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Synthesis and biological activity of peptide hydroxamate inhibitors of degradation of substance P analogues

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Summary — A series of hydroxamic acid derivatives of peptides related to fragments of substance P (SP) were synthesized. Methyl, ethyl or *N*-hydroxy-succinimide ester precursors of the desired peptides were prepared by using classical peptide synthesis methodology and these were reacted with excess hydroxylamine in either ethanol or *N,N*-dimethylformamide. The products were characterized by chromatographic methods, amino acid analysis and fast atom bombardment mass spectrometry. The inhibition of the degradation of the radiolabelled substrate desamino-[3-^{125}I-tyrosyl^5]SP(**5–11**) ([(^{125}I)BH^5]SP(**5–11**)) by these compounds in rat hypothalamus preparations was determined. The most potent inhibitors found were Boc-Phe-Phe-Phe-NHOH (**12d**, IC_{50} = 4 μM), Boc-Phe-Phe-Trp-NHOH (**9**, IC_{50} = 5 μM) and desamino-Tyr-Phe-Phe-Gly-NHOH (**22**, IC_{50} = 1.8 μM). A model describing the interaction of these compounds with the active site is proposed.

substance P / substance P analogue proteolysis / substance P degrading enzyme / inhibitors / peptide hydroxamic acids / active site of substance P degrading enzyme

Introduction

Substance P (SP), an undecapeptide with the sequence H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, is a putative neurotransmitter or neuro-modulator [1–7]. It has been shown that the C-terminal hexapeptide fragments and analogues are equipotent to the parent compound in most bioassays [4–7]. For example, the hexapeptide [pGlu^6]SP (**6–11**) is more potent than SP in contracting the guinea pig ileum (GPI) and in depolarizing spinal cord motoneurons and it is equipotent to SP in inducing K^+ release from rat parotid slices [4–7]. The transient nature of most *in vitro* and *in vivo* pharmacological effects stimulated by SP and [pGlu^6]SP (**6–11**) is in part due to fast proteolytic degradation by various enzymes. Lee *et al* [8] have reported the isolation and partial purification of a membrane-bound metalloendopeptidase from human brain, which cleaves SP between Phe^8 and Gly^9 with minor cleavage sites between Gln^6–Phe^7, and Phe^7–Phe^8. The characterization of the degradation patterns of [pGlu^6]SP (**6–11**) by rat parotid and hypothalamus slices has recently been reported and presents a similar cleavage pattern to that found for the human brain enzyme [9].

It has been reported that other membrane-bound brain metalloendopeptidases like neutral endopeptidase EC.3.4.24.11 converting enzyme (ACE) are capable of cleaving SP, yet it is not clear at present whether these peptidases also play significant roles in the inactivation of SP at the synaptic level [10, 11]. In order to extend the duration of SP activity, the following alternative approaches are applicable:

– the preparation of metabolically stable analogues which retain biological activity;

– the design and synthesis of potent specific inhibitors which would thus selectively block enzymatic inactivation.

In one of the most interesting examples of the application of the first approach, a new series of metabolically stable SP analogues were synthesized,

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Abbreviations: DCC: *N,N*-dicyclohexylcarbodiimide; DCU: *N,N'*-dicyclohexylurea; DMF: *N,N*-dimethylformamide; EtOAc: ethyl acetate; FAB-MS: fast atom bombardment mass spectrometry; HOBt: *N*-hydroxybenzotriazole; HONSu: *N*-hydroxy-succinimide; HPLC: high performance liquid chromatography; IBCF: isobutylchloroformate; IBH: iodo-Bolton-Hunter, desamino-iodo-tyrosyl-; NMM: *N*-methyl morpholine; -ONSu: succinimide ester; TLC: thin-layer chromatography; TFA: trifluoroacetic acid.

which led to the classification of third tachykinin receptor subtype designated as SP-N [12] and presently known as NK-3 [13].

Examples of the second approach are the various extremely potent protease inhibitors which have been reported recently. Among them are inhibitors of renin [14], ACE [15], neutral endopeptidase EC.3.4.24.11 [16] and others, some of which have promising therapeutic properties.

In the present work we prepared inhibitors of SP-degradation activity based on the knowledge of the main substrate cleavage sites [9].

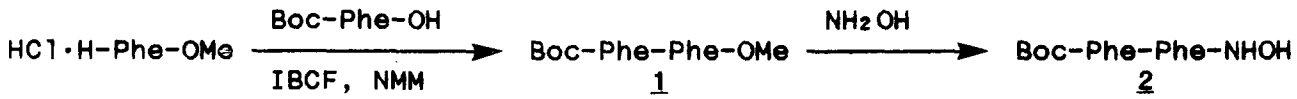
A structure–activity relationship study was carried out to identify the structures which had the best possible fit in the active site of the enzyme. The starting point was the delineation of a minimal sequence of the parent compound (SP) which had noticeable inhibitory effect on the degradation of SP. The protected tripeptide Boc-Phe-Phe-Gly-OEt was identified as a lead for further structural manipulation. On this basis a series of analogues consisting of short peptide sequences with a C-terminal hydroxamate group (an effective metal chelator present in known metalloprotease inhibitors) [17] was synthesized and their inhibitory effects were tested. The biological effects of some of these compounds have been previously reported briefly [18]; in the present paper we described in detail the synthetic pathways that led to the preparation of this family of compounds and their biological activities.

Results and discussion

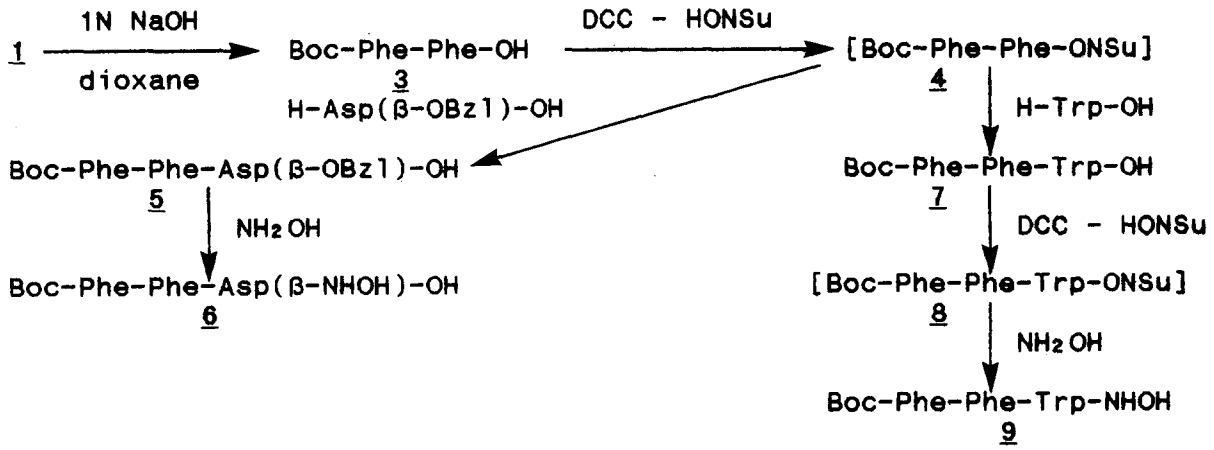
Synthesis

A series of short peptide analogues based on the sequence Phe–Phe were prepared. The general synthetic strategy called for the preparation of several ester precursors which reacted with hydroxylamine to give the desired products. Compound **1** (scheme 1) provided a convenient starting material for this purpose, and it was easily converted to the hydroxamic acid **2**.

Scheme 2 describes the preparation of the precursor compound **3** obtained by saponification of **1**. The



Scheme 1.



Scheme 2.

former protected dipeptide served as a common intermediate for the synthesis of 2 analogues **6** and **9**.

Analogue **6** was prepared by reacting the β-protected aspartic acid with the *N*-hydroxysuccinimide ester **4**, formed *in situ*. The reaction of the β-benzyl ester group of tripeptide **5** with hydroxylamine gave the monohydroxamic acid derivative. The same strategy was used to prepare compound **9**. The last step in the synthesis of the latter compound involved the activation of the carboxyl group of Trp and its conversion into the corresponding *N*-hydroxysuccinimide ester *via* DCC.

Scheme 3 summarizes the preparation of the 6 analogues **12a–f**.

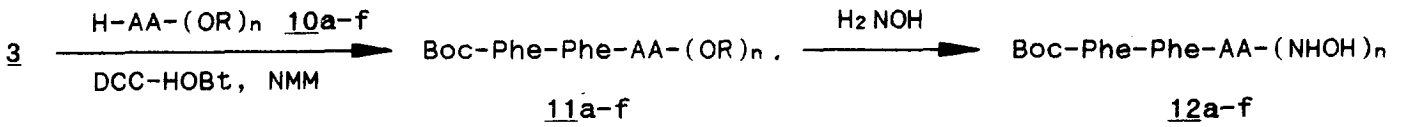
These were obtained by coupling the corresponding amino acid alkyl ester **10a–f** to the *N*-protected dipeptide **3** by the use of DCC and HOBt in DMF. The anticipated final products **12a–f** were obtained by reacting the protected tripeptide esters **11a–f** with an excess of hydroxylamine. The tetrapeptide hydroxamate **14** was prepared as described in scheme 4 starting from the intermediate **11a**. The iodo-Bolton–Hunter-tripeptide hydroxamate (compound **22**) was obtained by following the synthetic pathway outlined in scheme 5.

The physical constants of all final products can be found in table I. Reversed-phase HPLC analysis of all compounds listed in table I and their precursors did not reveal any 'ghost' peaks which could arise from diastereomeric mixture produced upon racemization during the course of the synthesis.

Biological activity

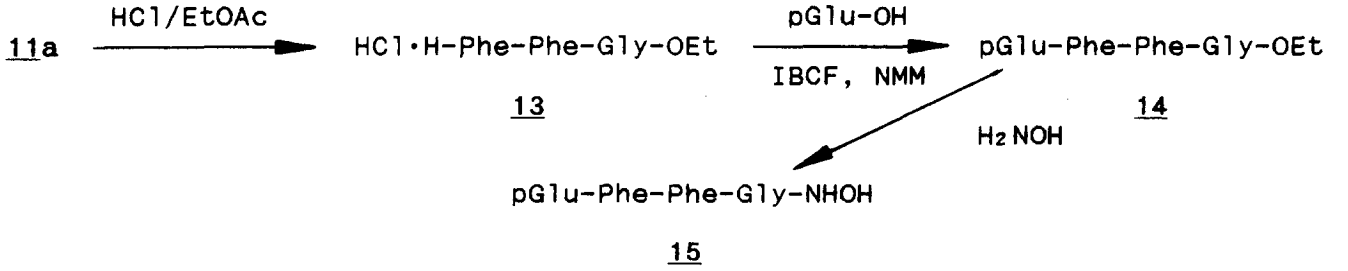
A fast screening method which was recently developed [19], allowed us to evaluate the inhibitory activities of the analogues prepared in the course of this work.

Briefly, the compounds to be assayed were incubated with rat diencephalon membrane preparations in the presence of [(^{125}I)IBH^5] SP (**6–11**) and the degradation rate of the radioiodinated substrate was monitored. This was achieved by using an ion-exchange

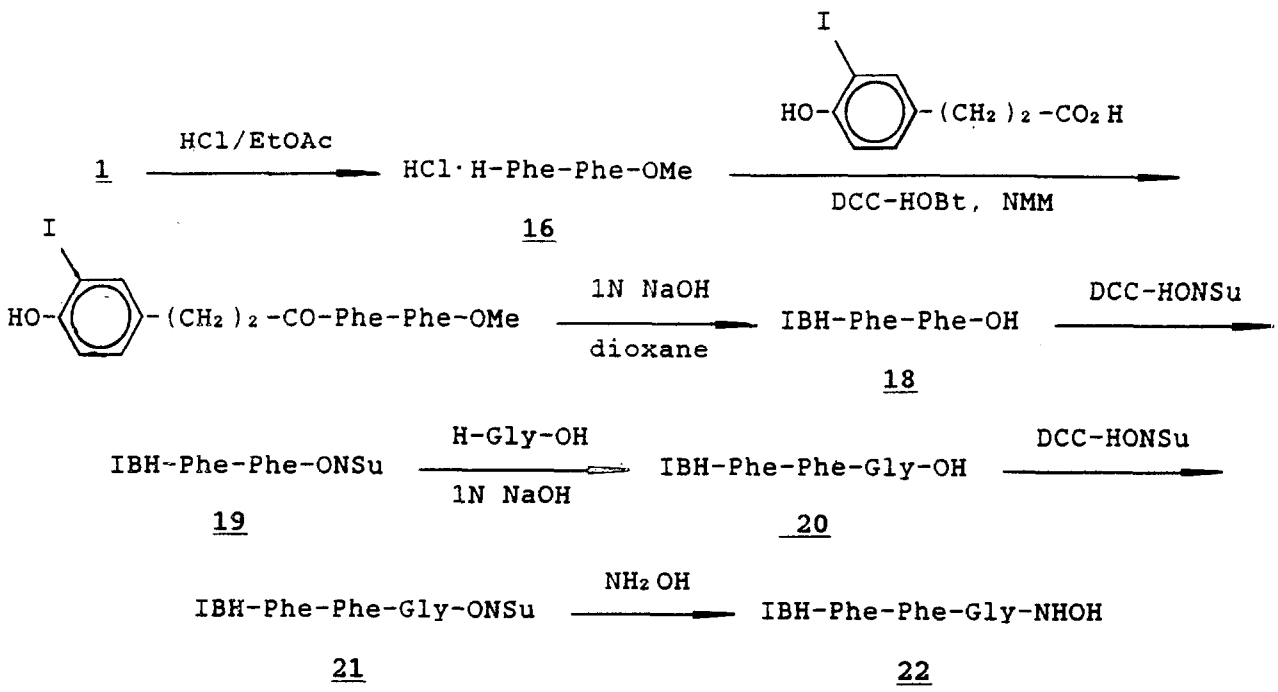


10-12	AA	R	n
a	Gly	Et	1
b	β-Ala	Me	1
c	GABA	Me	1
d	Phe	Me	1
e	$N(CH_2CO_2)_2$	Me	2
f	$NH-CH(CO_2)_2$	Et	2

Scheme 3.



Scheme 4.



Scheme 5.

chromatographic separation of the intact substrate from its proteolytic fragments, and quantitation of the remaining substrate associated radioactivity.

Substance P and short protected peptides related to the C-terminal sequence of substance P were tested as inhibitors of the degradation of [(^{125}I-BH] SP (**6–11**) in rat hypothalamus slice preparation [18].

These compounds were tested to identify the minimal sequence related to the C-terminal segment of the parent peptide which has inhibitory activity. The foregoing results provided a rational basis for the design of inhibitors of SP degradation. It was found that SP and some C-terminal fragments inhibit degradation of the substrate with an apparent K_i value of

Table I. Characterization of peptide hydroxamic acids synthesized in the present study.

Compound	*mp (°C)*	*TLC (R_f, eluent system)* [a]	*HPLC (k')*	*FAB MS [M + H]$^+$*
2	182–189	0.6 (a), 0.6 (d), 0.74 (e)	4.07 [b]	427
6	107–111	0.39 (a), 0.63 (c), 0.41 (f), 0.58 (g)	2.65 [d]	543
9	90–95	0.5 (a), 0.56 (b)	2.80 [d]	614
12a	92–97	0.19 (f), 0.76 (g)	1.76 [c]	485
12b	144–150	0.72 (b), 0.70 (c)	2.91 [d]	499
12c	63–64	0.84 (g)	2.69 [d]	513
12d	156–158	0.63 (b)	6.53 [d]	575
12e	108–112	0.3 (b), 0.74 (c)	1.83 [c]	558
12f	102–107	0.24 (b), 0.69 (c)	3.19 [c]	544
15	– [e]	–	3.22 [f]	496
22	181–183	0.4 (b)	4.07 [f]	663

[a]Spots gave a positive $FeCl_3$ reaction. The solvent systems used are described in the *Experimental protocols*. [b]Methanol/water, 0.05% TFA: 60/40 RP18. [c]Methanol/water, 0.05% TFA: 70/30 RP18. [d]Methanol/water, 0.05% TFA: 70/30 RP8. [e]Semi-solid. [f]Methanol/water, 0.05% TFA: 60/40 RP8.

around 200 μM. Peptides which do not have 2 consecutive phenylalanine residues (*eg* Boc-Phe-Gly-Leu-Met-NH_2 and Boc-Gly-Leu-Met-NH_2) were totally inactive. The shortest sequence that inhibited degradation with K_i values of the order of magnitude achieved by SP was Boc-Phe-Phe-Gly-OEt (K_i = 200 μM). Since it was found [8, 9] that the enzyme degrading SP is a metalloendopeptidase, we prepared analogues of part of the SP C-terminal sequence which contained a metal coordinating hydroxamic acid moiety close to the aromatic hydrophobic residues participating in the enzymatic recognition process. The inhibitory characteristics of these compounds are summarized in table II.

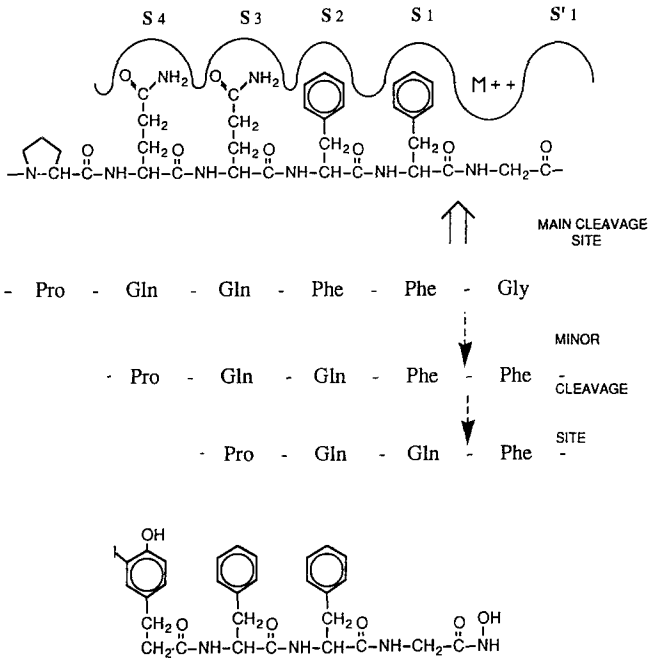
The scale of inhibitory potencies of the hydroxamic acids roughly parallels that of their ester precursors, the former being 2 orders of magnitude more active. For example, the rank order of inhibitory potencies at 200 μM of Boc-Phe-Phe-OMe (**1**) pGlu-Phe-Phe-Gly-OEt and Boc-Phe-Phe-Gly-OEt (**14**) was 16, 48 and 52%, respectively. This order parallels that of the EC_{50} of the corresponding hydroxamates (see table II). The effectiveness of Boc-Phe-Phe-Gly-NHOH as an inhibitor was not noticeably improved by elongation of branching of the backbone bearing the hydroxamic acid moiety. Inhibition was enhanced by replacement of the Gly residue by aromatic residues such as Phe or Trp. Replacement of the *N*-terminal Boc group of **12a** by a desamino-3-iodo-tyrosyl moiety further increased the inhibitory potency (compound **22**), whereas its replacement by a pyroglutamyl residue (compound **15**) proved deleterious towards inhibition.

Further improvement in inhibitory activity was achieved by combining both structural features, *ie*, 3 consecutive aromatic residues and a C-terminal glycine hydroxamate residue (analogue **22**).

Table II. Effect of hydroxamic acids derived from short fragments related to substance P C-terminal sequence on the degradation of [^{125}I-HB]SP (**6–11**) by a particulate fraction from rat diencephalon.

No	*Peptide*	IC_{50} *[μM]*
2	Boc-Phe-Phe-NHOH	58
12a	Boc-Phe-Phe-Gly-NHOH	13
12b	Boc-Phe-Phe-β-Ala NHOH	27
12c	Boc-Phe-Phe-GABA-NHOH	40
6	Boc-Phe-Phe-Asp(β-NHOH)OH	100
12e	Boc-Phe-Phe-N($CH_2CONHOH)_2$	25
12f	Boc-Phe-Phe-NH-CH($CONHOH)_2$	25
12d	Boc-Phe-Phe-Phe-NHOH	4
9	Boc-Phe-Phe-Trp-NHOH	4
15	pGlu-Phe-Phe-Gly-NHOH	35
22	IBH-Phe-Phe-Gly-NHOH	1.9

A hypothetical model of the active site of the SP-degrading peptidase is proposed based on structure–activity studies with SP related peptides and on the reported substrate specificity of this enzyme (scheme 6).



Scheme 6.

According to this model, subsites S_1, S_2, S_3, S_4 and possibly also S'_1 show a preference for aromatic or hydrophobic side-chains. The decreasing order of potencies in the inhibitor series IBH-Phe-Phe-Gly-NHOH (IC_{50} = 1.9 μM), Boc-Phe-Phe-Phe-NHOH or Boc-Phe-Phe-Trp-NHOH (IC_{50} = 4.0 μM), Boc-Phe-Phe-Gly-NHOH (IC_{50} = 13 μM) and Boc-Phe-Phe-NHOH (IC_{50} = 58 μM) is consistent with the model of interaction with the peptidase, involving binding of several consecutive aromatic amino acid side-chains to hydrophobic subsites on the enzyme, and coordination of the hydroxamic acid group with a metal ion at the active site.

Ongoing studies attempt to further improve the inhibitory potency of peptide derivatives related to IBH-Phe-Phe-NHOH by refining structural details considered to play a role in the interaction with the active site of peptidases related to SP degradation.

Experimental protocols

Melting points were measured on a Thomas–Hoover capillary melting point apparatus and are uncorrected. TLC was performed on silica gel plastic sheets by Macherey Nagel Co, Polygram Sil NH-S/UV 254 or RP18 precoated glass plates F 254 from Merck.

Elution systems were: a) CH_2Cl_2:MeOH 9:1; b) CH_2Cl_2:MeOH 4:1; c) CH_2Cl_2:MeOH 1:1; d) CH_2Cl_2:MeOH:HOAc 17:2:1; e) *n*-BuOH:pyridine–10% HOAc 5:3:11; f) MeOH:H_2O 7:3 (for RP18 plates); g) MeCN:H_2O 7:3 (for RP18 plates).

Visualization was carried out by the following means: 1), exposure to UV light at 254 nm; 2), ninhydrin 0.1% spray in EtOH; 3), fluorescamine 0.1% in acetone and exposure to UV light at 365 nm; 4), spray solution (a) 1% *t*-BuOCl in cyclohexane, spray solution (b) a 1:1 (v/v) mixture of 1% nBu_4NI (in MeOH) and 1% *o*-toluidine (in 50% aqueous MeOH); 5), $FeCl_3$, 1% solution in 1 N HCl. All final products were run in duplicate plates, one always being sprayed with the $FeCl_3$ reagent (5), and the second being subjected to the other visualization methods. A positive $FeCl_3$ reaction indicating the presence of the hydroxamic group was evidenced by the appearance of a deep purple spot on the TLC sheet.

HPLC was performed on a Spectra Physics SP 8000 or a Merck–Hitachi 655 LC system using the following columns: Merck Hibar Lichrosorb RP18 (7 μm mean particle size, 0.4 cm x 25 cm) or Lichrospher RP8 (5 μm mean particle size, 0.4 cm x 12.5 cm).

MS was performed by Dr K Eckhart at the laboratory of Prof H Schwartz, Technische Universitaet, Department of Chemistry, Berlin, using a FAB ion source. Elemental analyses were obtained at the Microanalytical Laboratory of the Institute of Chemistry of the Hebrew University.

Amino acid analyses were performed on an LKB 4400 apparatus coupled to a Spectra Physics SP 4100 printer plotter computing integrator, using 4 component sodium buffer systems and a standard 54-min program. Hydrolysis of samples for amino acid analysis was carried out on a 0.5-mg scale with constant boiling HCl solution (0.5 ml) which was degassed, sealed at high vacuum and heated for 20 h at 110°C. The hydrolysate was dried over KOH pellets at high vacuum and diluted in 0.5 ml of sodium citrate buffer (pH 2.2).

Materials

Phosphoramidon was purchased from the Peptide Research Institute (Osaka, Japan). Captopril was graciously provided to us by Dr MA Ondetti (Squibb Institute, Princeton, NJ) and [^{125}I-BH]SP (**6–11**) was prepared according to previously published procedures [18].

General coupling procedures

Method A. The mixed anhydride coupling was performed as described before [22].

Method B. The DCC mediated couplings were performed as previously described [22].

Method C. The *N*-protected amino acid or peptide (1 equiv) was dissolved in dioxane or DMF and stirred under cooling with DCC and HONSu (1 equiv) for a few hours. DCU was filtered off and to the filtrate was added an aqueous solution of the amino acid sodium salt (3 equiv). The mixture was left stirring at room temperature overnight. The solvent was partially evaporated and the aqueous residue was washed once with EtOAc which was subsequently discarded, and then the alkaline phase was brought to pH 2 with $KHSO_4$ (saturated solution). The aqueous phase was then extracted with EtOAc 3 times, this was washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure, to yield the desired products.

General deprotection procedure

This was carried out following known methods [22].

Boc-Phe-Phe-OMe ***1***

Boc-Phe-OH (15.9 g, 0.06 mol) and HCl·H-Phe-OMe (8.6 g, 0.04 mol) were reacted according to *Method A*. The dried product weighed 15.2 g (59.6%, mp 118°C). TLC: Rf 0.83 (c), 0.18 (f), 0.32 (g). Anal $C_{24}H_{30}N_2O_5$ (C, H, N).

Boc-Phe-NHOH ***2***

Compound **1** (0.42 g, 1.0 mmol) in EtOH abs solution (3 ml) containing NaOH (40 mg), was stirred for 2 days with a solution of NH_2OH in EtOH abs (6.4 ml). This solution was prepared by mixing NaOH (2.0 g) and NH_2OH·HCl (3.5 g) in EtOH abs for 3.5 h, followed by filtration of NaCl. A precipitate was formed which was collected and washed with cold 1 N HCl and ice water. After drying the product weighed 0.22 g (51.5%). Anal $C_{23}H_{29}N_3O_5$ (C, H, N).

Boc-Phe-Phe-OH ***3***

Compound **1** (5.0 g, 11.7 mmol) was dissolved in a water: dioxane 1:1 solution (100 ml). A 1 N NaOH solution (23 ml) was added to this and the mixture was stirred for 2 h at room temperature. The solvents were evaporated under reduced pressure and the residue was partitioned between water and EtOAc. After phase separation the organic layer was discarded and the aqueous phase was brought to pH 3 with a saturated $KHSO_4$ solution, and this was extracted into EtOAc. After drying the organic phase over $MgSO_4$ it was filtered and the solvent was evaporated at reduced pressure. The residue was dried at high vacuum over P_2O_5 for 2 days. It weighed 4.43 g (93%) mp 93–98°C. HPLC–RP18: k' (MeOH/H_2O, %) 5.0 (60/40), 2.3 (70/30). Anal $C_{23}H_{28}N_2O_5$ (C, H, N).

Boc-Phe-Phe-Asp(β-NHOH)-OH ***6***

Compound **4** (prepared *in situ* from dipeptide **3** and HONSu using DCC activation in dioxane) (0.3 g, 0.59 mmol) and H-(β-Bzl)Asp-OH (0.14 g, 0.65 mmol) were coupled according to general *Method C* in dioxane–1 N NaOH. The isolated product **5** weighed 0.14 g (38.4%). The partially blocked tripeptide **5** (0.1 g, 0.16 mmol) was stirred with a 1 M solution of NH_2OH in EtOH (0.48 ml, prepared as described above) with the addition of a solution of NaOH (0.3 mg) in EtOH (2 ml). The product was isolated as in **2** and after drying it weighed 40 mg (46%). Anal $C_{27}H_{34}N_4O_8$ (C, H, N).

Boc-Phe-Phe-Trp-NHOH ***9***

Compound **4** (0.14 g, 0.35 mmol) and H-Trp-OH (80.0 mg, 0.39 mmol) reacted according to general *Method C* in dioxane–1 N NaOH, giving **7** (60 mg, 0.1 mmol). This compound was initially treated with HONSu (10 mg, 0.1 mmol) and DCC (20 mg, 0.2 mmol) in DMF (5 ml), for 1.5 h. After filtration of DCU a solution of NH_2OH·HCl (14 mg, 0.2 mmol) in DMF (5 ml) containing Et_3N (14 mg, 0.2 mmol) was added, and the mixture was stirred overnight at room temperature. The solvent was evaporated at reduced pressure, the residue was mixed with EtOAc and this was washed twice with a saturated solution of $KHSO_4$. The solvent was evaporated after drying over $MgSO_4$; the dry product weighed 30 mg (49%). Anal $C_{34}H_{39}N_5O_6$·1.25 H_2O (C, H, N).

Boc-Phe-Phe-Gly-NHOH ***12a***
Compound **3** (0.68 g, 1.6 mmol) reacted with HCl·H–Gly–OEt (**10a**) (0.23 g, 1.6 mmol) according to general *Method B*. The product **11a** (0.48 g, 0.96 mmol) reacted with NH_2OH (1 M solution in EtOH) as described for **2**. The isolated product weighed 0.24 g (51.6%). Anal $C_{25}H_{32}N_4O_6 \cdot H_2O$ (C, H, N).

Boc-Phe-Phe-β-Ala-NHOH ***12b***
Compound **11b** was prepared by applying *Method B* to compound **3** (1.0 g, 2.4 mmol) and HCl·H–β-Ala-OMe (**10b**) (0.37 g, 2.4 mmol) in DMF (50 ml). Part of the product **11b** (0.6 g, 1.1 mmol); total yield: 0.88 g, 72%) was reacted with NH_2OH (1 M solution as in **2**). Work-up of the product followed the method as described for **2** yielded after drying 0.4 g (74%) of material. Anal $C_{26}H_{34}N_4O_6 \cdot 5\ H_2O$ (C, H, N).

Boc-Phe-Phe-GABA-NHOH ***12c***
Compound **11c** was prepared by using general *Method B* in DMF (5 ml). The coupling of compound **3** (0.7 g, 1.69 mmol) to HCl·H–GABA-OMe (**10c**) (0.26 g, 1.69 mmol) was carried out in DMF (5 ml). Of the product obtained **11c** (total yield: 0.68 g), 0.5 g (0.98 mmol) were reacted as described for compound **2** with a 0.7 M solution of NH_2OH in EtOH abs (4.03 ml) containing NaOH (58 mg). Yield 0.21 g (42%). Anal $C_{27}H_{36}N_4O_6$ (C, H, N).

Boc-Phe-Phe-Phe-NHOH ***12d***
Compound **3** (0.34 g, 0.82 mmol) was reacted with HCl·H-Phe-OMe (**10d**) (0.17 g, 0.82 mmol) according to general *Method B*, in DMF (3 ml), yielding 0.32 g (68%) of **11d**. This material (0.3 g, 0.5 mmol) was reacted with $HCl \cdot NH_2OH$ (0.73 g, 10 mmol) in DMF (10 ml), containing Et_3N (1.73 g, 12 mmol). After 4 days of stirring the solvent was removed under reduced pressure and the residue was partitioned between EtOAc and a saturated solution of $KHSO_4$. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. Cooling for several days at 4°C yielded a precipitate which was collected and dried. Weight: 0.18 g (63%).

Boc-Phe-Phe-N(CH_2*-CONHOH)*$_2$ ***12e***
Compound **3** (1.0 g, 2.4 mmol) was coupled to HCl·H-N(CH_2-CO_2CH_3)$_2$ (**10e**) (0.48 g, 2.4 mmol) in DMF (5 ml) according to general procedure B, yielding 0.59 g of **11e** (44%). This compound (0.3 g, 0.54 mmol) was reacted with 1 M EtOH solution of NH_2OH (1.62 ml, 1.62 mmol) as described for **2**. The product weighed 0.22 g (73%) after isolation and drying.

Boc-Phe-Phe-NHCH(CONHOH)$_2$ ***12f***
Compound **3** (1.0 g, 2.4 mmol) was coupled to $HCl \cdot H_2N$–$CH(CO_2C_2H_5)_2$ (**10g**) (0.51 g, 2.4 mmol) according to general *Method B* in DMF (5 ml). The product **11f** weighed 1.3 g (95%). Part of **11f** (0.6 g, 1.05 mmol) reacted with NH_2OH (3.1 ml in EtOH, 3.1 mmol) as described for **12e**. The product of this reaction which was isolated as described for **2** weighed 0.33 g (58%).

IBH-Phe-Phe-Gly-NHOH ***22***
The Boc protecting group was removed from compound **1** (1.8 g, 4.2 mmol) according to the standard procedure. The product **16** (1.32 g, 3.6 mmol) was coupled to 3-(*p*-hydroxy-*m*-iodo-phenyl) propionic acid (IBH–OH) [20] (1.06 g, 3.6 mmol) in DMF (10 ml) according to general *Method B*. The product **17** (1.47 g, 2.45 mmol) was saponified and isolated as described for **3** yielding 0.93 g (1.59 mmol) of **18**. This compound reacted with H·Gly–OH (0.13 g, 1.7 mmol) according to general *Method C* in dioxan-1 N NaOH to give **20** (0.46 g, 46%). Part of **20** (0.17 g, 0.26 mmol) reacted with HONSu (30 mg, 0.26 mmol) and DCC (54 mg, 0.26 mmol) in DMF (10 ml) for 2 h. After filtration of DCU, $NH_2OH \cdot HCl$ (22 mg, 0.31 mmol) was added followed by Et_3N (0.043 ml, 0.31 mmol) and the mixture was stirred for 3 days. After solvent removal at high vacuum, the residue was mixed with a saturated solution of $KHSO_4$ and with EtOAc. The aqueous layer was separated and extracted again with EtOAc. The organic phases were combined and extracted once with brine and then dried over $MgSO_4$, filtered and evaporated. The residue was precipitated from EtOAc: petroleum ether, and then from MeOH:H_2O. The product obtained weighed 0.13 g (76.5%). Anal $C_{29}H_{31}N_4O_6I \cdot 0.5\ H_2O$ (C, H, N). Amino acid analysis: Gly: 1, Phe: 2.

pGlu-Phe-Phe-Gly-NHOH ***15***
Compound **11a** (0.13 g, 0.3 mmol) was *N*-deprotected by the standard procedure. The product thus obtained, **13** (0.12 g, 0.27 mmol) was coupled to pGlu-OH (53 mg, 0.4 mmol) according to the general *Method A* in DMF (1 ml). The product of this reaction, **14** (30 mg, 0.059 mmol) was reacted with a 1 M solution of NH_2OH in EtOH (0.25 ml, 0.25 mmol) as described for **2** with the addition of NaOH (0.24 mg in 0.5 ml EtOH). The mixture was stirred overnight at room temperature. The solvent was evaporated at reduced pressure, and the residue was partitioned between a saturated solution of $KHSO_4$ and EtOAc. The phases were separated and the aqueous solution was extracted twice more with EtOAc. The organic phases were combined, washed with brine, dried over $MgSO_4$ and evaporated. The product was a semi-solid mass after drying, weighing 17 mg (60%). Amino acid analysis: Gly: 1, Phe: 2, Glu: 1.

Biological assays

Rat diencephalon membrane preparation
Diencephalons from 7 male albino rats were homogenized in 23 ml of ice-cold Hepes buffer (50 mM, pH 7.4), with 10 strokes of a Teflon-glass homogenizer at 800 rpm, and centrifuged at 100 *g* for 10 min. The supernatant was recentrifuged at 17 000 *g* for a further 10 min. The resulting pellet was suspended with a Dounce Homogenizer in cold buffer and centrifuged again at 17 000 *g* for 10 min. The final pellet was washed with cold buffer and resuspended in 5 ml of buffer (1–3 ml of protein).

Assay of peptidase acting on the C-terminal sequence of SP
The degradation of *N*-([^{125}I]-desaminoiodotyrosyl)SP (**6–11**) by a rat diencephalon membrane preparation and its inhibition by the above-described compounds was assayed as outlined in a previous publication [19]. High concentrations (10^{-6} M each) of phosphoramidon and captopril, potent inhibitors displaying affinities in the nanomolar range toward angiotensin converting enzyme and neutral endopeptidase EC.3.4.24.11, respectively [10, 20], were included in the assay in order to detect SP degrading activities distinct from these 2 enzymes. The IC_{50} values of all compounds were determined as described previously [18].

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