Metallopeptidase Inhibitors of Tetanus Toxin: A Combinatorial Approach

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The bacterial protein tetanus toxin (TeNt), which belongs to the family of zinc endopeptidases, cleaves synaptobrevin, an essential synaptic protein component of the neurotransmitter exocytosis apparatus, at a single peptide bond ($Gln^{76}-Phe^{77}$). This protease activity is a particularly attractive target for designing potent and selective synthetic inhibitors as a possible drug therapy for tetanus. β-Aminothiols mimicking Gln^{76} of synaptobrevin have been previously shown to inhibit the tetanus neurotoxin enzymatic activity in the 35−250 μM range. These compounds have now been modified to interact with S′ subsites of the TeNt active site, with the aim of increasing their inhibitory potencies. Combinatorial libraries of pseudotripeptides, containing an ethylene sulfonamide or an *m*-sulfonamidophenyl moiety as the P_1 side chain and natural amino acids in P_1' and P_2' positions, were synthesized. The best inhibitory activity was observed with Tyr and His as P_1' and P_2' components, respectively. This led to new inhibitors of TeNt with K_i values in the 3−4 μM range. These molecules are the most potent inhibitors of TeNt described so far.

Introduction

Tetanus neurotoxin (TeNt) is a 150-kDa protein produced by the anaerobic bacillus *Clostridium tetani*, which causes the lethal spastic paralysis of tetanus by blockade of inhibitory neurotransmitter release at central synapses.[1,2] It is a member of the clostridial neurotoxin family, which includes seven botulinum neurotoxins responsible for the flaccid paralysis of botulism.[1,2] TeNt is composed of two polypeptide chains joined by a single interchain disulfide bond.[1] The heavy chain (100 kDa) participates in binding to the nerve terminals, internalization, retrograde axonal transport, and membrane translocation of the toxic light chain (50 kDa) into the neuronal cytosol.[1,3] The light chain, after reduction of the disulfide bridge, is responsible for the inhibition of neurotransmitter release.[1]

TeNt belongs to the thermolysin family of Zn^{2+} metallopeptidases that contains the HExxH consensus sequence,[4−6] in which the two histidines are involved in zinc chelation whereas the glutamate is involved in catalysis.[7] This has been shown by site-directed mutagenesis experiments[8,9] and recently confirmed by the crystal structure determination of botulinum neurotoxin type A (BoNt/A).[10] TeNt has been shown to cleave a single 116-residue protein, VAMP/synaptobrevin, at the $Gln^{76}-Phe^{77}$ peptide bond[11] (Figure 1). This protein is an essential component of the neuro-exocytosis apparatus,[12−14] and its cleavage by TeNt leads to the blockade of inhibitory neurotransmitter release.[11] Such a narrow specificity, not common for other zinc peptidases,[6] has been recently explained by a cooperative mechanism, involving the binding to TeNt of synaptobrevin domains distal from the cleavage site for subsequent activation of proteolysis.[15−17] This probably explains why potent inhibitors of well-known zinc metallopeptidases such as captopril, thiorphan, or phosphoramidon have a weak inhibitory activity on TeNt, in the 10 mM range in vitro[18,19] or ex vivo.[20,21] As no efficient drug therapy is yet available for tetanus or botulism, we identified the zinc-dependent metallopeptidase activity of tetanus neurotoxin as a good target.

The best-described inhibitory potencies for TeNt were obtained recently[22] with glutaminethiol **1** ($K_i = 250 \pm 35$ μM) and its sulfonamide analogues **2** ($K_i = 100 \pm 5$ μM)) and **3** ($K_i = 35 \pm 5$ μM), expected to interact with the S_1 subsite of TeNt (Figure 2A). To improve the potency of these compounds, we report here the synthesis of analogues able to interact with additional subsites of the TeNt catalytic site. Given the critical importance of the primary amino group in compounds **1**−**3** for TeNt recognition,[22] these molecules were extended in order to interact with S′ subsites (Figure 2B). To have access to such compounds, the synthesis of the new fully protected β-amino-α-sulfanyl acids (Figure 2C) corresponding to synthons A, B, and C was achieved. These synthons were used in the solid-phase peptide synthesis of pseudotripeptides. The screening of a combinatorial library of such analogues toward TeNt allowed the preference of the S_1' and S_2' subsites to be determined.

Chemistry

Synthesis of Synthons A and B. The commercially available Boc-L-Gln(Trt)-OH (**4a**) and the (2*S*)-4-(*tert*-butylsulfamoyl)-2-(Cbz-amino)butanoic acid (**4b**) prepared from the Z-L-homocystine as previously described[22] were first homologated by the Arndt-Eistert reaction as described in Scheme 1.[23] Their free acid functions, activated as mixed anhydride derivatives,

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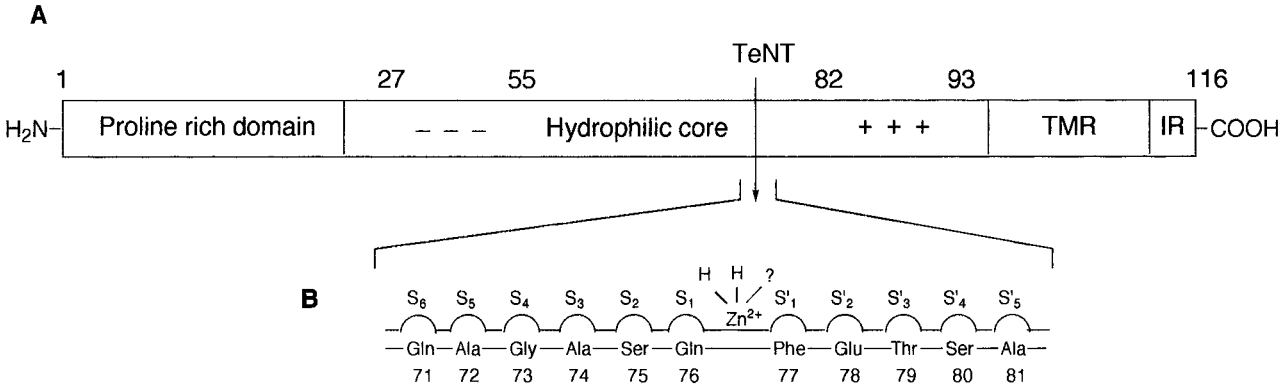


Figure 1. (A) Schematic representation of VAMP/synaptobrevin II. TMR indicates the transmembrane region and IR the intravesicular region. The cleavage site Gln^{76}—Phe^{77} is indicated by the arrow. Domains 27−55 and 82−93 represent respectively the acidic and basic clusters whose binding to tetanus neurotoxin is required to switch on its proteolytic activity.[16] (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 to S_6 on the left of the cleavage bond and S_1' to S_5' on the right.

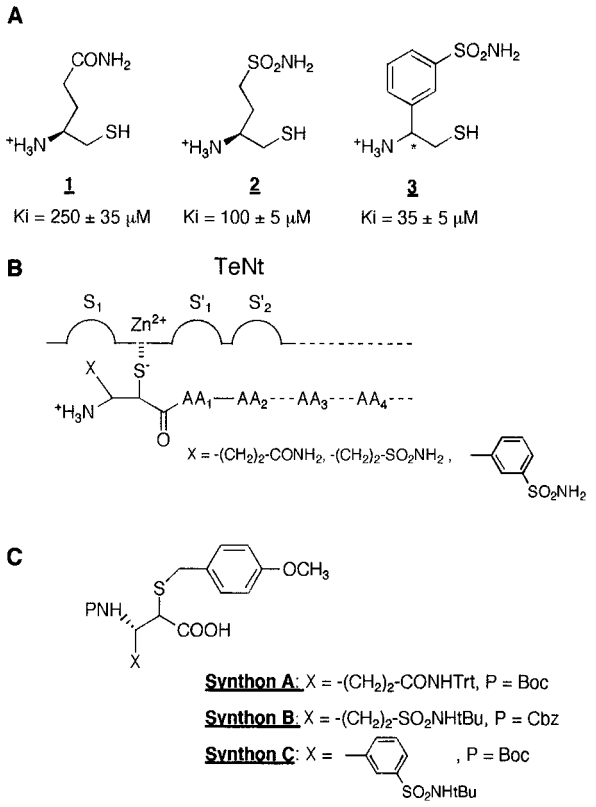
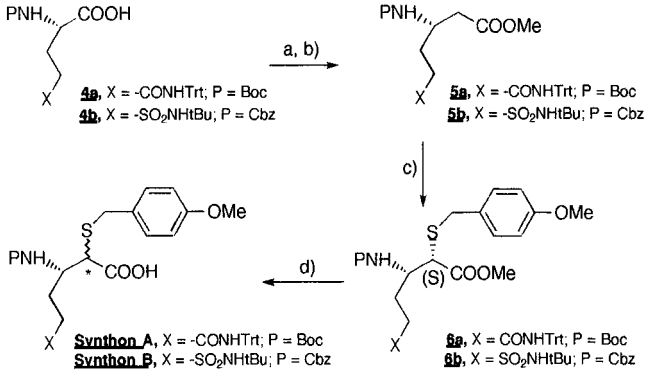


Figure 2. (A) β-Aminothiols bearing carboxamide or sulfonamide functionalities on aliphatic or aromatic side chains endowed with inhibitory activities against tetanus neurotoxin. (B) Extension of these β-aminothiols toward S′ subsites of TeNt. (C) Protected synthons required for the synthesis of such types of molecules.

were converted into the corresponding diazo ketones by action of diazomethane. These diazo ketones underwent the Wolff rearrangement in methanol with silver benzoate as a catalyst to afford the β-amino esters **5a** and **5b** (84% and 95% yields, respectively). The carbanions of these β-amino esters, formed by action of lithium diisopropylamide, were sulfenylated by the asymmetric 4-methoxybenzyl 2,4-dinitrophenyl disulfide according to the recent improvements reported in the synthesis of α-sulfenylated β-amino acids[24] (43% and 54% yields, respectively). The ester functions of resulting **6a** and

Scheme 1. Synthesis of Synthon A and Synthon B[a]

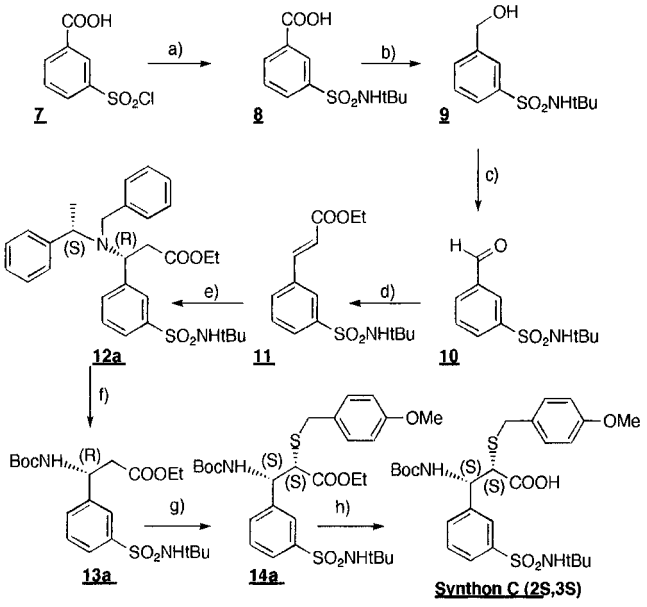


[a] (a) iBuOCOCl, NMM, CH_2N_2; (b) BzOAg, NEt_3, MeOH; (c) LDA, 4-methoxybenzyl 2,4-dinitrophenyl disulfide; (d) MeOH, NaOH.

6b were hydrolyzed by 1 M NaOH to yield the final synthons A and B, which were obtained as racemates on the C2 carbon bearing the thiol group.

Synthesis of Synthon C. Scheme 2 shows the pathway used in the synthesis of the *meta*-substituted sulfonamide analogues of β-aminophenylalanine (synthon C) obtained as a mixture of two diastereomers (2*SR*,3*S*) or as a pure diastereomer (2*S*,3*S*). The commercially available 3-(chlorosulfonyl)benzoic acid (**7**) was transformed into the *tert*-butylsulfonamide derivative **8** by *tert*-butylamine treatment (78% yield) and then transformed into the corresponding substituted benzaldehyde via a reduction of the free carboxylic group into the corresponding alcohol **9**[25] (90% yield), subsequently oxidized by the Swern method to afford the aldehyde **10**[26] (96% yield). Compound **10** was then converted by a Horner−Wadsworth−Emmons olefination[27] into an acrylate intermediate **11** (85% yield). A Michaël addition performed on compound **11** with the homochiral lithium (*S*)-benzyl(α-methylbenzyl)amide yielded the β-aminophenylalanine derivative **(α*S*,3*R*)-12a**[28] with an enantiomeric excess > 99% (87% yield). Hydrogenolysis under 5 atm in ethyl acetate in the presence of Boc_2O[28] afforded the *N*-Boc-protected ethyl ester β-aminophenylalanine analogue **(3*R*)-13a** (85% yield). The formation of the α-carbanion of **13a** with lithium diisopropylamide (LDA) followed by treatment with 4-methoxybenzyl 2,4-dinitrophenyl disulfide yielded

Scheme 2. Synthesis of Synthon C[a]

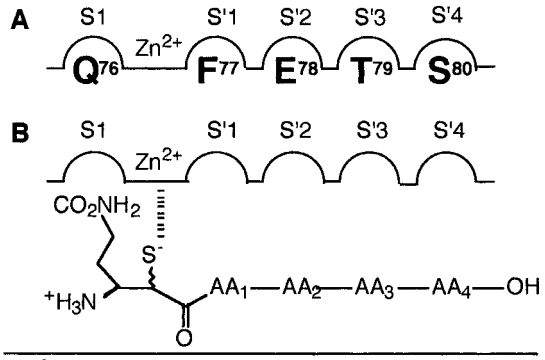


[a] (a) $tBuNH_2$; (b) $LiAlH_4$; (c) DMSO, $(COCl)_2$, NEt_3; (d) triethyl phosphonoacetate, NaH, DME, − 50 °C; (e) lithium (*S*)-(α-methylbenzyl)benzylamide, THF, −78 °C; (f) $Pd(OH)_2/C$ (1 equiv), H_2 (10 atm), Boc_2O (3.7 equiv), EtOAc, rt, 24 h; (g) LDA, 4-methoxybenzyl 2,4-dinitrophenyl disulfide; (h) BBTO, CH_3CN, 82 °C, 48 h.

the diastereomer **(2*S*,3*S*)-14a** (2*S*,3*S*/2*R*,3*S* = 85/15). The hydrolysis of the ethyl ester by bis(tributyltin)oxide (BBTO) gave the synthon C (2*S*,3*S*) without further racemization (70% yield), while its hydrolysis by 1 M NaOH in MeOH overnight at room temperature gave a 50/50 mixture of the two diastereomers (2*R*,3*S* and 2*S*,3*S*). To obtain the two other diastereomers (2*R*,3*R* and 2*S*,3*R*), the homochiral lithium (*R*)-benzyl(α-methylbenzyl)amide was used in the same synthetic pathway instead of the *S* isomer. In addition, the 2,4-dimethoxybenzylthio 4-methylphenylsulfonate was used as sulfenylating agent to obtain **14c** and **14d** with a thiol protected by a dimethoxybenzyl group.[30] The HPLC separation of both diastereomers after TFA deprotection and before HF cleavage becomes easier in this case than with the monomethoxybenzyl protecting group.

Syntheses of Substrate Mimics Derived from Compound 1. The syntheses of the thiol peptide derivatives spanning the various subsites defined as S_1, S_1', S_2', S_3', and S_4' of the tetanus toxin catalytic domain were performed by solid-phase peptide synthesis using synthon A, the protected amino acids corresponding to Phe, Glu, Thr, Ser, and the Fmoc/tBu strategy on Wang/HMP resin (Figure 3).[31] Cleavage from the solid support and deprotection of side-chain-protecting groups were achieved by using trifluoroacetic acid in the presence of scavengers.[32] This was followed by a final HF treatment[33] in order to obtain the various carboxamide thiols **(15−18)** which were then purified by reverse-phase HPLC.

Synthesis of Combinatorial Libraries of Thiol-Containing Inhibitors. A pseudotripeptide library, corresponding to 19 mixtures of thiol-containing peptides of formula (synthon B)-AA'_{1x}-AA'_{2x}, where AA'_{1x} and AA'_{2x} represent the 19 different natural amino acids (Cys omitted), was obtained by solid-phase peptide synthesis on 2-chlorotrityl chloride resin[34] by using a



n°	AA_1	AA_2	AA_3	AA_4	Ki
15	-Phe-				100±10 μM
16	-Phe-	-Glu-			90±10 μM
17	-Phe-	-Glu-	-Thr-		100±10 μM
18	-Phe-	-Glu-	-Thr-	-Ser-	80±10 μM

Figure 3. (A) Synaptobrevin sequence ^{76}QFETS in interaction with S and S′ subsites of tetanus neurotoxin. (B) Inhibition constants of pseudopeptides mimicking the synaptobrevin sequence ^{76}QF (**15**), ^{76}QFE (**16**), ^{76}QFET (**17**), and ^{76}QFETS (**18**), respectively.

previously described protocol[35,36] (Figure 4). The Divide/Couple/Recombine method[37] was used to synthesize these 19 sublibraries. A first amino acid mixture is prepared by combining the 19 different amino acids linked to the 2-chlorotrityl resin (Cys was omitted). This homogenized mixture is then split into 19 identical samples to which a known amino acid is coupled. This gives rise to 19 different mixtures, each of them containing 19 different dipeptides. The next step is the coupling of synthon B in each sample allowing the synthesis of 19 mixtures containing 19 couples of diastereomers. The 19 fractions of peptidyl resin were then cleaved from the resin by using a dichloromethane/trifluoroethanol mixture[38] (8/2). The deprotection of the TFA-labile protecting groups was achieved by using trifluoroacetic acid,[32] and the final deprotection step used to hydrolyze the benzylcarbamate group and the 4-methoxybenzyl group was performed with anhydrous hydrogen fluoride.[33] The 19 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 stoichiometry in the S_2' position, respectively (data not shown).

A second library corresponding to the parallel synthesis of 19 mixtures of two diastereomers of pseudotripeptides of formula (synthon B)-Tyr-AA'_{2x} was achieved on 2-chlorotrityl chloride resin with the same protocol described above.

Syntheses of Inhibitors Derived from the Libraries. The two diastereomers **19a** and **19b** (Figure 5A), issued from the best mixtures of the library (tyrosyl in S_1' and histidyl in S_2'), were purified and separated by RP-HPLC. To assign unambiguously the absolute configuration of the C2 carbon bearing the thiol group, the solid-phase synthesis of the diastereomer **19b** alone was performed by successive coupling of Fmoc-protected histidine, tyrosine, and the optically pure synthon B (2*S*,3*S*) obtained by hydrolysis of the methyl ester of **6b** by BBTO. Cleavage from the resin and side-chain deprotection were achieved with the same protocol described for the libraries.

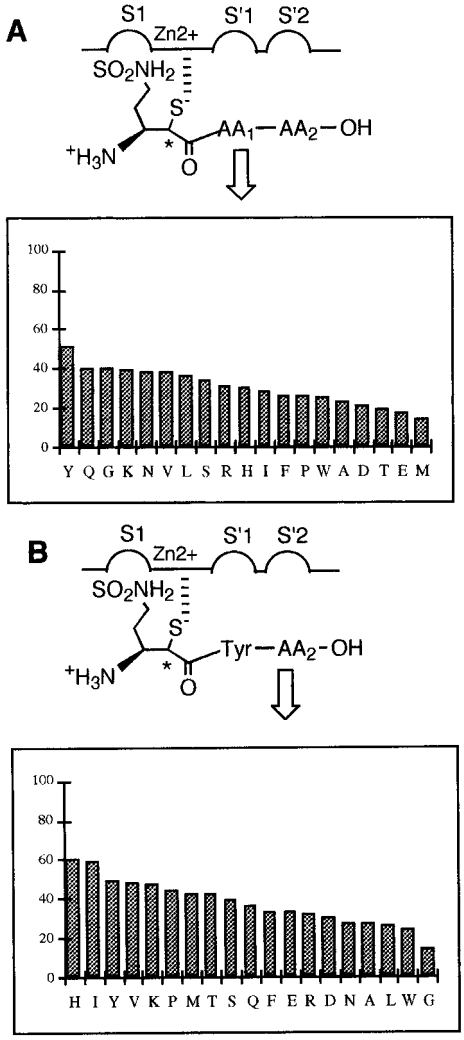
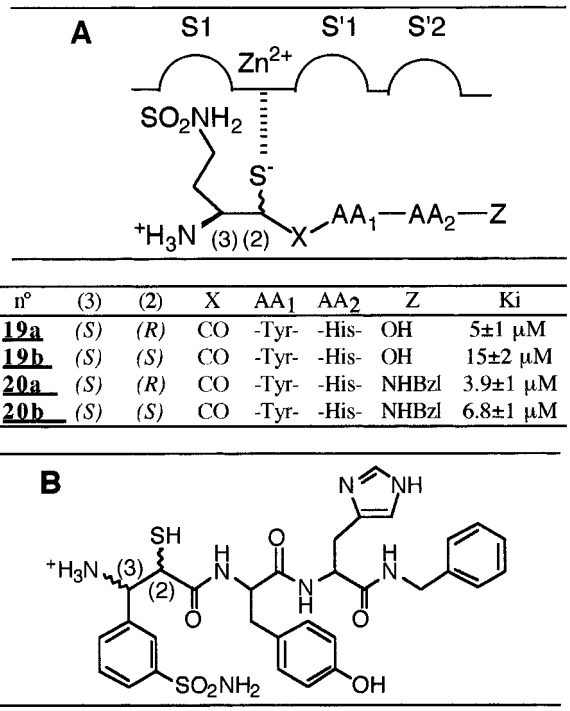


Figure 4. Histograms representing the percentage of inhibition obtained with 38 mixtures of pseudotripeptides derived from the β-aminothiol **2** (Figure 1) on tetanus neurotoxin zinc endoprotease activity. The general formula of these mixtures of compounds is given above the histogram. (A) Each mixture differs by a natural amino acid (except Cys) putatively interacting in S_1' which is represented as a one-letter code on the abscissa. A stoichiometric amount of each natural amino acid (except Cys) is present in S_2'. These mixtures were tested at 33 μM on TeNt. (B) A same tyrosyl residue is present in S_1'. Each mixture differs by the residue present in S_2' which corresponds to a natural amino acid (except Cys) indicated as a one-letter code on the abscissa. These mixtures were tested at 25 μM on TeNt.



n°	(3)	(2)	X	AA_1	AA_2	Z	Ki
19a	*(S)*	*(R)*	CO	-Tyr-	-His-	OH	5±1 μM
19b	*(S)*	*(S)*	CO	-Tyr-	-His-	OH	15±2 μM
20a	*(S)*	*(R)*	CO	-Tyr-	-His-	NHBzl	3.9±1 μM
20b	*(S)*	*(S)*	CO	-Tyr-	-His-	NHBzl	6.8±1 μM

n°	(3)	(2)	Ki
21a	(S)	(R)	3.0 ±0.9 μM
21b	(S)	(S)	9.6 ±0.9 μM
21c	(R)	(R)	6.0 ±0.9 μM
21d	(R)	(S)	4.0 ±0.9 μM

Figure 5. (A) Inhibition constants values of pseudotripeptides derived from the β-aminothiol **2** (Figure 2). The different absolute configurations of the C2 and C3 carbons of the β-amino acid synthon are indicated. (B) Inhibition constant values of pseudotripeptides derived from the β-aminothiol **3** (Figure 2). The different absolute configurations of the C2 and C3 carbons of the β-amino acid synthon are indicated.

Finally, synthon B (2*RS*,3*S*), and synthons C (2*RS*,3*S* and 2*RS*,3*R*) were coupled independently with the protected dipeptide H-Tyr(tBu)-His(Trt)-NHBzl. After deprotection by trifluoroacetic acid,[32] the resulting compounds were purified at this step by semipreparative HPLC in order to separate the couples of diastereomers and treated by anhydrous hydrogen fluoride[33] to afford compounds **20a**, **20b** (Figure 5A), **21a**, **21b**, **21c**, and **21d** (Figure 5B), respectively. To assign unambiguously the absolute configuration of the C2 carbon bearing the thiol, the syntheses of compounds **20b**, **21b**, and **21c** were achieved independently by using optically pure synthon B (2*S*,3*S*), synthon C (2*S*,3*S*), and synthon C (2*R*,3*R*). The ^{1}H NMR chemical shifts as well as the HPLC retention times of these compounds were compared to those of the corresponding couples of diastereomers in order to assign the absolute configuration of the chiral centers.

Biological Results

The various pseudopeptides **15**−**18** were tested on TeNt metallopeptidase activity in vitro.[39] The results are summarized in Figure 3. As compared to glutamine-thiol **1**, which had a K_i of 250 ± 35 μM (Figure 2A), the four compounds **15**−**18** exhibited increased inhibitory potencies with K_i values in the 80−100 μM range (Figure 3). Nevertheless, no significant difference was observed among them. Therefore, the pseudotripeptide was selected as a scaffold to investigate the S_1' and S_2' subsites of tetanus neurotoxin and to try increase the inhibitory potency. These molecules represent a good compromise in terms of size to generate structured diversity for exploring the S′ subsites.

For this purpose, a combinatorial library of pseudotripeptides derived from the sulfonamide analogue **2** was used. The results are summarized in Figure 4. Among the different mixtures corresponding to known amino acids putatively interacting with S_1' (Figure 4A), a significant preference for a tyrosyl residue (52% inhibition) was observed. Nevertheless, no great differences were observed for the other mixtures, with a 40% inhibition for Val, Asn, Gln, Gly, and Lys and from 15% to 35% inhibition for the others. Surprisingly, a Phe side

chain, mimicking the natural substrate synaptobrevin in the P_1' position, was not among the best inhibitors (30% inhibition). Although no clear preference for the S_1' subsite of TeNt was observed, the apparent best P_1' component, Tyr, was used to develop a second library of these pseudotripeptides possessing different natural amino acids in S_2'. This library, composed of mixtures of two diastereomers, was screened against TeNt metallopeptidase activity (Figure 4B). The best residues issued from this screening were His and Ile (60% and 55% inhibition, respectively). The other analogues demonstrated an inhibitory potency in the 30−50% range (except Gly, 15%). However, no great subsite specificity could be defined, even if a slight preference for aromatic or aliphatic side chains (His, Ile, Tyr, Val) was observed.

The best compound issued from both libraries, corresponding to a tyrosyl residue in S_1' and a histidyl residue in S_2', was purified and fully characterized. The corresponding two diastereomers **19a** and **19b** (Figure 5A) were separated by HPLC and screened independently on TeNt proteolytic activity. The compound **19a** corresponding to the 2*R*,3*S* absolute configuration was the best with a $K_i = 5 \pm 1$ μM as compared to $K_i = 15 \pm 2$ μM for **19b**.

Furthermore, the effect of the C-terminal substitution was explored on these compounds by adding a C-terminal benzamide. A slight improvement in K_i values was observed for compounds **20a** and **20b** ($K_i = 3.9 \pm 1$ and 6.8 ± 1 μM, respectively) (Figure 5A).

Finally, this tyrosyl-histidyl-benzylamide moiety was used to obtain the corresponding compounds derived from the *m*-sulfonamido-β-phenylalaninethiol **3** (Figure 5B). These molecules (**21a**, **21b**, **21c**, and **21d**) correspond to the four possible diastereomers. No significant improvement were obtained for compounds **21a** and **21b** possessing *S* absolute configuration at the level of the C3 carbon as compared to compounds **20a** and **20b** with K_i values equal to 3.0 ± 0.9 and 9.6 ± 0.9 μM, respectively (Figure 5). Interestingly, the compounds **21c** and **21d**, corresponding to the *R* absolute configuration at the level of the C3 carbon, did not demonstrate significant differences in terms of inhibitory potency ($K_{i\mathbf{21c}} = 6.0 \pm 0.9$ μM and $K_{i\mathbf{21d}} = 4.0 \pm 0.9$ μM) as compared to those with the *S* configuration.

Finally, the evaluation of the kinetics of action of one of these inhibitors was undertaken. The cleavage rate of the fluorescent substrate [Pya[88]]S39-88 by TeNt[39] was determined with concentrations of substrate ranging from 40 to 200 μM and with concentrations of compound **21c** ranging from 1 to 100 μM. The Lineweaver−Burk plot in Figure 6 represents the mean of three independent experiments performed in triplicate. This representation is particularly useful to determine a decrease of K_m and an unchanged V_{max} with increasing concentrations of inhibitors, which is the typical feature of a competitive inhibitor.

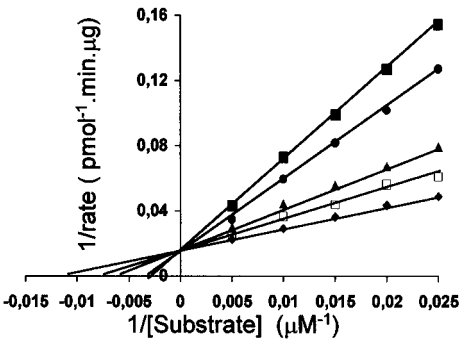


Figure 6. Lineweaver−Burk plot analysis of the inhibitory activity of compound **21c** for TeNt. The experiments were performed at 37 °C in 20 mM Hepes buffer (pH 7.4) containing 100 mM NaCl and 0.1 mM DTT. The enzymatic hydrolysis rates of the fluorescent substrate [Pya[88]]S39-88 at different concentrations (40, 50, 66, 100, 200 μM) were determined by RP-HPLC quantitation of the appearance of the fluorescent cleavage product [Pya[88]]S77-88 in the absence or presence of different concentrations (1, 10, 33, 100 μM) of inhibitor. Each point of the Lineweaver−Burk plot represents the mean of three independent experiments performed in triplicate.

Discussion

The inhibitory potencies recently obtained with β-glutaminethiol **1** ($K_i = 250$ μM) and *m*-sulfonamido-β-phenylalaninethiol **3** ($K_i = 35$ μM)[22] (Figure 2A) have shown that compounds capable of interacting with the S_1 subsite and the Zn metal atom are potent neurotoxin inhibitors. With the aim of improving the inhibitory potencies of these compounds, they were modified to incorporate moieties able to interact with the S′ subsites of TeNt (Figure 2). This approach has already been used successfully in the rational design of inhibitors of other Zn peptidases, in particular aminopeptidases.[23]

The improvement of the inhibitory potencies of the extended compounds **15**−**18** ($K_i = 80-100$ μM), as compared to their parent compound **1** ($K_i = 250$ μM) (Figure 2), confirmed the validity of this strategy. To optimize the interaction of these molecules with the S_1' and S_2' subsites of TeNt, the use of combinatorial chemistry techniques was chosen. Indeed combinatorial chemistry[40−42] has recently emerged as a powerful method, complementary to structure-based ligand design,[43,44] for the discovery and optimization of ligands for a variety of enzymes and receptors. Such methods reduce the need for repetitive syntheses of related compounds as well as laborious and expensive screening. Moreover they have been already successfully used in the design of inhibitors of zinc metallopeptidases such as angiotensin-converting enzyme,[45] matrix metalloproteinases,[46] and zinc endopeptidases 24.15[35] and 24.16.[36]

Thus a library of 19 mixtures of 38 pseudotripeptides, derived from the sulfonamide analogue of β-glutaminethiol and containing natural amino acids, was synthesized and screened against TeNt (Figure 4). The best compound issuing from this library possessed a tyrosyl and a histidyl residue putatively interacting with the S_1' and S_2' subsites of TeNt, respectively. Nevertheless, very few differences, in terms of percentage of inhibition, were observed (Figure 4), indicating an apparent low specificity of the TeNt subsites. This contrasts with the importance of subsite specificity observed for other zinc endopeptidases using the same combinatorial approach.[35,36] The best compound issuing from the libraries (Tyr-His) was purified, and the two diastereomers were separated (**19a** and **19b**). The best K_i value (5 ± 1 μM) was obtained with the compound whose absolute configuration is 2*R*,3*S* on the sulfonamide synthon (Figure 5A). A slight improvement was observed by adding a C-terminal benzylamide to these molecules especially on the 2*S*,3*S* diastereomer resulting in K_i

values that decrease from 15 to 6.8 μM. This tyrosyl-histidyl-benzylamide moiety was introduced in pseudotripeptides derived from the *m*-sulfonamido-β-phenylalaninethiol **3** whose K_i was 35 μM on TeNt[22] (Figure 2). The four corresponding diastereomers, obtained by introducing the different synthons of absolute configuration *S* or *R* on the C2 and C3 carbons, were synthesized and unambiguously assigned. Still no tremendous differences of activity were obtained with these compounds, their K_i values ranging from 3 to 9.6 μM.

Such a lack of specificity in the stereochemistry of residues at the level of S_1 subsite is unexpected for zinc peptidases.[6,47,48] To check if our molecules were interacting with the zinc atom of TeNt via the thiol group, we performed the synthesis of an analogue of compound **21** with a hydrogen replacing the free SH. This was achieved by coupling the corresponding acid synthon of *m*-sulfonamido-β-phenylalaninethiol **13b** to tyrosyl-histidyl-benzylamide (Scheme 2). This compound was devoid of any inhibitory activity even at 1 mM (data not shown). This clearly demonstrated that the thiol group was crucial for the activity of our compounds, probably via zinc chelation. Furthermore, the kinetics of action of compound **21c** was evaluated. The Lineweaver−Burk plot analysis of the experiments indicated the typical pattern observed for inhibitors competing with the substrate at the level of the active site.

The difficulty in improving the affinity of inhibitors for TeNt could be related to the mechanism of TeNt enzymatic activity. Indeed, it has recently been demonstrated that the shortest fragment of synaptobrevin behaving as a substrate contains 50 residues (sequence 39−88).[18,39,49] Thus, short synaptobrevin sequences surrounding the cleavage site ($G^{73}ASQFETSA^{81}$) are neither substrates nor inhibitors of TeNt proteolytic activity.[11,19] This appears to be due to the lack of N- and C-terminal acidic and basic sequences of synaptobrevin distal from the cleavage site.[15,17] The binding of these sequences to TeNt activates the proteolytic activity of TeNt probably by a transconformation,[16] as occurs in allosteric-type enzymatic mechanisms. Our inhibitors, designed to interact with the active site via zinc chelation and catalytic subsite recognition, are competing with the synaptobrevin substrate once it is already bound to TeNt via its N- and C-terminal domains. This is an unfavorable situation which could explain the micromolar inhibitory potencies observed. Nevertheless, the inhibitory potencies of our inhibitors were not modified by addition of peptidic fragments corresponding to these allosteric effectors[16] in the incubation medium (data not shown). This is not surprising because it has been recently shown that a small synaptobrevin sequence encompassing the cleavage site (^{73}GASQFETSA^{81}) is not cleaved by TeNt even in the presence of 1 mM S_1 (27−55) and S_2 (82−93) (unpublished results), while the sequence 56−81 is cleaved.[16] Thus, even residues localized in the 56−72 sequence of synaptobrevin are involved in the enzyme−substrate recognition, and S_1 and S_2 sequences of synaptobrevin are required but not sufficient for the hydrolysis to occur.

In conclusion, this study raises important points in the understanding of tetanus neurotoxin proteolytic activity. Moreover, the combinatorial approach reported here led to the design of new pseudotripeptides, with K_i values against TeNt endopeptidase activity in the 3−4 μM range. Such an inhibitory potency has never been reached until now for any clostridial neurotoxin. Ex vivo and in vivo studies of these compounds are now in progress.

Experimental Section

Inhibitory Potency. Enzymatic studies were performed using synaptobrevin [Pya^{88}]S39-88 as a substrate according to the protocol described by Solheihac et al.[39] with slight modifications.[22]

Chemistry. Solvents, reagents, and protected amino acids for peptide synthesis were obtained from Perkin-Elmer (les Ulis, France). High-performance liquid chromatography (HPLC) grade solvents and solvents were from SDS (Peypin, France). Melting points of the crystallized compounds were measured on an electrothermal apparatus and are reported uncorrected. Flash chromatography was carried out with Merck silica gel 60 (230−400 Mesh). TLC was performed on precoated silica gel plates (60 F_{254}, 0.2 mm thick; Merck). Plates were developed with UV light, iodine vapor, or ninhydrin. 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, flow rate 1 mL/min) with eluents A (H_2O, TFA 0.1% (v/v)) and B (CH_3CN/H_2O (7:3), TFA 0.09% (v/v)), on an Applied Biosystem 151A HPLC apparatus. The eluted peaks were monitored at 214 nm.

The structure of all the compounds was confirmed by ^{1}H NMR spectroscopy (Brüker AC 270 MHz or Brüker AM 400 MHz) in DMSO-d_6 or $CDCl_3$ or D_2O 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 Paris VI, were obtained (C, H, N) for all compounds. Optical rotations were mesured 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}. Mass spectral analyses were performed by Quad Service (Poissy, France) using the electrospray ionization (ESI) technique.

Abbreviations: BOP, (benzotriazolyl-1-yl)oxytris(dimethylamino)phosphonium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Pya, pyrenylalanine; BBTO, bis(tributyltin) oxide; TeNt, tetanus neurotoxin; BoNt/A, botulinum neurotoxin type A.

Solid-Phase Synthesis of the Library. Solid-phase syntheses were performed with an automated 357 Advanced Chemtech multiple peptide synthesizer using a previously described procedure.[35,36] The synthon B was incorporated as a building block during the solid-phase peptide synthesis.

Fully protected peptides were cleaved from 2-chlorotrityl resin with 5 mL of a mixture of TFE and CH_2Cl_2 (2/8) during 4 h.[38] Solutions of protected peptides were concentrated in vacuo. Protective groups were removed by 10 mL of TFA containing 0.5 mL of H_2O and 0.25 mL of triisopropylsilane.[32] Solutions of deprotected peptides were concentrated in vacuo and freeze-dried, and a final cleavage of the thiol protecting group (4-methoxybenzyl) and the benzyl carbamate was achieved by using 5 mL of anhydrous HF in the presence of *m*-cresol (100 μL) during 1 h at 0 °C.[33] After removal of HF in vacuo, the tripeptides were solubilized with 1.5 mL of TFA, precipitated with ether/hexane (1/1) (25 mL), and freeze-dried after addition of water. The correct stoichiometric content of peptide mixture in each expected amino acid was verified by quantitative amino acid analyses.

Construction of the Peptide Library by Combinatorial Chemistry. The protocol employed to synthesize the various mixtures of thiol-containing peptides of formula (synthon B)-AA'_{1x}-AA'_{2x}, where AA'_{1x} and AA'_{2x} represent 19 different natural amino acids (Cys was omitted) was previously described.[35,36]

Synthesis of (2S*R*,3*S*)-3-(Boc-amino)-2-[(4-methoxybenzyl)sulfanyl]-6-oxo(tritylamino)hexanoic Acid (Synthon A). Methyl (3*S*)-3-(Boc-amino)-6-oxo(tritylamino)-

hexanoate (5a). Compound **5a** was prepared by using a previously described procedure.[23] To a solution of compound Boc-Gln(Trt)-OH (**4a**) (4.48 g, 10 mmol) and *N*-methylmorpholine (1.2 mL, 11 mmol) in THF (50 mL) at −15 °C under nitrogen was added, over a 5-min period, isobutyl chloroformate (1.44 mL, 11 mmol). After the mixture stirred for 20 min at −20 °C, the *N*-methylmorpholine hydrochloride was removed by filtration and the filter cake was washed with a small portion of cold THF. The filtrate was treated with a cold (−78 °C) ethereal solution of diazomethane (prepared from 4.3 g (20 mmol) of diazald and distilled). The reaction mixture was allowed to reach room temperature and stirred for 1 h. After THF was removed in vacuo, the crude product was solubilized in ethyl acetate, washed with H_2O, $NaHCO_3$ (10%), and brine, and dried with Na_2SO_4. The solvent was then evaporated to afford the corresponding diazo ketone (4.45 g, 8.7 mmol). To a solution of this diazo ketone in anhydrous MeOH (20 mL) was added silver benzoate (119 mg, 0.52 mmol) in triethylamine (12 mL). The solution darkened, and rapid gas evolution was noted. After the mixture was stirred for 90 min, Celite and decolorizing carbon was added. After stirring, the mixture was filtered through Celite and the filtrate concentrated. The residue was dissolved in EtOAc and washed with water, 1 N $NaHCO_3$, 1 N $KHSO_4$, and brine. After drying (Na_2SO_4), the solvent was removed at reduced pressure and the crude product chromatographed (cyclohexane/CH_2Cl_2/AcOEt, 5/3/2) to give 4.35 g of compound **5a** as a colorless solid (84% yield): R_f (AcOEt:CH_2Cl_2, 1:3) = 0.58; ^{1}H NMR (DMSO-d_6) δ 1.32 (9H, s, tBu), 1.25−1.37 (1H, m, CH−CH_2−CH_2), 1.40−1.53 (1H, m, CH−CH_2−CH_2), 2.20 (2H, t, CH−CH_2−CH_2), 2.30 (2H, d, CH_2COOMe), 3.50 (3H, s, COOMe), 3.63−3.77 (1H, m, CH−CH_2−CH_2), 6.67 (1H, d, NH−Boc), 7.08−7.22 (15H, m, Trt), 8.50 (1H, s, NH-Trt). Anal. ($C_{31}H_{36}N_2O_5$) C, H, N.

Methyl (2*S*,3*S*)-3-(Boc-amino)-2-[(4-methoxybenzyl)-sulfanyl]-6-oxo(tritylamino)hexanoate (6a). This compound was prepared according to the procedure described in Bischoff et al.[24] To a solution of compound **5a** (1.032 g, 2 mmol) in tetrahydrofuran (20 mL) at −78 °C under nitrogen was added dropwise a solution of lithium diisopropylamide (1.6 M) (4.8 mL, 7.6 mmol) in THF/heptane/ethylbenzene. After 30 min of reaction time, a solution of 4-methoxy-2,4-dinitrophenyl disulfide (700 mg, 2.2 mmol) in 8 mL of THF was added, and the solution stirred for 2 h. The reaction was stopped by adding $KHSO_4$ (1 N) and extracted with ethyl acetate. The organic layer was washed by $KHSO_4$ (1 N) and brine, dried (Na_2SO_4), and then evaporated at reduced pressure. The crude product was chromatographed (AcOEt/CH_2Cl_2/cyclohexane, 2/3/5) to obtain 574 mg of compound **6a** (43% yield): R_f (AcOEt:CH_2Cl_2: cyclohexane, 20:30:50) = 0.27; ^{1}H NMR (DMSO-d_6) δ 1.32 (9H, s, tBu), 1.28−1.37 (1H, m, CH-CH_2−CH_2), 1.86−1.95 (1H, m, CH-CH_2−CH_2), 2.10−2.24 (2H, m, CH−CH_2-CH_2), 3.22 (1H, m, CH−S), 3.55 (3H, s, COOMe), 3.67 (5H, 2s, S-CH_2−C_6H_4-*OMe*), 3.70−3.78 (1H, m, *CH*-CH_2−CH_2), 6.72 (1H, d, *NH*-Boc), 6.79 (2H, d, H_3,H_5 PhOMe), 7.12 (2H, d, H_2,H_6 PhOMe), 7.10−7.23 (15H, m, Trt), 8.50 (1H, s, *NH*-Trt); MS (EI) 153, 243, 559, 669. Anal. ($C_{39}H_{44}N_2O_6S$) C, H, N.

(2*SR*,3*S*)-3-(Boc-amino)-2-[(4-methoxybenzyl)sulfanyl]-6-oxo(tritylamino)hexanoic Acid (Synthon A). Compound **6a** (334 mg, 0.5 mmol) was treated by 1 M NaOH in MeOH/H_2O (1/1). After classical workup, the solvent was removed in vacuo to afford 317 mg of synthon A (95% yield): mp = 118 °C; R_f (MeOH:CH_2Cl_2, 10:90) = 0.4; ^{1}H NMR (DMSO-d_6) δ 1.30 (1.39) (9H, s, tBu), 1.55−1.66 (1H, m, βCH_2(Gln)), 1.80−1.93 (1H, m, βCH_2(Gln)), 2.13−2.26 (2H, m, γCH_2(Gln)), 3.12 (3.18) (1H, d, CH−S), 3.68 (5H, s, CH_2−C_6H_5−OCH_3), 3.70−3.80 (1H, m, αCH(Gln)), 3.62(3.70) (1H, d, Boc*NH*), 6.80 (2H, d, H_3,H_5 PhOMe), 7.12 (2H, d, H_2,H_6 PhOMe), 7.10−7.25 (15 H, m, Trt), 8.48 (8.52) (1H, s, NHTrt); $\{\alpha\}_D$ = −9.4 (MeOH). Anal. ($C_{38}H_{42}N_2O_6S$) C, H, N.

***N*-[(2*SR*,3*S*)-3-Amino-5-carbamoyl-2-sulfanylpentanoyl]-L-phenylalanine (15).** Synthon A (100 mg) was coupled with H-Phe-OtBu using BOP as coupling agent as previously described.[50] After classical workup and deprotection by TFA[32] and anhydrous hydrogen fluoride,[33] 54 mg of crude product **15** was obtained by purification on a semipreparative HPLC with a C18 Vydac column (5 μM, 100 Å, 10 × 250 mm) (38% yield): retention time = 12.1 min (Nucleosil, C8, 150 × 4.6 mm, 5 μm, 100 Å, gradient 0−40% B in 15 min, flow rate = 1 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (DMSO-d_6) δ 1.37 (1H, m, β (Gln)), 1.50 (1H, m, β (Gln)), 2.08 (2H, t, γ (Gln)), 2.82 (1H, dd, β (Phe)), 3.06 (1H, dd, β (Phe)), 3.15 (1H, m, *CH*NH^{3+}), 3.51 (1H, d, *CH*SH), 4.40 (1H, m, α(Phe)), 7.00 (1H, s, $CONH_2$), 7.13−7.22 (5H, m, Ph), 7.40 (1H, s, $CONH_2$), 8.77 (1H, d, NH(Phe)); MS (ESI) $(M + H)^+$ m/z = 340.1. Anal. ($C_{15}H_{21}N_3O_4S$).

***N*-[(2*SR*,3*S*)-3-Amino-5-carbamoyl-2-sulfanylpentanoyl]-L-phenylalanyl-L-glutamic Acid (16).** Synthon A (150 mg) was coupled with H-Phe-Glu(tBu)-2-chlorotrityl resin using BOP as previously described.[50] After deprotection by trifluoroacetic acid[32] and anhydrous hydrogen fluoride,[33] the crude product **16** was obtained and purified by semipreparative HPLC on a Vydac C18 column (5 μM, 100 Å, 10 × 250 mm) (29% yield): retention time = 10.8 min (Nucleosil, C8, 150 × 4.6 mm, 5 μm, 100 Å, gradient 0−40% B in 15 min, flow rate = 1 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (DMSO-d_6) δ 1.29 (1H, m, β (Gln)), 1.43 (1H, m, β (Gln)), 1.78 (1H, m, β (Glu)), 1.84 (1H, m, β (Glu)), 2.04 (2H, t, γ (Gln)), 2.26 (2H, t, γ (Glu)), 2.69 (1H, dd, β (Phe)), 3.03 (1H, dd, β (Phe)), 3.09 (1H, m, *CH*NH_3+), 3.55 (1H, d, *CH*SH), 4.20 (1H, m, α(Glu)), 4.60 (1H, m, α(Phe)), 7.00 (1H, s, $CONH_2$), 7.12−7.27 (5H, m, Ph), 7.40 (1H, s, $CONH_2$), 7.93 (3H, br s, NH^{3+}), 8.40 (1H, d, NH(Glu)), 8.67 (1H, d, NH(Phe)); MS (ESI) $(M + H)^+$ m/z = 469.2. Anal. ($C_{20}H_{28}N_4O_7S$).

***N*-[(2*SR*,3*S*)-3-Amino-5-carbamoyl-2-sulfanylpentanoyl]-L-phenylalanyl-L-glutamyl-L-threonine (17).** Synthon A (150 mg) was coupled with H-Phe-Glu(tBu)-Thr(tBu)-2-chlorotrityl resin to give, by the procedure described for compound **16**, crude product **17** which was purified by semipreparative HPLC on C18 Vydac column (5 μM, 100 Å, 10 × 250 mm) (27% yield): retention time = 11.6 min (Nucleosil, C8, 150 × 4.6 mm, 5 μm, 100 Å, gradient 0−40% B in 15 min, flow rate = 1 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (DMSO-d_6) δ 1.02 (3H, d, γ (Thr)), 1.28 (1H, m, β (Gln)), 1.43 (1H, m, β (Gln)), 1.76 (1H, m, β (Glu)), 1.90 (1H, m, β (Glu)), 2.07 (2H, t, γ (Gln)), 2.28 (2H, t, γ (Glu)), 2.68 (1H, dd, β (Phe)), 3.03 (1H, dd, β (Phe)), 3.08 (1H, m, *CH*NH^{3+}), 3.54 (1H, d, *CH*SH), 4.11 (1H, m, β (Thr)), 4.16 (1H, m, α (Thr)), 4.40 (1H, m, α(Glu)), 4.60 (1H, m, α(Phe)), 4.93 (1H, br s, OH), 7.00 (1H, s, $CONH_2$), 7.10−7.20 (5H, m, Ph), 7.42 (1H, s, $CONH_2$), 7.83 (1H, d, NH(Thr)), 7.92 (3H, br s, NH^{3+}), 8.30 (1H, d, NH(Glu)), 8.67 (1H, d, NH(Phe)), 12.35 (2H, br s, COOH); MS (ESI) $(M + H)^+$ m/z = 570.3. Anal. ($C_{24}H_{35}N_5O_9S$).

***N*-[(2*SR*,3*S*)-3-Amino-5-carbamoyl-2-sulfanylpentanoyl]-L-phenylalanyl-L-glutamyl-L-threonyl-L-serine (18).** Synthon A (150 mg) was coupled with H-Phe-Glu(tBu)-Thr(tBu)-Ser(tBu)-2-chlorotrityl resin as described for compound **16**. Crude product **18** was purified by semipreparative HPLC on C18 Vydac column (5 μM, 100 Å, 10 × 250 mm) (32% yield): retention time = 13.9 min (Nucleosil, C8, 150 × 4.6 mm, 5 μm, 100 Å, gradient 0−40% B in 15 min, flow rate = 1 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (DMSO-d_6) δ 1.04 (3H, d, γ (Thr)), 1.25 (1H, m, β (Gln)), 1.42 (1H, m, β (Gln)), 1.73 (1H, m, β (Glu)), 1.89 (1H, m, β (Glu)), 2.03 (2H, t, γ (Gln)), 2.24 (2H, t, γ (Glu)), 2.65 (1H, dd, β (Phe)), 3.03 (1H, dd, β (Phe)), 3.04 (1H, m, *CH*NH^{3+}), 3.52 (1H, d, *CH*SH), 3.57 (1H, dd, β (Ser)), 3.68 (1H, dd, β (Ser)), 3.94 (1H, m, β (Thr)), 4.23 (1H, m, α (Ser)), 4.27 (1H, m, α (Thr)), 4.33 (1H, m, α(Glu)), 4.60 (1H, m, α(Phe)), 4.96 (2H, br s, OH(Ser) and OH(Thr)), 7.03 (1H, s, $CONH_2$), 7.10−7.24 (5H, m, Ph), 7.42 (1H, s, $CONH_2$), 7.78 (1H, d, NH(Thr)), 7.92 (2H, br s, NH_2), 7.93 (1H, d, NH(Ser)), 8.36 (1H, d, NH(Glu)), 8.69 (1H, d, NH(Phe)), 12.35 (2H, br s, COOH); MS (ESI) $(M + H)^+$ m/z = 657.4. Anal. ($C_{27}H_{40}N_6O_{11}S$).

Synthesis of (2*SR*,3*S*)-4-(*tert*-Butylsulfamoyl)-2-(Cbz-amino)butanoic Acid (Synthon B). Methyl (3*S*)-5-(*tert*-Butylsulfamoyl)-3-(Cbz-amino)pentanoate (5b). Compound **5b** was prepared from **4b** (20 g, 53.7 mmol) (prepared as previously described[23]) by the procedure described for **5a**.

Compound **5b** (20.1 g) was obtained as a colorless solid after flash chromatography (cyclohexane/CH_2Cl_2/AcOEt, 0.5/3/2) (95% yield): mp = 88 °C; R_f = 0.48 (AcOEt/CH_2Cl_2/cyclohexane, 1/1/1); ^{1}H NMR ($CDCl_3$) δ 1.29 (9H, s, tBu), 1.99 (2H, m, $CH_2CH_2SO_2NH$), 2.54 (2H, d, CH_2COOMe), 3.05 (2H, t, CH_2-SO_2NH), 3.61 (3H, s, COOMe), 4.02 (1H, m, CHCH$_2$COOMe), 5.03 (2H, s, CH_2Ph), 4.14 (1H, s, NHtBu), 5.37 (1H, d, BzlOCO*NH*), 7.28 (5H, m, Ph). Anal. ($C_{18}H_{28}N_2O_6S$) C, H, N.

Methyl (2*S*,3*S*)-5-(*tert*-Butylsulfamoyl)-3-(Cbz-amino)-2-[(4-methoxybenzyl)sulfanyl]pentanoate (6b). Compound **6b** was prepared from **5b** (1.5 g, 3.75 mmol) by using the procedure as described for **6a**. The crude residue obtained was chromatographed (AcOEt/CH_2Cl_2/cyclohexane, 2/3/5) to yield 1.1 g of compound **6b** (54% yield) (>95% ee according to HPLC monitoring): R_f (EtOAc/CH_2Cl_2/cyclohexane, 1/1/1) = 0.57; R_f (EtOAc/CH_2Cl_2/cyclohexane, 15/25/60) = 0.15; ^{1}H NMR ($CDCl_3$) δ 1.25 (9H, s, tBu), 1.86 to 2.30 (2H, m, CH-CH_2−CH_2), 2.96 (2H, t, CH−CH_2-CH_2), 3.29 (1H, 2d, CH−S), 3.64 (3H, s, COOMe), 3.72 (5H, s, S−CH_2−C_6H_4−OMe), 4.02 (1H, m, CH-CH_2−CH_2), 4.87 (1H, s, SO_2−NH), 4.95 (1H, d(AB), $CH_2C_6H_5$), 5.05 (1H, d(AB), $CH_2C_6H_5$), 5.50 (1H, d, *NH*-Z), 6.75 (2H, d, H_3,H_5 PhOMe), 7.14 (2H, d, H_2,H_6 PhOMe), 7.37 (5H, m, Ph); MS (ES) (M + H)$^+$ *m*/*z* = 539.3, (M + Na)$^+$ 561.3. Anal. ($C_{26}H_{36}N_2O_7S_2$) C, H, N.

(2*SR*,3*S*)-5-(*tert*-Butylsulfamoyl)-3-(Cbz-amino)-2-[(4-methoxybenzyl)sulfanyl]pentanoic Acid (Synthon B). The compound **6b** (0.55 g, 1 mmol) was hydrolyzed by 3 M NaOH in MeOH overnight at room temperature to afford 0.48 g of synthon B (91% yield): mp = 70 °C; R_f (EtOAc/AcOH/CH_2Cl_2, 30/2/70) = 0.24 and 0.32; retention time = 10.4 and 11.8 min (Chirose bond, C1, 250 × 4.6 mm, 5 μm, isocratic conditions: 30% of B″ with A″:phosphate buffer (0.05 M, pH = 6); B″:CH_3CN, flow rate = 1 mL/min); ^{1}H NMR ($CDCl_3$) δ 1.21 (9H, s, tBu), 1.79 to 1.93 (1H, m, CH-CH_2−CH_2), 1.96 to 2.10 (1H, m, CH-CH_2−CH_2), 2.97 (2H, t, CH−CH_2-CH_2), 3.70 (3H, s, OMe), 3.76 (2H, s, CH_2−CH_4−OMe), 4.04 (1H, m, CH-CH_2−CH_2), 5.01 (2H, s, CH_2−C_6H_5), 5.60 (1H, d, *NH*-Z), 6.72 and 6.77 (2H, d, H_3,H_5 PhOMe), 7.12 and 7.17 (2H, d, H_2,H_6 PhOMe), 7.25 (5H, br s, Ph); MS (ESI) (M + H)$^+$ *m*/*z* = 539.3, 561.3 (Na^+) Anal. ($C_{25}H_{34}N_2O_7S_2$) C, H, N..

N*-[(2*SR*,3*S*)-3-Amino-5-sulfamoyl-2-sulfanylpentanoyl]-L-tyrosyl-L-histidine (19a and 19b).** To 135 mg (250 μmol) of synthon B, 122 mg (320 μmol) of HATU, and 150 μmol of H-Tyr(tBu)-His(Trt)-2-chlorotrityl resin in 5 mL of NMP (*N*-methylpyrrolidone) was added at room temperature 200 μL of DIEA. After 6 h of stirring and negative Kaiser test, the peptidyl resin was successively washed with NMP and CH_2Cl_2. After deprotection by trifluoroacetic acid[32] and anhydrous hydrogen fluoride,[33] the crude products **19a** and **19b** were purified and separated by semipreparative HPLC on a C18 Vydac column (5 μM, 100 Å, 10 × 250 mm) (22% and 17% yields, respectively, λ = 214 nm): purity > 99%; retention time = 10.6 min (**19a**, **2*R*,3*S) and 11.5 min (**19b**, **2*S*,3*S***) (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å, gradient 0−40% B in 15 min, flow rate = 1 mL/min); ^{1}H NMR (D_2O) δ 2.12 (2H, m, CH-CH_2−CH_2), 2.96 (2H, dd, β(Tyr)), 3.08 (1H, dd, β(His)), 3.18 (1H, dd, β(His)), 3.33 (2H, t, CH−CH_2-CH_2), 3.70 (1H, d, CH-SH), 3.72 (1H, m, CH−CH_2−CH_2), 4.22 (1H, d, CH_2−C_6H_5), 4.37 (1H, d, CH_2−C_6H_5), 4.55 (1H, t, α(Tyr)), 4.60 (1H, t, α(His)), 6.82 (2H, d, H_3−H_5(Tyr)), 7.13 (2H, d, H_2−H_6(Tyr)), 7.14 (1H, s, H_4(His)), 7.19 (2H, d, C_6H_5), 7.37 (3H, m, C_6H_5), 8.51(1H, s, H_2(His)); MS (ESI) (M + H)$^+$ *m*/*z* = 529.1, (M + 2H)$^+$ 265.2. Anal. ($C_{20}H_{28}N_6O_7S_2$).

N*-[(2*SR*,3*S*)-3-Amino-5-sulfamoyl-2-sulfanylpentanoyl]-L-tyrosyl-*N*-benzyl-L-histidinamide (20a and 20b).** To 300 mg (0.57 mmol) of synthon B, 260 mg (0.68 mmol) of HATU, and 470 mg (0.68 mmol) of H-Tyr(tBu)-His(Trt)-NHBz in CH_2Cl_2 (5 mL) was added 350 μL of DIEA. After stirring for 6 h at room temperature and classical workup, 460 mg of crude protected product treated by trifluoroacetic acid[32] and anhydrous hydrogen fluoride[33] led to the mixture of **19a** and **19b.** The two diastereomers were separated by semipreparative HPLC on a C18 Vydac column (5 μM, 100 Å, 10 × 250 mm) to yield 84 mg of **20a** (18% yield) and 67 mg of **20b** (14% yield): retention time = 9.5 min (**20a**, **2*R*,3*S) and 9.9 min (**20b**, **2*S*,3*S*)** (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å, gradient 20−60% B in 15 min, flow rate = 1 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (D_2O) δ 1.93−2.13 (2H, m, CH_2−CH_2−SO_2NH), 2.87 and 3.08 (2H, dd, β(Tyr)), 2.98 (1H, dd, β(His)), 3.17 and 3.18 (1H, dd, β(His)), 3.29 and 3.31 (2H, t, CH_2-CH_2−SO_2NH), 3.66 and 3.68 (1H, d, CH-SH), 3.70 and 3.72 (1H, m, CH-CH_2−CH_2), 4.58 (1H, t, a(Tyr)), 4.63 (1H, t, a(His)), 6.78 and 6.82 (2H, d, H_3−H_5(Tyr)), 7.09 and 7.13 (2H, d, H_2−H_6(Tyr)), 7.14 and 7.23 (1H, s, H_4(His)), 8.48 and 8.51 (1H, s, H_2(His)); MS (ESI) (M + H)$^+$ *m*/*z* = 618.1. Anal. ($C_{27}H_{35}N_7O_6S_2$).

Synthesis of (2*SR*,3*SR*)-3-(Boc-amino)-3-[3-(*tert*-butylsulfamoyl)phenyl]-2-[(4-methoxybenzyl)sulfanyl]propanoic Acid (Synthon C). 3-(*tert*-Butylsulfamoyl)benzoic Acid (8). To a solution of 75 g (340 mmol) of 3-(chlorosulfonyl)-benzoic acid (**7**) (from Aldrich) in 1.5 L of CH_2Cl_2 was added at 0 °C 125 mL (1.19 mol, 0.8 M) of $tBuNH_2$. After 2 h stirring at room temperature, the final acid was extracted with NaOH (1 M) (three times) and the aqueous layer acidified with HCl (6 N). A white solid precipitated and was filtered. The filter cake was dried over P_2O_5 to obtain 68.36 g of **8** which was used without further purification (78% yield): R_f (AcOH/toluene, 3/7) = 0.45; ^{1}H NMR (DMSO-d_6) δ 1.05 (9H, s, tBu), 7.65 (1H, s, SO_2NH), 7.65 (1H, t, Ar), 8.00 (1H, d, Ar), 8.08 (1H, d, Ar), 8.33 (1H, s, Ar). Anal. ($C_{11}H_{15}NO_4S$) C, H, N.

***N*-*tert*-Butyl-3-(hydroxymethyl)benzenesulfonamide (9).** A 12.94 g (50.3 mmol) portion of 3-(*tert*-butylsulfamoyl)benzoic acid was treated with 3 molar equiv of $LiAlH_4$[25] in THF to afford 10.9 g of **9** (90% yield): R_f (AcOOH/toluene, 3/7) = 0.24; ^{1}H NMR (DMSO-d_6) δ 1.04 (9H, s, tBu), 4.52 (2H, d, CH_2OH), 5.36(1H, t, CH2O*H*), 7.45 (1H, s, SO_2NH), 7.46 (1H, d, Ar), 7.46 (1H, d, Ar), 7.64 (1H, dd, Ar), 7.77 (1H, s, Ar). Anal. ($C_{11}H_{17}NO_3S$) C, H, N.

***N*-*tert*-Butyl-3-(oxomethyl)benzenesulfonamide (10).** Compound **10** was synthesized by applying the Swern procedure[26] with slight modification. Briefly, to a solution of 4.7 mL (53.7 mmol) of oxalyl chloride (freshly distilled) in 100 mL of CH_2Cl_2 was added dropwise, at −60 °C, under N_2, a solution of 9.5 mL (134 mmol) of DMSO in 20 mL of CH_2Cl_2. The reaction was stirred for 5 min, and the alcohol **9** in 4 mL of DMSO and 30 mL of CH_2Cl_2 was added within 5 min. Stirring was continued for an additional 30 min at the same temperature; 31.2 mL (224 mmol) of triethylamine was then added, and the reaction mixture was stirred for 5 min at −65 °C and then allowed to warm to room temperature. After 1 h of stirring, water was added and the aqueous layer was reextracted with additional CH_2Cl_2. The organic layers were combined, washed with $KHSO_4$ (1 N), $NaHCO_3$ (10%), and brine, and dried over Na_2SO_4 to give after concentration in vacuo 10.45 g of compound **10** (96% yield): R_f (AcOEt/cyclohexane, 1/2) = 0.32; ^{1}H NMR (DMSO) δ 1.05 (9H, s, tBu), 7.70 (1H, s, SO_2NH), 7.78 (1H, t, Ar), 8.10 (2H, d, Ar), 8.29 (1H, s, Ar), 10.05 (1H, s, C*H*O). Anal. ($C_{11}H_{15}NO_3S$) C, H, N.

Ethyl 3-[3-(*tert*-Butylsulfamoyl)phenyl]propenoate (11). A 20.4 mL (102 mmol) portion of triethyl phosphonoacetate was added dropwise to a suspension of sodium hydride (3 g, 104 mmol) in dry DME (200 mL) using a previously described protocol.[27] After the mixture stirred for 30 min at 45 °C, 16.8 g (69.6 mmol) of aldehyde **10** was added rapidly, and the mixture was stirred for 1 h at 50 °C. The reaction was poured into 250 mL of ice−water and extracted with ethyl acetate (2 × 250 mL). The ethyl acetate solution was washed with water and brine, dried over Na_2SO_4, filtered, and evaporated in vacuo; 20.56 g of compound **11** (85% yield) was isolated as a white solid after crystallization in ether/heptane (1/1): R_f (AcOEt/cyclohexane, 4/6) = 0.40; ^{1}H NMR (DMSO) δ 1.05 (9H, s, tBu), 1.20 (3H, t, CH_3−CH_2), 4.15 (2H, q, CH_3-CH_2), 6.65 (1H, *J* = 16 Hz, d, ϕCH=CH), 7.50 (1H, s, SO_2NH), 7.58 (1H, t, Ar), 7.68 (1H, t, CH=*CH*-COOEt), 7.82 (1H, d, Ar), 7.91 (1H, d, Ar), 8.10 (1H, s, Ar). Anal. ($C_{15}H_{21}NO_4S$) C, H, N.

Ethyl (3*R*)-3-[(*S*)-*N*-Benzyl-1-phenylethylamino]-3-[3-(*tert*-butylsulfamoyl)phenyl]propanoate (12a) and Ethyl (3*S*)-3-[(*R*)-*N*-Benzyl-1-phenylethylamino]-3-[3-(*tert*-butylsulfamoyl)phenyl]propanoate (12b). Both diastereo-

isomers were synthesized by applying the procedure previously described.[28,51] Briefly, to a solution of 4 g (18.9 mmol) of commercially available (*S*)-(−)-*N*-benzyl-α-methylbenzylamine (99%) (Aldrich) in 70 mL of dry THF was added, at 0 °C, 11.8 mL (18.9 mmol) of nBuLi (1.6 M in hexane) in 30 mL of THF. The reaction mixture was stirred for 15 min and cooled to −78 °C. A solution of 2.36 g (7.56 mmol) of **11** in 30 mL of THF was added dropwise to the reaction mixture which was stirred for 30 min; 30 mL of $KHSO_4$ (1 M) was then added. After evaporation of THF, the aqueous residue was extracted with EtOAc. The ethyl acetate layer was extracted with $KHSO_4$ (1 N), $NaHCO_3$ (10%), and brine, dried over Na_2SO_4, and evaporated in vacuo. The product was then purified by flash chromatography on a silica gel column using AcOEt/CH_2Cl_2/cyclohexane (1.5/1.5/7) as eluent to obtain 3.28 g of compound **12a** (87% yield) (>99% ee according to HPLC monitoring). (*R*)-(+)-*N*-Benzyl-α-methylbenzylamine was used to obtain **12b**: R_f (AcOEt/cyclohexane, 3/7) = 0.41; ^{1}H NMR ($CDCl_3$) δ 1.02 (3H, t, CH_2CH_3), 1.13 (9H, s, tBu), 1.20 (3H, t, CH-CH_3), 2.57 (2H, m, CH_2−COOEt), 3.64 (2H, d, $CH_2\phi$), 3.84−3.95 (3H, m, CH_3-CH_2 and CH_3-*CH*), 4.47 (1H, dd, *CH*-CH_2-COOEt), 4.51 (1H, s, SO_2NH), 7.11−7.35 (10H, m, 2ϕ), 7.39 (1H, dd, Ar), 7.51 (1H, d, Ar), 7.71 (1H, d, Ar), 7.90 (1H, s, Ar). **12a:** $\{\alpha\}_D$ = +5.6 (MeOH). Anal. ($C_{30}H_{38}N_2O_4S$) H, N; C: calcd, 68.94; found, 68.44.

Ethyl (3*R*)-3-(Boc-amino)-3-[3-(*tert*-butylsulfamoyl)-phenyl]propanoate (13a) and Ethyl (3*S*)-3-(Boc-amino)-3-[3-(*tert*-butylsulfamoyl)phenyl]propanoate (13b). A 3.28 g (6.28 mmol) portion of **12a** (respectively **12b**) and 4.8 g of Boc_2O were solubilized into 60 mL of AcOEt containing 4.4 g of $Pd(OH)_2$/C (20%). The reaction mixture was vigorously stirred under 20 bar of H_2 at 25 °C for 18 h. After filtration of the catalyst on Celite, the filtrate was evaporated in vacuo and purified on a silica gel column using AcOEt/cyclohexane (1/3) as eluent to obtain 2.28 g of compound **13a** (respectively **13b**) (85% yield): R_f (AcOEt/cyclohexane, 3/7) = 0.21; ^{1}H NMR ($CDCl_3$) δ 1.13 (3H, t, CH_2CH_3), 1.15 (9H, s, *tBu*-NH-SO_2-), 1.35 (9H, s, Boc), 2.76 (2H, m, CH_2−COOEt), 3.99 (2H, q, CH_3-CH_2), 4.60 (1H, s, SO_2NH), 5.08 (1H, m, *CH*-CH_2-COOEt), 5.58 (1H, m, *NH*-Boc), 7.38 (1H, dd, Ar), 7.40 (1H, d, Ar), 7.72 (1H, d, Ar), 7.77 (1H, s, Ar). **13a:** $\{\alpha\}_D$ = +24.0 (MeOH). **13b:** $\{\alpha\}_D$ = −23.5 (MeOH). Anal. ($C_{20}H_{32}N_2O_6S$) H, N; C: calcd, 56.06; found, 56.65.

Ethyl (2*S*,3*S*)-3-(Boc-amino)-3-[3-(*tert*-butylsulfamoyl)-phenyl]-2-[(4-methoxybenzyl)sulfanyl]propanoate (14a) and Ethyl (2*R*,3*R*)-3-(Boc-amino)-3-[3-(*tert*-butylsulfamoyl)phenyl]-2-[(2,4-dimethoxybenzyl)sulfanyl]propanoate (14b). A 0.5 g (1.17 mmol) portion of **13a** was treated according to the procedure described for compound **5a**. The crude residue was purified on a silica gel column, using EtOAc/cyclohexane (1/3) as eluent, to yield 331 mg of compound **14a** (>85% ee according to HPLC monitoring) (49% yield): R_f (AcOEt/cyclohexane, 3/7) = 0.31; ^{1}H NMR ($CDCl_3$) δ 1.08 (3H, t, CH_2CH_3), 1.12 (9H, s, *tBu*-NH-SO_2-), 1.36 (9H, s, Boc), 3.40 (1H, m, *CH*-COOEt), 3.75 (3H and 2H, s, OMe and ϕ-CH_2−S), 3.96 (2H, q, CH_3-CH_2), 4.51 (1H, s, SO_2NH), 5.00 (1H, m, BocNH-*CH*), 6.11 (1H, m, *NH*-Boc), 6.82 (2H, d, ϕ-OMe), 7.22 (2H, d, ϕ-OMe), 7.25 (1H, d, Ar), 7.30 (1H, t, Ar), 7.50 (1H, s, Ar), 7.68 (1H, d, Ar). Anal. ($C_{28}H_{40}N_2O_7S_2$) C, H, N.

According to a previously described protocol,[30] to a solution of 1,1,1,3,3,3-hexadimethyldisilazane (6.4 mL, 3.5 molar equiv, 30.2 mmol) in THF (70 mL) at 0 °C was added a solution of *n*-butyllithium (2.5 M solution in hexane, 11 mL, 3.2 molar equiv). After stirring at this temperature for 10 min, the mixture was cooled to −78 °C and a solution of 3.7 g of **13b** (8.64 mmol) in 40 mL of THF was added dropwise. The stirred mixture was maintained at 30 °C for 2 h and then cooled to −78 °C and a solution of 2,4-dimethoxybenzylthio 4-methylphenyl sulfonate (3.7 g, 10.37 mmol) in 30 mL of THF was added. After stirring at this temperature for 1 h, the reaction mixture was poured into saturated aqueous ammonium chloride solution (50 mL), and the mixture obtained was extracted with diethyl ether (2 × 300 mL). The combined extracts were washed with water and brine, dried over Na_2SO_4, and evaporated in vacuo; 3.11 g of **14b** (59% yield) was obtained after flash chromatography: R_f (AcOEt/cyclohexane, 1/4) = 0.15; ^{1}H NMR ($CDCl_3$) δ 1.08 (3H, t, CH_2CH_3), 1.12 (9H, s, tBu-NH−SO_2), 1.35 (9H, s, Boc), 3.56 (1H, m, CH−COOEt), 3.79 (6H and 2H, s, OMe and ΦCH_2S), 4.00 (2H, q, CH_3-CH_2), 4.51 (1H, s, SO_2NH), 5.06 (1H, m, Boc-NH−CH), 6.13 (1H, m, NH−Boc), 7.30−7.42 (3H, m, *F*(OMe)), 7.58−7.78 (4H, m, Ar).

(2*SR*,3*S*)-3-(Boc-amino)-3-[3-(*tert*-butylsulfamoyl)-phenyl]-2-[(4-methoxybenzyl)sulfanyl]propanoic Acid and (2*SR*,3*R*)-3-(Boc-amino)-3-[3-(*tert*-butylsulfamoyl)phenyl]-2-[(2,4-dimethoxybenzyl)sulfanyl]propanoic Acid. To a stirred solution of bis(tributyltin) oxide (BBTO) (5.3 mL, 10.33 mmol, 3 molar equiv) in 20 mL of CH_3CN was added 2 g of compound **14a** (3.44 mmol) solubilized in 6 mL of CH_3CN. The mixture was refluxed for 48 h, and 50 mL of CH_3CN was added. The organic phase was acidified with HCl (2 N), and the mixture was stirred for 30 min. After evaporation of CH_3CN and classical workup, the crude residue was purified on a silica gel column using AcOEt/cyclohexane/AcOH (45/55/2.5) as eluent; 1.3 g of synthon C (2*S*,3*S*) was thus obtained (70% yield): retention time = 11.8 min (2*S*,3*S*) (Chirose bond, C1, 250 × 4.6 mm, 5 mm, isocratic at 30% of B″ with A″:phosphate buffer (0.05 M, pH = 6); B″:CH_3CN, flow rate =1 mL/min).

A 0.33 g (0.57 mmol) portion of **14a** was hydrolyzed as described for synthon A to give 310 mg of a racemic mixture of synthon C (2*SR*,3*S*) (98% yield): R_f (CH_2Cl_2/MeOH/AcOOH, 96/3/1) = 0.16; retention time = 11.8 min (2*S*,3*S*) and 10.7 min (2*R*,3*S*) (Chirose bond, C1, 250 × 4.6 mm, 5 μm, isocratic at 30% of B″ with A″:phosphate buffer (0.05 M, pH = 6); B″:CH_3CN, flow rate = 1 mL/min); ^{1}H NMR ($CDCl_3$) δ 1.11 and 1.12 (9H, s, *tBu*-NH-SO_2-), 1.30 and 1.35 (9H, s, Boc), 3.40 and 3.45 (1H, m, *CH*-COOEt), 3.72 and 3.75 (3H and 2H, s, OMe and ϕ-CH_2−S), 3.65 (1H, s, SO_2NH), 5.05 and 5.32 (1H, m, BocNH-*CH*), 5.37 and 6.20 (1H, m, *NH*-Boc), 6.75 and 6.80 (2H, d, ϕ-OMe), 7.12 and 7.20 (2H, d, ϕ-OMe), 7.30 (1H, d, Ar), 7.40 (1H, t, Ar), 7.81 and 7.70 (1H, s, Ar), 7.68 and 7.75 (1H, d, Ar). 2*S*,3*R*: $\{\alpha\}_D$ = +29.8 (MeOH). 2*S*,3*S*: $\{\alpha\}_D$ = −29.2 (MeOH). Anal. ($C_{26}H_{36}N_2O_7S_2$) C, H, N.

A 0.35 g (0.57 mmol) portion of **14b** was saponified as described for synthon A to give 310 mg of 2*SR*,3*R* (98% yield): R_f (CH_2Cl_2/MeOH/AcOOH, 96/3/1) = 0.16; retention time = 15.7 min (2*R*,3*R*) and 17.4 min (2*S*,3*R*) (Chirose bond, C1, 250 × 4.6 mm, 5 mm, isocratic at 30% of B with A:phosphate buffer (0.05 M, pH = 6); B:CH_3CN, flow rate = 1 mL/min); ^{1}H NMR (DMSO-d_6) δ 0.98 and 1.00 (9H, s, *tBu*-NH-SO_2-), 1.24 and 1.28 (9H, s, Boc), 3.53 and 3.63 (1H, d, *CH*-COOH), 3.61, 3.66, 3.68, 3.71, and 3.77 (6H and 2H, s, OMe and ϕ-CH_2−S), 4.81 (1H, m, BocNH-*CH*), 6.32 and 6.40 (1H, dd, ϕ-OMe), 6.40 and 6.47 (H, d, ϕ-OMe), 6.87 and 7.10 (1H, d, ϕ-OMe), 7.41−7.65 (5H, m, Ar and NH), 7.75 and 7.80 (1H, s, Ar). Anal. ($C_{27}H_{38}N_2O_8S_2$) C, H, N.

***N*-[(2*RS*,3*S*)-3-Amino-3-(3-sulfamoylphenyl)-2-sulfanylpropanoyl]-L-tyrosyl-*N*-benzyl-L-histidinamide (21a and 21b).** To 300 mg (0.57 mmol) of synthon C (2*SR*,3*S*), 260 mg (0.68 mmol) of HATU, 470 mg (0.68 mmol) of H-Tyr(tBu)-His(Trt)-NHBz, and 350 μL of DIEA was added 5 mL of CH_2Cl_2. After stirring for 6 h at room temperature and classical workup, the residue was treated by trifluoroacetic acid[32] to obtain 480 mg of crude product which was purified by semipreparative HPLC on C18 Vydac column (5 μM, 100 Å, 10 × 250 mm) in order to separate the two diastereomers: retention time = 12.85 and 14.35 min (Nucleosil, C18, 150 × 4.6 mm, 5μm, 100 Å, gradient 10−90% B in 30 min, flow rate = 1.5 mL/min).

Both diastereomers were then treated independently by anhydrous hydrogen fluoride[33] with 2% *m*-cresol to yield **21a** (28% yield) and **21b** (20% yield): retention time = 9.6 min **(21a)** and 10.7 min **(21b)** (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å, gradient 10−50% B in 15 min, flow rate = 1.5 mL/min, λ = 214 nm); purity > 99%.

***N*-[(2*R*,3*S*)-3-Amino-3-(3-sulfamoylphenyl)-2-sulfanylpropanoyl]-L-tyrosyl-*N*-benzyl-L-histidinamide (21a):** retention time = 9.6 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm,

100 Å, gradient 10−50% B in 15 min, flow rate = 1.5 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (D_2O + DTT) δ 2.44 (1H, dd, β(Tyr)), 2.63 (1H, dd, β(Tyr)), 3.07 (1H, dd, β(His)), 3.17 (1H, dd, β(His)), 3.96 (1H, d, *CH*-SH), 4.19 (1H, t, α(Tyr)), 4.20 (1H, d, CH_2−C_6H_5), 4.34 (1H, d, CH_2−C_6H_5), 4.58 (1H, t, α(His)), 4.60 (1H, d, H_2NCH), 6.69 (2H, d, H_3−H_5(Tyr)), 6.82 (2H, d, H_2−H_6(Tyr)), 7.14 (1H, s, H_4(His)), 7.19 (2H, d, H_2−H_6(Ar)), 7.33−7.40 (3H, m, H_3−H_4−H_5(Ar)), 7.61 (2H, d, $PhSO_2NH_2$), 7.84 (1H, s, $PhSO_2NH_2$), 7.96 (1H, m, $PhSO_2NH_2$), 8.51 (1H, s, H_2(His)); MS (ESI) $(M + H)^+$ m/z = 666.2. Anal. ($C_{31}H_{35}N_7O_6S_2$).

***N*-[(2*S*,3*S*)-3-Amino-3-(3-sulfamoylphenyl)-2-sulfanylpropanoyl]-L-tyrosyl-*N*-benzyl-L-histidinamide (21b):** retention time = 10.7 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å, gradient 10−50% B in 15 min, flow rate = 1.5 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (D_2O + DTT) δ 2.88 (2H, m, β(Tyr)), 3.10 (2H, d, β(His)), 4.15 (1H, d, CH_2−C_6H_5), 4.17 (1H, d, *CH*-SH), 4.36 (1H, d, CH_2−C_6H_5), 4.50 (1H, t, α(Tyr)), 4.53 (1H, t, α(His)), 5.00 (1H, d, H_2NCH), 6.77 (2H, d, H_3−H_5(Tyr)), 7.01 (2H, d, H_2−H_6(Tyr)), 7.14 (1H, s, H_4(His)), 7.16 (2H, d, H_2−H_6(Ar)), 7.33−7.40 (3H, m, H_3−H_4−H_5(Ar)), 7.69 (2H, d, $PhSO_2NH_2$), 7.88 (1H, s, $PhSO_2NH_2$), 7.99 (1H, m, $PhSO_2NH_2$), 8.46 (1H, s, H_2(His)); MS (ESI) $(M + H)^+$ m/z = 666.2. Anal. ($C_{31}H_{35}N_7O_6S_2$).

Both diastereoisomers were synthesized by the procedure described for compounds **21a** and **21b** using synthon C (2*SR*,3*R*) (23% yield for **21c** and 19% yield for **21d**).

***N*-[(2*R*,3*R*)-3-Amino-3-(3-sulfamoylphenyl)-2-sulfanylpropanoyl]-L-tyrosyl-*N*-benzyl-L-histidinamide (21c):** retention time = 9.6 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å, gradient 10−50% B in 15 min, flow rate = 1.5 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (D_2O + DTT) δ 2.78 (1H, ABX, β(Tyr)), 2.88 (1H, ABX, β(Tyr)), 2.93 (2H, d, β(His)), 4.05 (1H, d, *CH*-SH), 4.10 (1H, d, CH_2−C_6H_5), 4.30 (1H, d, CH_2−C_6H_5), 4.30 (1H, t, α(His)), 4.33 (1H, t, α(Tyr)), 4.65 (1H, d, H_2NCH), 6.76 (2H, d, H_3−H_5(Tyr)), 7.02 (2H, d, H_2−H_6(Tyr)), 7.07 (1H, s, H_4(His)), 7.12 (2H, d, H_2−H_6(Ar)), 7.35−7.40 (3H, m, H_3−H_4−H_5(Ar)), 7.70 (2H, d, $PhSO_2NH_2$), 7.86 (1H, t, $PhSO_2NH_2$), 7.96 (1H, s, $PhSO_2NH_2$), 8.45 (1H, s, H_2(His)); MS (ESI) $(M + H)^+$ m/z = 665.7. Anal. ($C_{31}H_{35}N_7O_6S_2$).

***N*-[(2*S*,3*R*)-3-Amino-3-(3-sulfamoylphenyl)-2-sulfanylpropanoyl]-L-tyrosyl-*N*-benzyl-L-histidinamide (21d):** retention time = 9.6 min (Nucleosil, C18, 150 × 4.6 mm, 5 μm, 100 Å, gradient 10−50% B in 15 min, flow rate = 1.5 mL/min, λ = 214 nm); purity > 99%; ^{1}H NMR (D_2O + DTT) δ 2.75 (2H, m, β(Tyr)), 3.08 (1H, d, β(His)), 3.17 (1H, d, β(His)), 4.11 (1H, d, *CH*-SH), 4.22 (1H, d, CH_2−C_6H_5), 4.35 (1H, d, CH_2−C_6H_5), 4.38 (1H, t, α(Tyr)), 4.57 (1H, t, α(His)), 4.86 (1H, d, H_2NCH), 6.74 (2H, d, H_3−H_5(Tyr)), 6.94 (2H, d, H_2−H_6(Tyr)), 7.14 (1H, s, H_4(His)), 7.19 (2H, d, H_2−H_6(Ar)), 7.34−7.40 (3H, m, H_3−H_4−H_5(Ar)), 7.54 (1H, d, $PhSO_2NH_2$), 7.65 (1H, t, $PhSO_2NH_2$), 7.87 (1H, s, $PhSO_2NH_2$), 7.98 (1H, d, $PhSO_2NH_2$), 8.50 (1H, s, H_2(His)); MS (ESI) $(M + H)^+$ m/z = 665.6. Anal. ($C_{31}H_{35}N_7O_6S_2$).

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