibitor. This is an unusual result for a zinc endopeptidase, as for most other enzymes of this group, the introduction of a zinc-coordinating group on molecules able to interact with at least one subsite of the catalytic domain has given lead compounds, which have been further optimized by structure–activity relationship analyses to give efficient inhibitors.[27,31,35–36] For instance, for angiotensin-converting enzyme (ACE) and for neprilysin (NEP), the sulfanyl analogues of Phe-Trp and Phe-Leu had inhibitory activities in the micromolar range.[37,38]

The peptide sequence of synaptobrevin at the N-terminus of the scissible bond (AGASQ), putatively interacting with the corresponding subsites of TeNt active site, was also modified by substituting the COOH terminal by a sulfanyl-methylene group (Table 1B). Weak activities were observed from pentapeptide to dipeptide, while the β-amino-glutamine-thiol **12** gave complete inhibition at 1 mM. Further experiments determined a K_i value of 250 μM for this compound (Table 4). Such types of inhibitor are well-known to inhibit efficiently aminopeptidase activities. Indeed, β-amino-glutamate-thiol, β-amino-methionine-thiol, and β-amino-lysine-thiol have K_i values ranging from 10^{-7} to 10^{-9} M for aminopeptidase A,[39] aminopeptidase N,[31] and aminopeptidase B,[30] respectively. Given the strict endopeptidase character of tetanus neurotoxin, this finding was quite surprising. Nevertheless, the requirement of a free primary amino group, common for aminopeptidase substrates or inhibitors, was further verified for TeNt by introducing an acetyl (**14**) or a

Table 4. Exploration of the TeNt Light Chain S_1 Subsite with Various β-Amino-thiols[a]

no.	S1 — H, H, ? / Zn^{2+} — S'1	Ki (μM)	no.	S1 — H, H, ? / Zn^{2+} — S'1	Ki (μM)
(12)	$CONH_2$; S^-; H_2N—CH—CH_2 (S)	250 ± 35	(53)	COOH (phenyl); S^-; H_2N—CH—CH_2 *	250 ± 40
(37)	COOH; S^-; H_2N—CH—CH_2 (S)	800 ± 50	(29a)	$CONH_2$ (phenyl); S^-; H_2N—CH—CH_2 *	125 ± 25
(46)	SO_3Na; S^-; H_2N—CH—CH_2 *	300 ± 60	(29b)	$CON(Me)_2$ (phenyl); S^-; H_2N—CH—CH_2 *	250 ± 45
(54)	SO_3Na; S^-; H_2N—CH_2—CH—CH_2 *	500 ± 45	(55)	SO_3Na (phenyl); S^-; H_2N—CH—CH_2 *	40 ± 5
(24)	SO_2NH_2; S^-; H_2N—CH—CH_2 *	100 ± 5	(36)	SO_2NH_2 (phenyl); S^-; H_2N—CH—CH_2 *	35 ± 5

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

methyl (**38**), which almost completely abolished inhibitory activity (Table 2). The requirement of its correct positioning was also verified with compound **54** by introducing a methylene between the primary amino group and the β-carbon of the thiol leading to a significant decrease in inhibitory efficiency (Table 4). This is unexpected for a strict endopeptidase. Two hypotheses could account for this behavior: (i) either an accessible glutamate (or aspartate) stabilizing this primary amino group by formation of hydrogen bonds is present in the active site of the enzyme, as demonstrated for APN;[40] or (ii) these inhibitors bind the zinc ion as bidentates involving both sulfanyl and amino groups.

The next step in this study was to optimize the nature and the positioning of the zinc chelating group. The sulfanyl group was found to be the most potent, as compared to other well-known zinc ligands such as hydroxamates, carboxylates, and phosphonates (Table 2). This was not unexpected because the sulfanyl group is one of the most efficient zinc ligands.

Finally, to improve the affinities of these β-amino-thiols for TeNt through efficient interactions with the putative S_1 subsite, various compounds previously synthesized and studied in our laboratory for aminopeptidase inhibition[26,27] and having different side chains have been tested (Tables 3 and 4). From these results, it can be concluded that the best interactions with the S_1 subsite are obtained with hydrophilic side chains bearing acid or amide moieties in the following decreasing order of efficacy $SO_2NH_2 > SO_3H > CONH_2 >$ COOH. Furthermore, these functional groups have to be preferentially in γ positions of linear alkyl side chains, or even better in the meta position of a phenyl ring. The conformational restriction imposed by the phenyl ring, as compared to the linear alkyl chain, suggests a particularly well-fitted interaction in this case, between the acid or amide functional group and a putative hydrogen-bond acceptor group present in the S_1 subsite of TeNt. Moreover, inversion of the absolute configuration in **41** and **43** (compare respectively to **37** and **42**) does not seem to be important in terms of inhibitory potency.

In addition, unlike most of the endopeptidases studied such as NEP, ACE, thermolysin, E.C. 24-15, and E.C. 24-16, TeNt does not recognize hydrophobic side chains at the S_1 subsite.

In conclusion, the present study is a preliminary step toward the characterization of the active site of this new type of zinc endopeptidase and the design of potent and selective inhibitors of TeNt. These data demonstrate the crucial role of the S_1 subsite in the active site cleft of TeNt, which seems to have some analogies with the S_1 subsite of acidic aminopeptidases such as aminopeptidase A, although it is well-known that TeNt does not have any aminopeptidase activity. The structure–activity relationship analysis performed on diversely substituted β-amino-thiols has established the critical position of a sulfanyl, a primary amino group, and a sulfonamide moiety for TeNt inhibition. This led to the design of β-amino-(4-sulfamoylphenyl)glycine-thiol **36**, which with a K_i value of 35 μM is the first described relatively efficient in vitro inhibitor of TeNt proteolytic activity. Cell culture experiments are now in progress

in our laboratory in order to determine the membrane permeability of these β-amino-thiols. To be efficient in vivo, these inhibitors should be capable of entering the neuronal cytosol to inhibit the proteolytic activity of the toxin at its site of action.

Experimental Section

Chemistry. High-performance liquid chromatography (HPLC) grade solvents and solvents were from Solvants Documentation Synthese (Peypin en provence, France). Fmoc amino acids, solvents, and other reagents for solid-phase peptide synthesis (SPPS) were obtained from Perkin-Elmer. Chromatography was carried out with Merck silica gel Si 60 (40−63 μm). TLC was performed on silica gel (60 F 254, 0.2 mm thick; Merck). The final products were purified on an Applied Biosystem 151A HPLC apparatus with a C_{18} Vydac (ref 2178TP510) and acetonitrile gradients performed with buffers A (H_2O, TFA 0.1% (v/v)) and B (CH_3CN/H_2O (7:3), TFA 0.09% (v/v)). The absorbance of eluted peaks was monitored at 214 nm. The purity of the final compounds was also checked by HPLC using a reverse-phase column (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) with the same elution buffers.

The structure of all the compounds was confirmed by ^{1}H NMR spectroscopy on a Bruker AC 270-MHz or Bruker AM 400-MHz spectrometer in DMSO-d_6 or $CDCl_3$ solutions (5 × 10^{-3} M) using HMDS as internal reference. The signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Satisfactory elemental analyses, performed at the university of P & M Curie Paris VI (Jussieu, Paris), were obtained (C, H, N) for all compounds. Mass spectral analyses for all the final compounds were achieved by Quad Service (Poissy, France) using the electrospray ionization technique (ESI). Optical rotations were measured on a Perkin-Elmer 241 polarimeter (1.0-dm cell) for MeOH solutions at 20 °C. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Melting points of the crystallized compounds were measured on an electrothermal apparatus and are reported uncorrected.

Abbreviation: Pya, pyrenylalanine.

General Procedure for Solid-Phase Peptide Synthesis. Protected peptide assemblies were carried out in NMP using either Wang/HMP resin or 2-chlorotrityl chloride resin on a ABI 431 peptide synthesizer (Perkin-Elmer) using the Fmoc/tBu strategy. Fmoc deprotection was achieved using 20% piperidine in NMP. Residues were coupled with 10-fold molar excess of Fmoc-amino acids using standard activation by DCC/HOBt in NMP. With HMP resin, the first amino acid could be coupled to the HMP resin by using DCC with DMAP catalysis, whereas the loading of 2-chlorotrityl chloride resin was performed by using DIEA in dry CH_2Cl_2. The amino acid side chain protecting groups used were 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg; Trt for Asn, Gln, and His; Boc for Lys; tBu for Asp, Glu, Ser, Thr, and Tyr. For HMP resin cleavage and peptide deprotection, the dry peptidyl resins were treated by TFA/H_2O/triisopropylsilane mixtures (92.5/5/2.5) for 2 h at room temperature.[41,42] For fully protected peptide cleavage from 2-chlorotrityl chloride resin, the dry peptidyl resins were stirred for 4 h with TFE/CH_2Cl_2 (2:8).[43]

Preparation of $HSCH_2$-CH(-CH_2Ph)-CO-NH-CH(R1)-CO-Thr-Ser-Ala-OH (Compounds 1−5). The various peptidyl-resins were prepared according to the solid-phase peptide synthesis procedure at a 50-μmol scale on HMP resin using (2*SR*)-3-(acetylsulfanyl)-2-benzylpropanoic acid in the last coupling step. This synthon was prepared as previously described.[44]

To deprotect the thiol group, the crude peptides were dissolved in degassed MeOH (2 mL/mmol) under inert atmosphere and 1 N NaOH (3 equiv) was added at 0 °C. The mixture was stirred for 3 h at room temperature. After acidification with HCl (2 N), the organic layers were evaporated, diluted in H_2O, and extracted with degassed EtOAc. The organic layers were washed with H_2O and brine, dried over Na_2SO_4, and evaporated to dryness to obtain the different compounds SH-free which were purified by HPLC.

R_1 = -CH_2-CH_2COOH (1): retention time = 14.6 and 15.4 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (26% yield); ^{1}H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, CHγ(Thr)), 1.21 (3H, d, CHγ(Ala)), 1.74 (1H, m, CHβ(Glu)), 1.89 (1H, m, CHβ(Glu)), 2.20 (2H, t, CHγ(Glu)), 2.38−2.74 (4H, m, *SH*, *CH₂*S, *CH*CH₂S), 2.85 (2H, dd, *CH₂*Ph), 3.58 (2H, m, CHβ(Ser)), 3.97 (1H, m, CHβ(Thr)), 4.10−4.40 (4H, m, CHα(Ala), CHα(Thr), CHα(Ser), CHα(Glu)), 7.03−7.20 (5H, m, Ar), 7.60 (A) 7.70 (B) (1H, d, NH(Thr)), 7.82 (1H, m, NH(Ser)), 8.00 (1H, m, NH(Ala)), 8.16 (A) 8.23 (B) (1H, d, NH(Glu)).

R_1 = -CH_3 (2): retention time = 15.8 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (40% yield); ^{1}H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, CHγ(Thr)), 1.20 (3H, d, CHγ(Ala)), 2.30−2.72 (4H, m, *SH*, *CH₂*S, *CH*CH₂S), 2.88 (2H, dd, *CH₂*Ph), 3.58 (2H, m, CHβ(Ser)), 3.97 (1H, m, CHβ(Thr)), 4.09−4.36 (4H, m, CHα(Ala), CHα(Thr), CHα(Ser), CHα(Ala)), 7.06−7.23 (5H, m, Ar), 7.60 (A) 7.70 (B) (1H, d, NH(Thr)), 7.78 (1H, m, NH(Ser)), 8.00 (1H, m, NH(Ala)), 8.11 (A) 8.22 (B) (1H, d, NH(Ala)).

R_1 = -CH_2Ph (3): retention time = 21.4 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (34% yield); ^{1}H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, CHγ(Thr)), 1.20 (3H, d, CHγ(Ala)), 2.40−2.80 (4H, m, *SH*, *CH₂*S, *CH*CH₂S), 3.00 (2H, dd, *CH₂*Ph), 3.60 (2H, m, CHβ(Ser)), 4.00 (1H, m, CHβ(Thr)), 4.15 (1H, m, CHα(Ala)), 4.30 (2H, m, CHα(Thr), CHα(Ser)), 4.58 (1H, m, CHα(Phe)), 6.91−7.25 (5H, m, Ar), 7.80 (1H, d, NH(Thr)), 7.90 (1H, m, NH(Ser)), 8.03 (1H, m, NH(Ala)), 8.26 (1H, d, NH(Phe)).

R_1 = -CH_2-CH_2-$CONH_2$ (4): retention time = 13.4 and 14.3 min (A, B) (Nucleosil, C_{18}, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−90% B in 30 min, flow rate 0.8 mL/min) (38% yield); ^{1}H NMR (DMSO-d_6 + TFA) δ 1.00 (3H, d, CHγ(Thr)), 1.20 (3H, d, CHγ(Ala)), 1.75 (1H, m, CHβ(Gln)), 1.89 (1H, m, CHβ(Gln)), 2.10 (2H, t, CHγ(Gln)), 2.33−2.72 (4H, m, *SH*, *CH₂*S, *CH*CH₂S), 2.86 (2H, dd, *CH₂*Ph), 3.58 (2H, m, CHβ(Ser)), 3.97 (1H, m, CHβ(Thr)), 4.10−4.30 (4H, m, CHα(Ala), CHα(Thr), CHα(Ser), CHα(Gln)), 7.06−7.21 (5H, m, Ar), 7.67 (A) 7.81 (B) (1H, d, NH(Thr)), 7.82 (1H, m, NH(Ser)), 8.05 (1H, m, NH(Ala)), 8.22 (1H, d, NH(Glu)).

3,3′-(Disulfanediyl)bis[(2*SR*)-2-benzylpropanoic acid] (5). The 3,3′-(disulfanediyl)bis[(2*SR*)-2-benzylpropanoic acid] was prepared as previously described.[44]

Preparation of Symmetric Disulfides [Ac-Gln-Ala-Gly-Ala-Ser-*Gln*-(CH_2S-)]-$_2$ and [H_2N-CH(R_2)-CONH-CH(CH_2-CH_2CONH_2)-CH_2S]-$_2$ (6−11). The various protected commercially available BocHN-CH(R′2)-COOH as well as the protected peptide Ac-Gln(Trt)-Ala-Gly-Ala-Ser(tBu)OH, synthesized according to the general procedure of solid-phase peptide synthesis on 2-chlorotrityl chloride resin, were coupled to the symmetric disulfide [H_2N-CH(CH_2CH_2CONHTrt)-CH_2S]-$_2$ **(13)** using BOP as coupling agent.[45] The protected peptides were treated by TFA/H_2O/triisopropylsilane mixtures (92.5/5/2.5) for 2 h at room temperature as previously described[41,42] and purified by HPLC.

[Ac-Gln-Ala-Gly-Ala-Ser-*Gln*-(CH_2S-)]-$_2$ (6): retention time = 8.92 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 10−50% B in 15 min, flow rate 0.8 mL/min) (37% yield); ^{1}H NMR (DMSO-d_6) δ 1.17 (6H, d, 2 × CH_3β(Ala)), 1.50−1.65 (2H, m, CH_2β(Gln)), 1.68−1.84 (2H, m, CH_2β(Gln)), 1.78 (3H, s, *CH₃*-CO), 1.95−2.05 (4H, m, 2 × CH_2γ(Gln)), 2.74 (2H, m, *CH₂*S), 3.5−3.7 (4H, m, CH_2α(Gly) and CH_2β(Ser)), 3.83 (1H, m, *CH*CH₂S), 4.1−4.25 (4H, m, CHα(Gln), CHα(Ala), CHα(Ala) and CHα(Ser)), 6.70 (2H, s, $CONH_2$), 7.16 (1H, s, $CONH_2$), 7.22 (1H, s, $CONH_2$), 7.63 (1H, d, NH), 7.87 (1H, d, NH), 7.95 (2H, t, NH), 8.06 (1H, d, NH), 8.13 (1H, t, NH).

R_2 = -CH_2Ph (7): retention time = 16.42 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−80% B in 30 min, flow rate 0.8 mL/min) (41% yield); ^{1}H NMR (DMSO-d_6) δ 1.6−1.75 (2H, m, CH_2β(Gln)), 2.05 (2H, m, CH_2γ(Gln)), 2.6−2.75

(2H, dd, CH_2S), 2.9−3.05 (2H, dd, CH_2Ph), 3.9 (2H, m, CHα(Phe) and CHα(Gln)), 6.75 and 7.15 (2H, s, $CONH_2$), 7.1−7.3 (5H, m, Ar), 8.1 (3H, s, NH_3^+), 8.4 (1H, d, −CONH−).

R_2 = -$(CH_2)_2$COOH (8): retention time = 6.64 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−40% B in 15 min, flow rate 0.8 mL/min) (36% yield); ^{1}H NMR (DMSO-d_6) δ 1.6−1.75 (2H, m, CH_2β(Gln)), 2.1 (4H, m, CH_2γ(Gln) and CH_2β(Glu)), 2.3 (2H, m, CH_2γ(Glu)), 2.8 (2H, dd, CH_2S), 3.95 (2H, m, CHα(Glu), CHα(Gln)), 7.95 (1H, d, −CONH−), 8.25 (3H, s, NH_3^+).

R_2 = -CH$(CH_3)_2$ (9): retention time = 14.18 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−40% B in 15 min, flow rate 0.8 mL/min) (40% yield); ^{1}H NMR (DMSO-d_6) δ 0.85 (6H, m, CH_3δ), 1.4−1.8 (5H, m, CH_2β(Gln), CH_2β(Leu), and CHγ(Leu)), 2.05 (2H, t, CH_2γ(Gln)), 2.7−2.9 (2H, dd, CH_2S), 3.6 (1H, m, CHα(Leu)), 3.95 (1H, m, CHα(Gln)), 6.7−7.15 (2H, s, $CONH_2$), 8.1 (3H, s, NH_3^+), 8.35 (1H, d, −CONH−).

R_2 = -$(CH_2)_4NH_2$ (10): retention time = 3.26 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−30% B in 15 min, flow rate 0.8 mL/min) (29% yield); ^{1}H NMR (DMSO-d_6) δ 1.3−1.8 (8H, m, CH_2β(Gln), CH_2β(Lys), CH_2γ(Lys), CH_2δ(Lys)), 2.05 (2H, t, CH_2γ(Gln)), 2.7 (2H, m, CH_2ε(Lys)), 2.7−2.9 (2H, dd, CH_2S), 3.65, 3.95 (2H, m, CHα(Lys) and CHα(Gln)), 6.75−7.2 (2H, s, $CONH_2$), 7.7−8.1 (6H, s, NH_3^+ and NH_3^+(Lys)), 8.4 (1H, d, −CONH−).

R_2 = -CH_2OH (11): retention time = 5.15 min (Nucleosil, C_8, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−30% B in 15 min, flow rate 0.8 mL/min) (33% yield); ^{1}H NMR (DMSO-d_6) δ 1.6−1.75 (2H, m, CH_2β(Gln)), 2.05 (2H, t, CH_2γ(Gln)), 2.7−2.85 (2H, dd, CH_2S), 3.55 (1H, m, CHα(Ser)), 3.7 (2H, m, CH_2β(Ser)), 3.9 (1H, m, CHα(Gln)), 5.45 (1H, m, CH_2OH), 6.7−7.15 (2H, s, $CONH_2$), 8.05 (3H, s, NH_3^+), 8.3 (1H, d, −CONH−).

Preparation of 5,5′-(Disulfanediyl)bis[(4*S*)-4-amino-*N*-tritylpentanamide] (13) and 5,5′-(Disulfanediyl)bis[(4*S*)-4-aminopentanamide)] (12). The carboxylic acid function of (4*S*)-BocGln(Trt)-OH was transformed into alcohol by formation of the mixed anhydride with isobutyl chloroformate in DME at −15 °C followed by a reduction with sodium borohydride as previously described[24] yielding (4*S*)-4-(Boc-amino)-5-hydroxy-*N*-tritylpentanamide as a white solid (70% yield). Thioacetylation of this compound was then performed by a Mitsunobu reaction[25] to afford (4*S*)-5-(acetylsulfanyl)-4-(Boc-amino)-*N*-tritylpentanamide as a white solid (40% yield).

Deprotection of the acetyl group was achieved by using 1 N NaOH in methanol at room temperature for 3 h, and I_2 was then added until a persistent yellow color was obtained. The excess of iodine was reduced by $Na_2S_2O_3$, and the solution was evaporated in vacuo. The aqueous residue was acidified with 2 N HCl to pH 2−3 and extracted with EtOAc. After classical workup, 5,5′-(disulfanediyl)bis[(4*S*)-4-(*N*-Boc-amino)-*N*-tritylpentanamide] was obtained as a white solid (87% yield).

To a solution of 5,5′-(disulfanediyl)bis[(4*S*)-4-(*N*-Boc-amino)-*N*-tritylpentanamide] in acetic acid was added 6.5 equiv of boron trifluoride diethyl etherate.[46] After stirring for 1 h, the mixture was treated with a solution of NH_4OH (28%), the pH was adjusted to 9−10 with $KHCO_3$, and the aqueous layer was extracted with CH_2Cl_2. The organic layers were washed with brine, dried over Na_2SO_4, and evaporated in vacuo to afford 5,5′-(disulfanediyl)bis[(4*S*)-4-amino-*N*-tritylpentanamide] **(13)** (82% yield): retention time = 24.3 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−90% B in 30 min, flow rate 1.5 mL/min); ^{1}H NMR (DMSO-d_6) δ 1.8 (2H, m, CH_2β), 2.4 (2H, m, CH_2γ), 2.95 (2H, m, CH_2S), 3,15 (1H, m, CHα), 7.15 (15H, m, Trt), 8.30 (3H, s, NH_3^+), 8.65 (1H, s, CON*H*Trt); $[\alpha]_D$ = +82.0 (*c* 0.87, MeOH); SM (ES) $(M + H)^+$ *m*/*z* = 780.3. Anal. ($C_{48}H_{50}N_4O_2S$).

5,5′-(Disulfanediyl)bis[(4*S*)-4-(*N*-Boc-amino)-*N*-tritylpentanamide] was treated by a TFA/H_2O/triisopropylsilane mixture (92.5/5/2.5) for 2 h at room temperature as previously described.[41,42] 5,5′-(Disulfanediyl)bis[(4*S*)-4-aminopentanamide] **(12)** was thus obtained as a yellow oil by precipitation in diethyl ether/hexane (1/1) (98% yield): retention time = 9.97 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−40% B in 15 min, flow rate 0.8 mL/min); ^{1}H NMR (DMSO-d_6) δ 1.8 (2H, m, CH_2β), 2.2 (2H, m, CH_2γ), 2.9 (2H, m, CH_2S), 3.3 (1H, m, CHα), 6.9−7.4 (2H, s, $CONH_2$), 8.1 (3H, s, NH_3^+); SM (ES) $(M + H)^+$ *m*/*z* = 295.2. Anal. ($C_{10}H_{22}N_4O_2S_2$).

5,5′-(Disulfanediyl)bis[(4*S*)-4-(acetylamino)pentanamide] (14). $[H_2N\text{-}CH(CH_2CH_2CONHTrt)\text{-}CH_2S]_{-2}$ **(13)** was coupled to anhydride acetic acid in DMF with 2 equiv of DIEA. The *N*-tritylamide was deprotected as previously described[41,42] and purified by semipreparative HPLC on a C18 Vydac column (29% yield): retention time = 10.37 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å; gradient 0−40% B in 15 min, flow rate 0.8 mL/min); ^{1}H NMR (DMSO-d_6) δ 1.5−1.7 (2H, m, CH_2β), 1.7 (3H, s, CH_3CONH), 2.0 (2H, t, CH_2γ), 2.75 (2H, m, CH_2S), 3.85 (1H, m, CHα), 6.66 (1H, s, $CONH_2$), 7.2 (1H, s, $CONH_2$), 7.7 (1H, d, CH_3CON*H*).

(2*S*)-2-Amino-N^1-hydroxypentanediamide (15). BocGln(Trt)OH was coupled with benzyloxyamine hydrochloride (1 molar equiv) using BOP as coupling agent[45] to afford (2*S*)-N^1-benzyloxy-2-(Boc-amino)-N^5-tritylpentanediamide: $[\alpha]_D$ = −17.8 (*c* 0.95, MeOH) (91% yield).

The benzyl protecting group was cleaved by hydrogenolysis with 10% Pd/C as catalyst to yield (2*S*)-2-(Boc-amino)-N^1-hydroxy-N^5-tritylpentanediamide, and the trityl group was cleaved as previously described[41,42] to afford (2*S*)-2-amino-N^1-hydroxypentanediamide **(15)** (36% yield): R_f = 0.23 in propanol-2/NH_4OH/H_2O (7/1/1); ^{1}H NMR (DMSO-d_6) δ 1.55 (2H, m, $CH_2$$CH_2CONH_2$), 2.01 (2H, t, $CH_2$$CONH_2$), 2.56 (1H, m, H_3^+N*CH*), 7.00 (1H, s, $CONH_2$), 7.34 (1H, s, $CONH_2$), 7.73 (3H, br s, NH_3^+), 8.90 (1H, s, OH), 10.64 (1H, s, NH); SM (ES) $(M + H)^+$ *m*/*z* = 162.0. Anal. ($C_5H_{11}N_3O_3$).

(3*S*)-3-(Boc-amino)-6-oxo-(tritylamino)hexanoic Acid (16). To 0.5 M (*S*)-BocGln(Trt)-OH in THF was added *N*-methylmorpholine (1.1 molar equiv), followed by isobutyl chloroformate (1.1 molar equiv) at −15 °C. After 20 min of stirring at this temperature, the white precipitate was filtered off and washed with THF. To this solution was added an ethereal solution of diazomethane (2.0 molar equiv), and the yellow reaction mixture stirred at room temperature during 1 h. After evaporation of the excess diazomethane and removal of the solvent under reduced pressure, the diazoketone was taken up in EtOAc, washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude diazoketone, dissolved in dioxane was gradually added into a stirring mixture of Ag_2O (0.18 equiv), anhydrous Na_2CO_3 (0.96 equiv), $Na_2S_2O_3 \cdot 5H_2O$ (0.23 equiv), H_2O heated at 50 °C. After 1 h at reflux, the reaction mixture was cooled, diluted with water, filtered, and extracted with three portions of diethyl ether. The aqueous phase was acidified with $KHSO_4$ (1 N), extracted with ethyl acetate, washed with brine, dried with Na_2SO_4, and evaporated in vacuo (75% yield): R_f = 0.57 in AcEt/CH_2Cl_2 (1/3); ^{1}H NMR (DMSO-d_6) δ 1.33 (9H, s, tBu), 1.39−1.58 (2H, m, $CH_2$$CH_2$CONHTrt), 2.16−2.27 (4H, m, CH_2CONHTrt and CH_2COOH), 3.69 (1H, m, BocNH*CH*), 6.61 (1H, d, Boc*NH*), 7.06−7.25 (15H, m, Trt), 8.50 (1H, s, *NH*Trt), 12.1 (1H, br s, COOH); SM (ES) $(M + H)^+$ *m*/*z* = 503.7. Anal. ($C_{30}H_{34}N_2O_5$) C, H, N.

6,6′-(Disulfanediyl)bis[(4*S*)-4-aminohexanamide] (17). This compound was synthesized from **16** using the procedure described for compound **12** (94% yield): ^{1}H NMR (DMSO-d_6 + TFA) δ 1.90 (2H, m, $CH_2$$CH_2CONH_2$), 2.30 (2H, t, $CH_2$$CONH_2$), 2.90 (1H, ABX *J* = 16, 6 Hz, CH_2S), 3.08 (1H, ABX *J* = 16, 6 Hz, CH_2S), 3.44 (1H, m, BocNH*CH*), 8.03 (3H, br s, NH_3^+); SM (ES) $(M + H)^+$ *m*/*z* = 323.7. Anal. ($C_{12}H_{26}N_4O_2S_2$).

(3*S*)-3-Amino-N^1-hydroxyhexanediamide (18). This compound was synthesized from **16** using the procedure described for compound **15** (33% yield): ^{1}H NMR (DMSO-d_6) δ 1.68 (2H, m, $CH_2$$CH_2CONH_2$), 2.26 (2H, t, $CH_2$$CONH_2$), 2.40 (2H, t, CH_2CONHOH), 3.30 (1H, m, H_3^+N*CH*), 7.12 (1H, s, $CONH_2$), 7.60 (1H, s, $CONH_2$), 7.84 (3H, br s, NH_3^+), 8.92 (1H, s, OH), 10.66 (1H, s, NH); SM (ES) $(M + H)^+$ *m*/*z* = 176.0. Anal. ($C_6H_{13}N_3O_3$).

(3*S*)-3-Amino-6-oxo-aminohexanoic Acid (19). This compound was obtained by deprotection of the tritylamide of compound **16** as previously described[41,42] (92% yield): R_f = 0.34 in propanol-2/NH_4OH/H_2O (7/1/1); ^{1}H NMR (DMSO-d_6) δ 1.73

(2H, m, *CH*$_2$CH$_2$CONH$_2$), 2.15 (2H, t, *CH*$_2$CONH$_2$), 2.57 (2H, ABX J = 16, 6 Hz, *CH*$_2$COOH), 3.35 (1H, m, BocNH*CH*), 6.86 (1H, s, CO*NH*$_2$), 7.33 (1H, s, CO*NH*$_2$), 7.86 (3H, br s, *NH*$_3^+$); SM (ES) (M + H)$^+$ m/z = 161.3. Anal. ($C_6H_{12}N_2O_3$).

4,4′-(Disulfanediyl)bis[methyl (2*SR*)-2-(Cbz-amino)butanoate] or DL-(*Z*)-Homocystine-OMe (20). This compound was synthesized as previously described[32] (96% yield): R_f = 0.46 in AcEt/CH$_2$Cl$_2$ (1/9); ^{1}H NMR (DMSO-d_6) δ 1.83–2.09 (2H, m, *CH*$_2$CH$_2$S), 2.68 (2H, t, *CH*$_2$S), 3.58 (3H, s, COO*CH*$_3$), 4.13 (1H, m, *CH*COOMe), 4.98 (2H, s, *CH*$_2$ Ph), 7.28 (5H, m, Ph), 7.75 (1H, d, Bzl OCO*NH*). Anal. ($C_{26}H_{32}N_2O_8S_2$) C, H, N.

Methyl (2*SR*)-4-(*tert*-Butylsulfamoyl)-2-(Cbz-amino)-butanoate (21). To a cold (0 °C) solution of (*Z*)-homocystine-OMe **(20)** in MeOH (0.7 mL/mmol) and CCl$_4$ (3.5 mL/mmol) was bubbled Cl$_2$ (gas) for 1 h[32] in order to obtain methyl (2*SR*)-2-(Cbz-amino)-4-(chlorosulfonyl)butanoate. To a solution of the latter in CH$_2$Cl$_2$ (1.8 mL/mmol) was added *tert*-butylamine. After evaporation and classical workup, the residue was purified on a silica gel column, using cHex/CH$_2$Cl$_2$/EtOAc (5/3/2) as eluent (77% yield): R_f = 0.42 in AcEt/CH$_2$Cl$_2$ (1/9); ^{1}H NMR (DMSO-d_6) δ 1.18 (9H, s, tBu), 1.88–2.13 (2H, m, *CH*$_2$-CH$_2$SO$_2$NH), 2.85–3.10 (2H, m, *CH*$_2$SO$_2$NH), 3.58 (3H, s, COO*CH*$_3$), 4.21 (1H, m, *CH*COOMe), 4.99 (2H, s, CH$_2$Ph), 6.86 (1H, s, NHtBu), 7.29 (5H, m, Ph), 7.85 (1H, d, BzlOCO*NH*). Anal. ($C_{17}H_{26}N_2O_6S$) C, H, N.

(3*SR*)-*N*-*tert*-Butyl-3-(Cbz-amino)-4-hydroxybutanesulfonamide (22). The N-protected amino ester **21** (1 equiv) was dissolved in EtOH/H$_2$O (1/1). NaBH$_4$ (4 equiv) was added, and the mixture was heated at 50 °C for 6 h. The reaction was stopped with 1 N HCl, and the mixture was extracted with EtOAc. After classical workup, (3*SR*)-*N*-*tert*-butyl-3-(Cbz-amino)-4-hydroxybutanesulfonamide was obtained (74% yield): R_f = 0.18 in MeOH/CH$_2$Cl$_2$ (4/96); ^{1}H NMR (DMSO-d_6) δ 1.20 (9H, s, tBu), 1.64–1.99 (2H, m, *CH*$_2$CH$_2$SO$_2$NH), 2.90 (2H, t, *CH*$_2$SO$_2$NH), 3.30 (2H, m, *CH*$_2$OH), 3.50 (1H, m, *CH*CH$_2$OH), 4.72 (1H, t, CH$_2$*OH*), 4.97 (2H, d, CH$_2$Ph), 6.76 (1H, s, NHtBu), 7.11 (1H, d, BzlOCO*NH*), 7.29 (5H, m, Ph). Anal. ($C_{16}H_{26}N_2O_5S$) C, H, N.

(3*SR*)-*N*-*tert*-Butyl-3-(Cbz-amino)-4-(acetylsulfanyl)butanesulfonamide (23). The thioacetylation of (3*SR*)-*N*-*tert*-butyl-3-(Cbz-amino)-4-hydroxybutanesulfonamide was then performed by a Mitsunobu reaction[25] to afford (3*SR*)-*N*-*tert*-butyl-3-(Cbz-amino)-4-(acetylsulfanyl)butanesulfonamide as a white solid (60% yield): R_f = 0.24 in Et$_2$O/cyclohexane (75/25); ^{1}H NMR (DMSO-d_6) δ 1.17 (9H, s, tBu), 1.70–1.90 (2H, m, *CH*$_2$CH$_2$SO$_2$NH), 2.28 (3H, s, SCO*CH*$_3$), 2.82 (1H, ABX J = 13.6 Hz, CH*CH*$_2$SCOCH$_3$), 2.89 (2H, t, *CH*$_2$SO$_2$NH), 3.03 (1H, ABX J = 13.6 Hz, CH*CH*$_2$SCOCH$_3$), 3.60 (1H, m, *CH*CH$_2$-SCOCH$_3$), 4.98 (2H, s, CH$_2$Ph), 6.80 (1H, s, SO$_2$*NH*), 7.29 (5H, m, Ph), 7.37 (1H, d, BzlOCO*NH*). Anal. ($C_{18}H_{28}N_2O_5S_2$) C, H, N.

(3*SR*)-3-Amino-4-sulfanylbutanesulfonamide (24). The deprotection of the sulfonyl group was performed as described for compounds **1**–**5**. Then, the cleavage of the trityl group[41,42] was followed by HF treatment.[47] (3*SR*)-3-Amino-4-sulfanylbutanesulfonamide **(24)** was thus obtained as a yellow oil (98% yield): ^{1}H NMR (DMSO-d_6) δ 2.00 (2H, m, *CH*$_2$CH$_2$SO$_2$NH$_2$), 2.73 (2H, m, *CH*$_2$SH), 3.07 (2H, m, *CH*$_2$SO$_2$NH$_2$), 3.30 (1H, m, *CH*CH$_2$SH), 6.85 (3H, brs, NH$_3^+$); SM (ES) (M + H)$^+$ m/z = 185.4. Anal. ($C_4H_{12}N_2O_2S_2$).

DL-Diethyl (Boc-amino)-(3-carboxyphenyl)glycinate (25). DL-(3-Carboxyphenyl)glycine, prepared as previously described,[27] was refluxed in EtOH with 5 equiv of SOCl$_2$ (100%). The resulting compound was N-protected by a *tert*-butyloxycarbonyl group with a procedure previously described[48] to obtain DL-diethyl (Boc-amino)(3-carboxyphenyl)-glycinate **(25)** using Boc$_2$O, NEt$_3$ in DMF (100% yield): R_f = 0.61 in EtOAc/heptane (1/1); ^{1}H NMR (DMSO-d_6) δ 1.07 (3H, t, CHCOOCH$_2$*CH*$_3$), 1.26 (3H, t, C$_6$H$_4$COOCH$_2$*CH*$_3$), 1.33 (9H, s, tBu), 4.04 (2H, q, CHCOO*CH*$_2$CH$_3$), 4.27 (2H, q, C$_6$H$_4$-COO*CH*$_2$CH$_3$), 5.22 (1H, d, *CH*COOCH$_2$CH$_3$), 7.46 (1H, t, Ar), 7.60 (1H, d, Ar), 7.85 (2H, d, Ar and Boc*NH*), 7.93 (1H, s, Ar). Anal. ($C_{18}H_{25}NO_6$) C, H, N.

3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]benzoic Acid (26). The α-amino ester **25** (1 equiv) was reduced in dry EtOH/THF (8 mL/mmol) at 0 °C by NaBH$_4$ (4 equiv) and LiCl (4 equiv) as previously described.[49] This compound (1 equiv) was dissolved in MeOH (2 mL/mmol), and 1 N NaOH (3 equiv) was added at 0 °C. After classical treatment, 3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]benzoic acid **(26)** (83% yield) was obtained: R_f = 0.58 in CH$_2$Cl$_2$/MeOH/AcOH (9/1/0.5); ^{1}H NMR (DMSO-d_6) δ 1.30 (9H, s, tBu), 3.45 (2H, m, *CH*$_2$OH), 4.51 (1H, q, *CH*CH$_2$OH), 4.27 (1H, br s, CH$_2$*OH*), 7.30 (1H, d, Boc*NH*), 7.37 (1H, t, Ar), 7.47 (1H, d, Ar), 7.75 (1H, d, Ar), 7.83 (1H, s, Ar). Anal. ($C_{14}H_{19}NO_5$) C, H, N.

Preparation of 3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]benzamide (27a) and 3-[(2*SR*)-2-(Boc-amino)-1-hydroxyethyl]-*N*,*N*-dimethylbenzamide (27b). To a cold (−15 °C) solution of **26** (1 equiv) in DMF were successively added *N*-methylmorpholine (1.1 equiv) and ^{i}BuOCOCl (1.1 equiv). After 15 min, the precipitated *N*-methylmorpholine hydrochloride was removed by filtration. For preparation of **27a**, a solution of NH$_3$ (2 M) in DME (60 equiv) was added at −15 °C. For preparation of **27b**, a solution of dimethylamine (3 equiv) and triethylamine (3 equiv) in DMF was added at −15 °C. In both cases the mixture was stirred at room temperature for 4 h. After evaporation in vacuo, the residue was washed with H$_2$O and brine, dried over Na$_2$SO$_4$, and evaporated in vacuo. The product **27a** was purified by flash chromotography on a silica gel column, using CH$_2$Cl$_2$/MeOH/AcOH (9/1/0.5) as eluent (46% yield): R_f = 0.37 in CH$_2$Cl$_2$/MeOH/AcOH (9/1/0.5); ^{1}H NMR (DMSO-d_6) δ 1.31 (9H, s, tBu), 3.45 (2H, t, *CH*$_2$OH), 4.50 (1H, q, *CH*CH$_2$OH), 4.77 (1H, t, CH$_2$*OH*), 7.20 (1H, d, BOCNH), 7.28 (1H, s, CO*NH*$_2$), 7.32 (1H, d, Ar), 7.36 (1H, t, Ar), 7.67 (1H, d, Ar), 7.76 (1H, s, Ar), 7.87 (1H, s, CO*NH*$_2$). Anal. ($C_{14}H_{20}N_2O_4$) C, H, N.

The product **27b** was purified by flash chromatography on a silica gel column using CH$_2$Cl$_2$/MeOH/AcOH (9/1/0.5) as eluent (68% yield): R_f = 0.60 in CH$_2$Cl$_2$/EtOAc/AcOH (9/1/0.5); ^{1}H NMR (DMSO-d_6) δ 1.32 (9H, s, tBu), 2.85 (3H, s, CON-(CH$_3$)*CH*$_3$), 2.93 (3H, s, CON(*CH*$_3$)CH$_3$), 3.45 (2H, d, *CH*$_2$OH), 4.50 (1H, m, *CH*CH$_2$OH), 7.17–7.34 (5H, m, Ar and Boc*NH*). Anal. ($C_{16}H_{24}N_2O_4$) C, H, N.

Preparation of 3-[(2*SR*)-1-(Acetylsulfanyl)-2-(Boc-amino)ethyl]benzamide (28a) and 3-[(2*SR*)-1-(Acetylsulfanyl)-2-(Boc-amino)ethyl]-*N*,*N*-dimethylbenzamide (28b). The free hydroxy compound **27a** (respectively **27b**) (1 equiv) was dissolved in DMF (0.2 mmol/mL); then TEA (2.7 molar equiv) and methanesulfonyl chloride were added at −10 °C.[50] The reaction mixture was stirred at room temperature for 3 h. The DMF was evaporated, and the residue was taken up in EtOAc and treated by classical workup. To a solution of the mesylate (1 equiv) in DMF (5 mL/mmol) was added at 0 °C 3 equiv of potassium thioacetate. After stirring overnight at room temperature and evaporation to dryness, the residue was taken up in EtOAc, washed with water and brine, dried, and evaporated in vacuo.

Compound 28a: R_f = 0.21 in *n*-heptane/EtOAc/AcOH (5/5/0.5) (71% yield); ^{1}H NMR (DMSO-d_6) δ 1.29 (9H, s, tBu), 2.27 (3H, s, SCO*CH*$_3$), 2.97 (1H, dd, *CH*$_2$SCOCH$_3$), 3.15 (1H, dd, *CH*$_2$SCOCH$_3$), 4.54 (1H, q, *CH*CH$_2$SCOCH$_3$), 7.33 (1H, s, CO*NH*$_2$), 7.36 (1H, t, Ar), 7.42 (1H, d, Ar), 7.53 (1H, d, Boc*NH*), 7.70 (1H, d, Ar), 7.80 (1H, s, Ar), 7.93 (1H, s, CO*NH*$_2$). Anal. ($C_{16}H_{22}N_2O_4S$) C, H, N.

Compound 28b: R_f = 0.21 in *n*-heptane/EtOAc/AcOH (5/5/0.5) (38% yield); ^{1}H NMR (DMSO-d_6) δ 1.30 (9H, s, tBu), 2.28 (3H, s, SCO*CH*$_3$), 2.83 (3H, s, CON(CH$_3$)*CH*$_3$), 2.90 (3H, s, CON(*CH*$_3$)CH$_3$), 3.00 (1H, dd, *CH*$_2$SCOCH$_3$), 3.15 (1H, dd, *CH*$_2$-SCOCH$_3$), 4.54 (1H, m, *CH*CH$_2$SCOCH$_3$), 7.19–7.38 (4H, m, Ar), 7.53 (1H, d, BocNH). Anal. ($C_{18}H_{26}N_2O_4S$) C, H, N.

Preparation of 3,3′-[(Disulfanediyl)bis((2*SR*)-2-aminoethylene)]bis(benzamide) (29a) and 3,3′-[(Disulfanediyl)-bis((2*SR*)-2-aminoethylene)]bis(*N*,*N*-dimethylbenzamide) (29b). Deprotection of the acetyl and Boc groups of compounds **28a**,**b** was obtained as described for compound **12**.

Compound 29a: R_f = 0.14 in CH$_2$Cl$_2$/MeOH/AcOH (5/5/0.5) (82% yield); ^{1}H NMR (DMSO-d_6) δ 3.30 (2H, m, *CH*$_2$S), 4.50

(1H, m, *CH*CH_2S), 7.43 (1H, s, CO*NH_2*), 7.46 (1H, t, Ar), 7.56 (1H, d, Ar), 7.85 (1H, d, Ar), 7.99 (2H, s, CO*NH_2* and Ar), 8.58 (3H, br s, *NH_3^+*); SM (ES) (M + H)$^+$ m/z = 391.6. Anal. ($C_{18}H_{22}N_4O_2S_2$).

Compound 29b: R_f = 0.33 in 2-propanol/NH_4OH (9/0.5) (52% yield); ^{1}H NMR (DMSO-d_6) δ 2.82 (3H, s, CON(CH_3)-*CH_3*), 2.92 (3H, s, CON(*CH_3*)CH_3), 3.23 (2H, m, *CH_2*S), 4.49 (1H, m, *CH*CH_2S), 7.33−7.50 (4H, m, Ar), 8.50 (3H, br s, NH_3^+); SM (ES) (M + H)$^+$ m/z = 447.5. Anal. ($C_{22}H_{30}N_4O_2S_2$).

Preparation of 3-(*N*-Benzylsulfamoyl)benzoic Acid (30). To a cold (0 °C) solution of 3-(chlorosulfonyl)benzoic acid (Aldrich) in CH_2Cl_2 was added benzylamine (3.5 equiv). The mixture was stirred for 30 min. After evaporation in vacuo and acidification with $KHSO_4$ (1 N), the aqueous residue was extracted three times with EtOAc (95% yield): R_f = 0.38 in toluene/AcOH (17/3); ^{1}H NMR (DMSO-d_6) δ 3.97 (2H, d, *CH_2* Ph), 7.13−7.24 (5H, m, Ph), 7.63 (1H, t, Ar), 7.94 (1H, d, Ar), 8.08 (1H, d, Ar), 8.26 (1H, s, Ar), 8.29 (1H, t, SO_2*NH*), 13.4 (1H, br s, COOH). Anal. ($C_{14}H_{13}NO_4S$) C, H, N.

***N*-Benzyl-3-formylbenzenesulfonamide (31).** The carboxylic acid function of 3-(*N*-benzylsulfamoyl)benzoic acid **(30)** was transformed into alcohol to obtain *N*-benzyl-3-(hydroxymethyl)benzenesulfonamide by formation of the mixed anhydride with isobutyl chloroformate in DME at −15 °C followed by a reduction with sodium borohydride as previously described[24] (94% yield). Swern oxidation was performed with a cold (−78 °C) solution of oxalyl chloride (1.2 equiv) and dimethyl sulfoxide (3 equiv) diluted with dichloromethane.[51] After 5 min at −78 °C was added a solution of alcohol (1 equiv) in CH_2Cl_2 with dimethyl sulfoxide (3 equiv); stirring was continued for an additional 15 min. Triethylamine (5 equiv) was added, and the mixture was stirred for 5 min and then allowed to reach room temperature. After classical workup, *N*-benzyl-3-formylbenzenesulfonamide (96% yield) was obtained **(31)**: R_f = 0.43 in toluene/AcOH (17/3); ^{1}H NMR (DMSO-d_6) δ 4.00 (2H, d, CH_2 Ph), 7.18 (5H, br s, Ph), 7.74 (1H, t, Ar), 8.02 (1H, d, Ar), 8.08 (1H, d, Ar), 8.20 (1H, s, Ar), 8.35 (1H, t, SO_2*NH*), 10.03 (1H, s, C*H*O). Anal. ($C_{14}H_{13}NO_3S$) C, H, N.

***N*-Benzyl-3-[(*SR*)-[(diphenylmethyl)amino]-cyanomethyl]benzenesulfonamide (32).** To a solution of aldehyde **31** (1 equiv) and KCN (1.01 equiv) in freshly distillated MeOH were added aminodiphenylmethane (1.15 equiv) and acetic acid (2.3 equiv). The mixture was stirred at reflux overnight. After evaporation in vacuo, the residue was purified by flash chromatography on a silica gel column, using EtOAc/cyclohexane (22/78) as eluent (63% yield): R_f = 0.33 in EtOAc/cyclohexane (1/2); ^{1}H NMR (DMSO-d_6) δ 3.97 (2H, d, *CH_2*Ph), 4.28 (1H, ABX J = 12.3 Hz, Ph(Ph)CH*NH*), 4.70 (1H, d J = 12 Hz, *CH*CN), 5.03 (1H, d J = 3 Hz, Ph(Ph)*CH*NH), 7.12−7.50 (15, m, 3 × Ph), 7.58 (1H, t, Ar), 7.72−7.74 (2H, m, Ar), 7.93 (1H, s, Ar), 8.24 (1H, t, *NH*Bzl). Anal. ($C_{28}H_{25}N_3O_2S$) C, H, N.

***N*-Benzyl-3-[(*SR*)-[(diphenylmethyl)amino]-(methoxycarbonyl)methyl]benzenesulfonamide (33).** The nitrile group of **32** was transformed into a carboxamide group by HCl-(g) in anhydrous MeOH (75%) in order to obtain *N*-benzyl-3-[(2*SR*)-2-[(diphenylmethyl)amino]ethanamido]benzenesulfonamide. The carboxamide was combined with a 15-fold excess (by weight) of Amberlite IR-120 acidic resin in methanol.[52] The mixture was gently stirred and warmed (60 °C) for 2 days. The product was recovered by collecting the resin in a column and eluting slowly with a mixture of MeOH/NEt_3 (2/1) as eluent (67% yield): R_f = 0.52 in CH_2Cl_2/MeOH/AcOH (9/0.2/0.2); ^{1}H NMR (DMSO-d_6) δ 3.42 (1H, ABX J = 10, 5 Hz, Ph(Ph)CH*NH*), 3.58 (3H, s, $COOCH_3$), 3.94 (2H, d, *CH_2*Ph), 4.23 (1H, d J = 10 Hz, *CH*CN), 4.70 (1H, d J = 5 Hz, Ph(Ph)*CH*NH), 7.13−7.37 (15, m, 3 × Ph), 7.49 (1H, t, Ar), 7.52 (1H, d, Ar), 7.68 (1H, d, Ar), 7.76 (1H, s, Ar), 8.16 (1H, t, *NH*Bzl). Anal. ($C_{29}H_{28}N_2O_4S$) C, H, N; C: calcd, 69.58; found, 70.01.

***N*-Benzyl-3-[(2*SR*)-2-[(diphenylmethyl)amino]-1-hydroxyethyl]benzenesulfonamide (34).** Reduction of ester of **33** was performed using the procedure described for compound **26**.[49] The product was purified by flash chromatography on a silica gel column, using EtOAc/cyclohexane (15/85) as eluent (96% yield): R_f = 0.31 in EtOAc/cyclohexane (3/7); ^{1}H NMR (DMSO-d_6 + TFA) δ 3.82 (2H, t, *CH_2*OH), 3.96 (1H, t, *CH*CH_2-OH), 4.05 (1H, t, CH_2*OH*), 3.98 (2H, d, *CH_2*Ph), 5.35 (1H, s, Ph(Ph)*CH*N), 7.03−7.62 (15H, m, 3 × Ph) and (2H, m, Ar), 7.80 (1H, d, Ar), 7.87 (1H, s, Ar), 8.17 (1H, t, *NH*Bzl). Anal. ($C_{28}H_{28}N_2O_3S$) C, H, N.

Preparation of *N*-Benzyl-3-[(2*SR*)-1-(acetylsulfanyl)-2-[(diphenylmethyl)amino]ethyl]benzenesulfonamide (35). This compound was prepared from **34** according to the procedure described for **23**. The product was purified on a silica gel column using Et_2O/cyclohexane (1/9) as eluent (43% yield): R_f = 0.36 in EtOAc/*n*-heptane (3/7); ^{1}H NMR (DMSO-d_6 + TFA) δ 2.10 (3H, s, SCO*CH_3*), 3.39 (1H, t, *CH*CH_2-$SCOCH_3$), 3.80 (1H, ABX J = 12.4 Hz, CH*CH_2*$SCOCH_3$), 4.16 (1H, ABX J = 12.4 Hz, CH*CH_2*$SCOCH_3$), 4.00 (2H, s, *CH_2*Ph), 5.31 (1H, s, Ph(Ph)*CH*N), 7.05−7.54 (20H, m, 3 × Ph) and (2H, m, Ar), 7.82 (1H, d, Ar), 7.92 (1H, s, Ar), 8.16 (1H, t, *NH*Bzl). Anal. ($C_{30}H_{30}N_2O_3S_2$) C, H, N.

3-[(2*SR*)-2-Amino-1-sulfanylethyl]benzenesulfonamide (36). This compound was prepared from **35** according to the procedure described for **24**. The single modification was that HF cleavage was run at room temperature: retention time = 18.4 min (C_{18} Nucleosil column, 0→80% B in 30 min, flow 1.2 mL/min); ^{1}H NMR (DMSO-d_6) δ 2.54 (1H, t, CH_2*SH*), 2.90−3.04 (2H, m, *CH_2*SH), 4.42 (1H, m, *CH*CH_2SH), 7.42 (2H, s, SO_2*NH_2*), 7.62 (2H, s, Ar), 7.80 (1H, s, Ar), 7.90 (1H, s, Ar), 8.50 (3H, br s, *NH_3^+*); SM (ES) (M + H)$^+$ m/z = 233.0. Anal. ($C_8H_{12}N_2O_2S_2$).

Compounds **37**,[26] **38**,[27] **39**,[28] **40**,[26] **41**,[27] **42**,[26] **43**,[26] **44**,[26] **45**,[30] **46**,[26] **47**,[26] **48**,[31] **49**,[27] **50**,[27] **51**,[27] **52**,[27] **53**,[27] **54**,[53] and **55**[53] were prepared as previously described.

Inhibitory Potency. Enzymatic studies were performed using the fluorescent synaptobrevin derivative [Pya^{88}]S 39-88 as a substrate according to the protocol described by Solheihac et al.,[34] with slight modifications; 250 ng of TeNt-L chain (Pasteur-Meyrieux, France), purified according to the procedure of Solheihac et al.,[34] was preincubated for 30 min at 37 °C with increasing concentrations of inhibitor in 90 μL of 20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol. A 10-μL solution of 100 μM [Pya^{88}]S 39-88 in buffer was then added (10 μM final concentration), and the mixture was kept for 60 min at 37 °C in the dark. The reaction was stopped by adding 50 μL of 0.2 M HCl.

The fluorescent cleavage product was separated from the fluorescent substrate by reverse-phase HPLC, on a Nucleosil C_8 column (300 Å, 7 μm, 70 × 4 mm) with a LC-10AS Shimadzu apparatus and quantified by a RF-35 Shimadzu fluorimeter detector (on line) (excitation 343 nm, emission 377 nm).

The elution buffers used were A (H_2O, TFA 0.05% (v/v)) and B (CH_3CN/H_2O (9:l, v/v), TFA 0.038% (v/v)). The metabolites were eluted with a gradient of 28−50% in B for 2.5 min at a flow rate of 1 mL/min, followed by a gradient of 50−100% in B for 0.5 min, and isocratic at 100% of B for 2 min. In these conditions, the retention times of the fluorescent cleavage product and the fluorescent substrate peaks were respectively 3.4 and 5.7 min.

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