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Synthesis and biological activities of neurokinin pseudopeptide analogues containing a reduced peptide bond

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Summary — A series of pseudopeptides, analogues of neurokinin selective agonists, in which a peptide bond was replaced by a (CH_2NH) bond were synthesized. The biological activities of these compounds were determined on selective pharmacological preparations: the dog carotid artery for NK-1, the rabbit pulmonary artery devoid of endothelium for the NK-2 and the rat portal vein for the NK-3 receptors. The results reported in this study indicate that insertion of a pseudopeptide bond in various positions of these selective agonists resulted in a great decrease in potency compared to the parent compounds. Furthermore, the selectivity of agonists is maintained by the use of a methylene amino group in position 9–10 (Sar) for the NK-1 or in position 7–8 (MePhe) for the NK-3 selective compound. The selectivity is greatly diminished for the NK-2 analogues.

Résumé — Synthèses et activités biologiques de pseudopeptides analogues des neurokinines contenant une liaison amide réduite. *Plusieurs analogues des agonistes sélectifs des neurokinines ont été synthétisés. Dans ces composés, l'une des liaisons amides a été remplacée par une liaison isostère (-CH_2NH-). Les activités biologiques ont été mesurées sur des préparations sélectives de chaque sous-type de récepteur: l'artère carotide de chien pour NK-1, l'artère pulmonaire de lapin dépourvue d'endothélium pour NK-2 et la veine porte de rat pour NK-3. Les résultats montrent que l'insertion d'une liaison pseudopeptidique dans diverses positions des agonistes sélectifs conduit généralement à une diminution de l'activité. La sélectivité est relativement conservée lorsque le groupement méthylène-amino est situé en position 9–10 (Sar) pour l'agoniste NK-1 ou en position 7–8 (MePhe) pour le composé sélectif NK-3. La sélectivité est de beaucoup diminuée pour les agonistes NK-2.*

neurokinin agonists / pseudopeptides / reduced peptide bond / structure–activity studies / selective preparations

Introduction

The 3 neuropeptides, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are widely distributed in the central and peripheral nervous system of mammals. These neurokinins (fig 1) share the same C-terminal sequence -Phe-X-Gly-Leu-Met-NH_2, and are involved in many important physiological functions exerting their effects by activating 3 distinct receptor types NK-1, NK-2 and NK-3 [1].

	1	2	3	4	5	6	7	8	9	10	11
SUBSTANCE P	H-Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met-NH_2
	1	2	3	4	5	6	7	8	9	10	
NEUROKININ A	H-His	Lys	Thr	Asp	Ser	Phe	Val	Gly	Leu	Met-NH_2	
NEUROKININ B	H-Asp	Met	His	Asp	Phe	Phe	Val	Gly	Leu	Met-NH_2	

Fig 1. Structures of neurokinins.

Since naturally occurring peptides are not selective enough for pharmacological, biochemical and histochemical studies, new selective agonists (fig 2) have been recently described [2, 3].

Despite the high affinity of these peptides on mono-receptor tissue preparations, the need for metabolically stable analogues for *in vivo* studies prompted us to synthesize pseudopeptides with primary structures derived from those of the most selective agonists.

We describe the synthesis by solution and solid phase methods of peptides in which some amide

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Abbreviations: DMF, dimethylformamide; DIEA, *N,N*-diisopropyl *N*-ethylamine; NMM, *N*-methylmorpholine; DCC, *N,N'*dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; EtOAC, ethyl acetate; IBCF, isobutylchloroformate. 'Ψ' has been used to represent a pseudopeptide bond according to AF Spatola (*Chemistry and Biochemistry of Amino Acids, Peptides and Proteins* (1983) (Weinstein B, ed) Marcel Dekker Inc, NY, vol 7, 268–357). Other abbreviations used are those recommended by the IUPAC–IUB Commission (*Eur J Biochem* (1984), 138, 9–37).

		n°
NK-1 receptor	Ac-Arg-Phe-Phe-Sar-Leu-Met(O_2)-NH_2	*24*
NK-2 receptor	H-Asp-Ser-Phe-Val-Gly-Leu-Nle-NH_2	*25*
NK-3 receptor	H-Asp(-Phe-Phe-MePhe-Gly-Leu-Met-NH_2)-OH	*26*

Fig 2. Selective neurokinin receptor agonists.

bonds (-CONH), more sensitive to enzymatic degradation [4] were replaced by the isosteric and stable methylene amino group (-CH_2NH) (fig 3). This modification has been successful in other studies affording potent and enzyme resistant agonists [5] or even valuable antagonists [6].

The biological activities of these peptides were evaluated on selective pharmacological preparations, the dog carotid artery (DCA) for NK-1, the rabbit pulmonary artery (RPA) devoid of endothelium for the NK-2 and the rat portal vein (RPV) for the NK-3 receptors.

Results and discussion

Synthesis

Pseudopeptides Ac-Arg-Phe-Phe-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 **9**, Asp-Ser-Phe-Val-Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2 **17** and βAsp-Phe-Phe-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2 **23** were synthesized in solution according to figures 4, 5 and 6, respectively. They were prepared stepwise by the mixed anhydride

NK-1 receptor

	n°
Ac-Arg-Phe-Phe-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2	*9*
Ac-Arg-Phe-Ψ(CH_2-NH)-Phe-Sar-Leu-Met(O_2)-NH_2	*27*

NK-2 receptor

	n°
H-Asp-Ser-Phe-Val-Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2	*17*
H-Asp-Ser-Phe-Ψ(CH_2-NH)-Val-Gly-Leu-Nle-NH_2	*28*
H-Asp-Ser-Phe-Val-Ψ(CH_2-NH)-Gly-Leu-Nle-NH_2	*29*

NK-3 receptor

	n°
H-Asp(-Phe-Phe-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2)-OH	*23*
H-Asp(-Phe-Ψ(CH_2-NH)-Phe-MePhe-Gly-Leu-Met-NH_2)-OH	*30*
H-Asp(-Phe-Phe-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2)-OH	*31*

Fig 3. Structure of neurokinin pseudopeptide analogs.

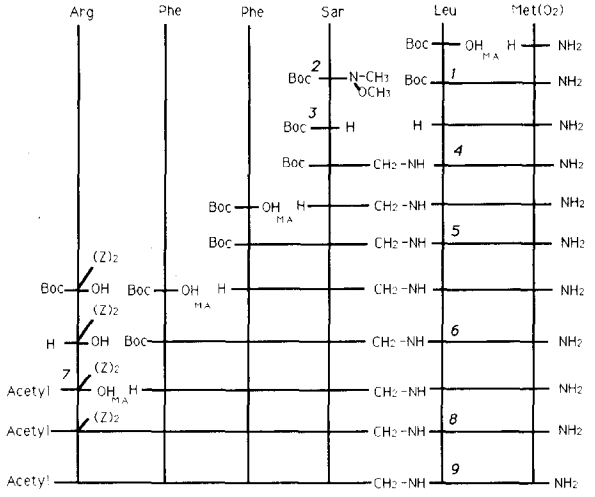

Fig 4. Solution synthesis of pseudopeptide **9**.

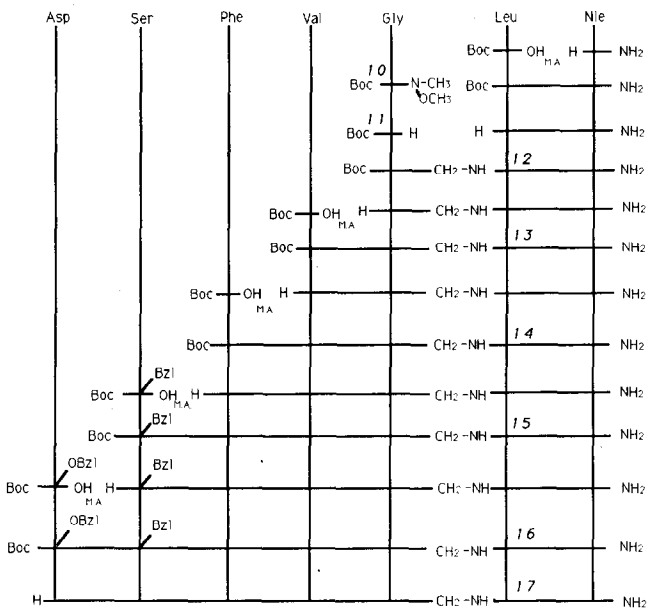

Fig 5. Solution synthesis of pseudopeptide **17**.

method [7]. The Boc-amino acid *N,O*-dimethylhydroxamates were obtained by the same method and were converted into the corresponding aldehydes, in the presence of $LiAlH_4$ [8]. The (-CH_2NH) bond was built up by condensation of the aldehydes with the α-amino function of the deprotected dipeptides in the presence of $NaBH_3CN$ as reducing reagent in a methanol–acetic acid mixture [9]. No attempt was made to isolate 'dimeric' contaminants resulting from the condensation of an excess of aldehyde.

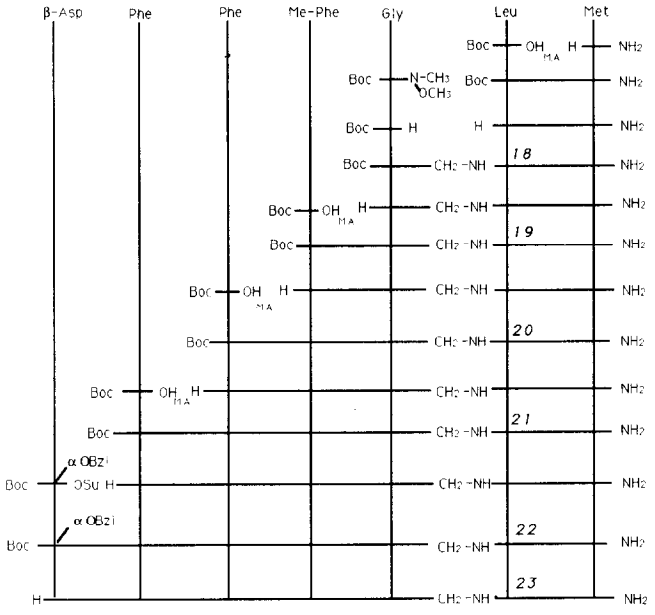

Fig 6. Solution synthesis of pseudopeptide **23**.

The pseudopeptides (**27**, **28**, **29**, **30** and **31**) were obtained by the solid phase methodology (fig 7). Boc-amino acids were coupled using the symmetrical anhydride method [10]. The coupling and deprotection steps during the synthesis were monitored by the qualitative Kaiser ninhydrin test [11]. The methylbenzhydrylamine resin (MBHA) was used to obtain final peptides with a C-terminal carboxamide group. After removal of the last Nα-Boc protecting group, the resin was dried overnight *in vacuo*. Peptides were cleaved from the resin with liquid HF in the presence of anisole and ethyl sulfide. The introduction of the Boc aldehyde was performed by the method proposed by Sasaki and Coy [12]. The crude product was purified by gel filtration on Sephadex G-10, then, by preparative HPLC with an acetonitrile gradient in 0.1% TFA. Purity of the final peptides was determined by mass spectrometry, by TLC in 2 solvent systems and by analytical HPLC. Amino acid analysis gave the expected composition although *N*-methylamino acids and amino acids with isostere bonds could not be detected.

Biological activities

The peptides were tested as agonists on 3 pharmacological preparations described as selective mono-receptor preparations:

– the dog carotid artery (DCA) with intact endothelium is very selective to SP and provides a good preparation to study the NK-1 receptor [13];

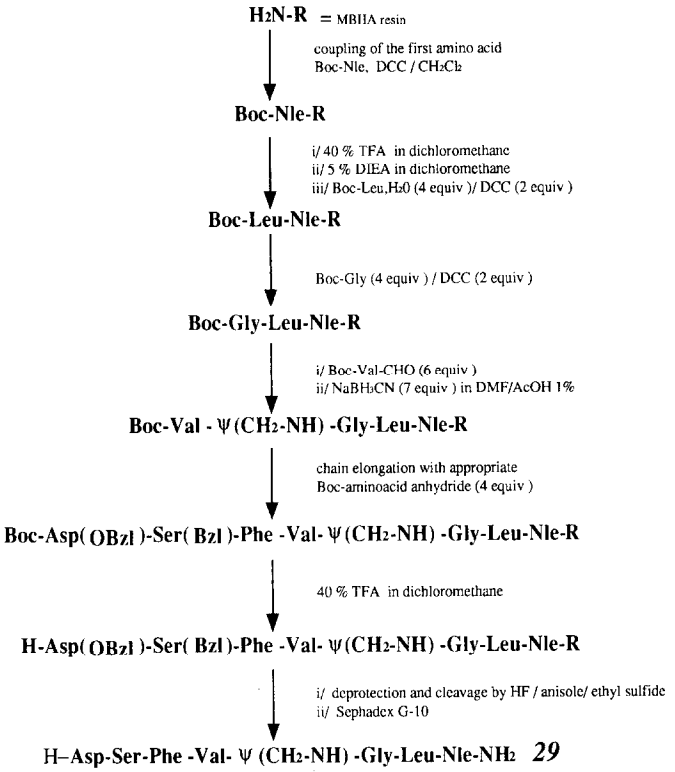

Fig 7. Synthesis of pseudopeptides by solid-phase method.

– the rabbit pulmonary artery (RPA) without endothelium is a preparation that responds by contraction to NKA and contains only the NK-2 receptor type [14];

– the third preparation, sensitive to NKB is the rat portal vein (RPV), which is considered as selective for the NK-3 receptor type [15]. The tissues were suspended in 10-ml organ baths, filled with oxygenated Krebs solution at 37°C. The changes of tension were recorded isometrically on a Grass system.

The values of pD_2 (apparent affinity evaluated from the concentration of agonists that produced 50% of the maximal effect [16]) were used to characterize and compare the various compounds. Relative affinities of agonists were calculated and displayed in percentage of those of each neurokinin in the adequate preparation. No antagonist activity could be observed with these peptides.

The results, summarized in table I, indicate that the replacement of the amide bond in the positions 7–8 (compound **27**) and 9–10 (compound **9**) in the structure of the selective NK-1 agonist decreased the affinity. Some selectivity was observed with the replacement in position 9–10 (compound **9**). These results point out the essential role played by Phe^7 for activation of the NK-1 receptor.

Table I. Biological activities of neurokinin pseudopeptide analogs on selective preparations. pD_2 (apparent affinity) = – log (EC_{50}) is evaluated from the concentration of agonist that produced 50% of the maximal effect (EC_{50}); $P < 0.05$, $n = 6$. RA = relative activity in percent of that of SP or NKA or NKB in DCA (dog carotid artery for NK-1), RPA (rabbit pulmonary artery for NK-2) and RPV (rat portal vein for NK-3), respectively.

Peptide	*No*	*DCA*		*RPA*		*RPV*	
		pD_2	*RA*	pD_2	*RA*	pD_2	*RA*
Substance P (SP)*		10.0	100	6.1	4.0	5.8	1.4
[Ac-Arg^6, Sar^9, Met(O_2)^{11}] SP(6–11)*	24	10.2	174	Inact		Inact	
[Ac-Arg^6, $^7\Psi^8$, CH_2-NH, Sar^9, Met(O_2)^{11}] SP(6–11)	27	7.3	0.2	5.2	0.1	Inact	
[Ac-Arg^6, Sar^9, $^9\Psi^{10}$, CH_2-NH, Met(O2)^{11}] SP(6–11)	9	8.7	5.8	Inact		Inact	
Neurokinin A (NKA)*		9.4	25	8.2	100	6.4	6.0
[Nle^{10}] NKA (4–10)*	25	7.0	< 1.0	7.9	49	Inact	
[$^8\Psi^9$, CH_2-NH, Nle^{10}] NKA (4–10)	17	6.1	< 1.0	5.4	0.2	Inact	
[$^6\Psi^7$, CH_2-NH, Nle^{10}] NKA (4–10)	28	6.9	< 1.0	Inact		Inact	
[$^7\Psi^8$, CH_2-NH, Nle^{10}] NKA (4–10)	29	6.1	< 1.0	6.0	< 1.0	Inact	
Neurokinin B (NKB)*		5.8	1.4	6.4	5.8	7.7	100
[βAsp^4, MePhe^7] NKB (4–10)*	26	7.0	< 1.0	5.1	< 1.0	7.6	76
[βAsp^4, MePhe^7, $^8\Psi^9$, CH_2-NH] NKB (4–10)	23	6.6	< 1.0	Inact		Inact	
[β-Asp^4, $^5\Psi^6$, CH_2-NH, MePhe^7] NKB (4–10)	30	6.7	< 1.0	Inact		6.2	3.2
[β-Asp^4, MePhe^7, $^7\Psi^8$, CH_2-NH] NKB (4–10)	31	6.6	< 1.0	Inact		6.4	5.4

*See [2].

For the NK-2 analogues, the selectivity is greatly diminished and the results show the importance of the peptide bonds in the interior positions 6–7 (compound **28**), 7–8 (compound **29**) and 8–9 (compound **17**).

In contrast, the introduction of a (-CH_2-NH-) bond in the positions 5–6 (compound **30**) and 7–8 (compound **31**) in the NK-3 analogues maintained some activity and selectivity which are abolished by the replacement in position 8–9 (compound **23**).

Insertion of a pseudopeptide bond in various positions of these selective agonists resulted in a great decrease in potency as compared to the parent compounds. These modifications were investigated with the aim of improving the stability of selective agonists to enzymic degradation [4]. They did not improve the agonist or antagonist activities. As recently reported, the introduction of a methylene amino group into [Leu^{11}] substance P in position 9–10 led to an agonist whereas the substitution in the position 10–11 led to an antagonist or to weak agonists in the other positions [17]. Similar substitutions in peptides analogs of tetragastrin also led to antagonist activities [6]. On the other hand [$^9\Psi^{10}$,CH_2-NH,Leu^{10}] neurokinin A (4–10) is an agonist for the NK-2 receptor with enhanced specificity [18]. Thus, the pseudopeptide bond replacements that result in antagonist properties seem entirely unpredictable.

The relationship between the structure and the activity and selectivity of the model selective agonists is very close, since moderate modifications can abolish both activity and selectivity. As mentioned before, the inclusion of a (-CH_2-NH-) bond in linear peptides would bring additional degrees of freedom as compared to the amide bond which may induce a favourable conformation [19, 20]. In addition, it should be postulated that a decrease of affinity and selectivity may also result from the protonation of the methylene amino group.

Experimental procedures

Solid phase synthesis (compounds **27–31**) was performed as described by Drapeau *et al* [21] in the laboratory of D Regoli. Introduction of the peptide reduced bond was performed according to Sasaki *et al* [12]. The peptides were purified by high performance liquid chromatography (HPLC). Purity criteria (FAB-MS, HPLC and TLC) are reported in table II.

Synthesis by solution method was performed as follows. Reagents and solvents used for the reactions were of analytical grade. Amino acids, all of L-configuration, were purchased from Fluka or Bachem. Boc-protected derivatives were prepared according to Keller *et al* [22]; Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) was purchased from Pierce. Melting points were taken on a Leitz apparatus and are reported corrected. Optical rotations were measured with a Perkin–Elmer 121 precision polarimeter (± 0.002°). Fast atom bombardment (FAB) (MH^+) mass spectra were performed by the Service de Spectrométrie de Masse de l'Institut de Chimie des Substances Naturelles (ICSN) on an MS 80 RF Kratos spectrometer. ^1H NMR spectra were recorded on a Brucker WB 200 spectrometer at 200 MHz or on a Brucker AM400 spectrometer at 400 MHz. Thin-layer chromatography (TLC) was performed on analytical precoated Merck silica gel 60 F_{254} plates. The following solvent systems were used (by vol): (A),

Table II. Characterization of pseudopeptides **27–31**.

Peptide	*No*	*FAB-Mass spectrum*[a]	*Elution time*[b] *(min)*	*Rf* A^c	*Rf* B^d
[Ac-Arg^6, $^7\Psi^8$, CH_2-NH, Sar^9, Met$(O_2)^{11}$] SP (6–11)	27	843	11.4	0.61	0.26
[$^6\Psi^7$, CH_2-NH, Nle^{10}] NKA (4–10)	28	735	11.4	0.60	0.41
[$^7\Psi^8$, CH_2-NH, Nle^{10}] NKA (4–10)	29	735	11.6	0.59	0.42
[β-Asp^4, $^5\Psi^6$, CH_2-NH, MePhe^7] NKB (4–10)	30	876	14.5	0.73	0.55
[β-Asp^4, MePhe^7, $^7\Psi^8$, CH_2-NH] NKB (4–10)	31	876	15.2	0.75	0.52

[a]M+H values. [b]Peptides were eluted for 2 min with isocratic conditions (95% solvent A: H_2O, TFA 0.05%; 5% solvent B: CH_3CN, TFA 0.05%), then with a linear gradient (35% solvent A and 65% solvent B in 15 min); column 218-TP104 VYDAC, C-18 reverse phase, 10 μm; flow 2 ml/min. [c]TLC in butanol / acetic acid / water / pyridine 15:3:3:10. [d]TLC in butanol / acetic acid / water 4:1:1.

EtOAc; (B), $CHCl_3$/MeOH,9:1; (C), $CHCl_3$/MeOH,8:2; (D), $CHCl_3$/MeOH/AcOH,9:1:0.5; (E), 1-BuOH/AcOH/H_2O,4:1:1. Column chromatography was performed on silica gel 60, 230–400 mesh ASTM (Merck).

Spots were detected with ninhydrin or by a spray of *o*-tolidine after chlorination [23] or with 2,4-dinitrophenylhydrazine for the aldehydes [24]. Reversed phase HPLC was performed on a SFCC C18 nucleosil (5 μm) column (4.6 mm id x 250 mm) by gradient elution with the following solvent system: A = 5% CH_3CN in H_2O and 0.1% TFA, B = 0.1% TFA in CH_3CN. The flow rate was 1 ml/min, and the detection wavelength was 254 nm.

To a stirred solution of Boc-amino acid or Boc-peptide (1 eq) in DMF was added an equivalent of NMM. The mixture, cooled to – 20°C, was allowed to react 5 min with IBCF (1 eq). A cooled solution (– 20°C) of the amino component (trifluoroacetate or hydrochloride) (1.1–1.5 eq) in DMF and NMM (1.1–1.5 eq) was added rapidly and allowed to react for 1–3 h at – 15°C. The reaction was monitored by TLC. After completion, DMF was removed under reduced pressure (5 mmHg), the crude product was dissolved in EtOAc, washed successively with a 5% aqueous sodium bicarbonate solution, H_2O, 5% aqueous citric acid solution, H_2O, and brine. The organic layer was dried over Na_2SO_4 and processed for purification by crystallization or chromatography on a silica gel column or by HPLC.

Boc-protected peptides were treated by TFA (10 eq, 40% in CH_2Cl_2), 2% anisol and 0.2% ethanedithiol, 1 h at 0°C. After evaporation of the solvents, the residue was triturated in anhydrous diethyl ether and dried *in vacuo* over KOH pellets. Purity of deprotected peptides was checked by TLC.

*Boc-Leu-Met(O_2)-NH_2 **1***

Boc-Leu, H_2O (3.75 g, 15 mmol) dissolved in DMF (30 ml), NMM (1.65 ml, 15 mmol) was reacted for 5 min at – 15°C with IBCF (1.96 ml, 15 mmol), with TFA, Met(O_2)-NH_2 (25) (4.85 g, 16.5 mmol) in DMF (20 ml), NMM (1.82 ml, 16.5 mmol) 1 h at – 15°C, then 4 h at 0°C. Completion of the reaction was monitored by TLC. The mixture was processed according to the general procedure, recrystallized from a mixture of EtOAc and hexane. Yield: 5.13 g (85%). mp = 173–174°C. $[\alpha]_D^{20}$ = – 19.0 (c 1.0, DMF). Rf(B) = 0.23. Rf(C) = 0.52. MH^+ = 394.

*Boc-Sar-N,O-dimethylhydroxamate **2***

Boc-Sar OH (3.78 g, 20 mmol) dissolved in CH_2Cl_2 (40 ml) was reacted with IBCF (2.6 ml, 20 mmol) in the presence of NMM (2.2 ml, 20 mmol) at – 15°C for 5 min. A solution of *N-O*-dimethylhydroxylamine hydrochloride (2.15 g, 22 mmol, 20 ml) was added, followed by NMM (2.3 ml, 22 mmol). The reaction mixture was stirred at – 15°C for 1 h, then at 4°C for 16 h. After evaporation, the residue was dissolved in EtOAC. The solution was washed with 5% citric acid, H_2O, 5% sodium bicarbonate, H_2O, then dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography with a mixture of EtOAc/hexane 1:1 as eluant, to yield a colorless oil. Yield: 3.79 g (82%). Rf(A) = 0.45. MH^+ = 233. ^1H NMR ($CDCl_3$) δ = 1.6 (s, 9H, Boc), 2.9 (s, 3H, – CH_3), 3.2 (s, 3H, -CH_3), 3.7 (s, 3H, O-CH_3), 4.15 (s, 2H, -CH_2-).

*Boc-sarcosinal **3***

Compound **2** (1.16 g, 5 mmol) was dissolved in anhydrous tetrahydrofuran (40 ml) and the reaction mixture was cooled down to 0°C. $LiAlH_4$ (0.96 g, 25 mmol) was added portionwise over a period of 30 min. After 2 h, no more starting material could be detected by TLC. Ether (200 ml) was added followed by a cold aqueous 20% citric acid solution (200 ml). The mixture was vigorously stirred during 30 min. The organic layer was collected and the aqueous phase extracted again with ether (3 x 100 ml). The ether extracts were pooled, washed with an aqueous 10% citric acid solution (3 x 25 ml), H_2O (25 ml), a saturated sodium bicarbonate solution (3 x 25 ml), H_2O (25 ml), brine (25 ml), and dried over anhydrous Na_2SO_4. The solution was concentrated *in vacuo* to yield a colorless oil that was used rapidly for the next reaction. Yield: 0.78 g (90%). Rf(A) = 0.57. MH^+ = 174. ^1H NMR ($CDCl_3$) δ = 1.8 (s, 9H, Boc), 3.05 (s, 3H, -N-CH_3), 4.15 (s, 2H, -CH_2-), 9.7 (s, H, -CHO).

*Boc-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 **4***

Boc-sarcosinal **3** (0.78 g, 4.5 mmol) was dissolved in a mixture of MeOH/AcOH 99:1 (20 ml) containing TFA, Leu-Met-$(O_2)NH_2$ (1.55 g, 3.8 mmol). $NaBH_3CN$ (0.477 g, 7.6 mmol) was added portionwise over a period of 30 min. After an additional 1 h, the reaction mixture was cooled in an ice-H_2O bath and a saturated sodium bicarbonate solution (150 ml) was added under stirring and extracted again with EtOAc (4 x 100 ml). The organic layer was collected, washed with H_2O (20 ml), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified on a silica gel column with CH_2Cl_2/MeOH 9:1 as eluant to yield a colorless oil. 1.35 g (79%) $[\alpha]_D^{20}$ = – 3.9 (c 1.0, DMF). Rf(B) = 0.16. Rf(C) = 0.50. MH^+ = 451.

Coupling of an excess of **3** (2 to 3 equiv) led to a product (88%) which corresponds to *N,N*-di-(Boc-N(CH_3)-(CH_2)$_2$)-Leu-Met(O2)-NH_2 as characterized by mass spectrometry (MH^+ = 608); Rf(D) = 0.55. No 'dimeric' adducts could be observed with the coupling of the other Boc-aminoaldehydes.

Boc-Phe-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 ***5***
Boc-Phe-OH (1.193 g, 4.5 mmol) dissolved in CH_2Cl_2 (20 ml), NMM (0.5 ml, 4.5 mmol) was reacted 5 min at – 1.5°C with IBCF (0.53 ml, 4.5 mmol). A solution of TFA, Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 (1.3 g, 2.8 mmol) in DMF (20 ml) was added, followed by NMM (0.3 ml, 2.8 mmol). The reaction mixture was stirred at – 15°C for 1 h, then at 4°C for 16 h. The solvents were evaporated under reduced pressure (5 mmHg). The residue was purified by silica gel column chromatography with a mixture of CH_2Cl_2/MeOH 95:5. Yield: 1.26 g (75%). mp = 103°C (dec); $[\alpha]_D^{20}$ = 14.5 (c 1.2, DMF). Rf(B) = 0.2. MH^+ = 598.

Boc-Phe-Phe-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 ***6***
Boc-Phe-OH (0.45 g, 1.7 mmol) dissolved in DMF (10 ml), NMM (0.19 ml), 1.7 mmol was reacted at – 15°C with IBCF (0.19 ml, 1.7 mmol) for 5 min. A solution of the deprotected compound **5** (0.86 g, 1.4 mmol) in DMF (5 ml) was added, followed by NMM (0.15 ml, 1.4 mmol). The reaction mixture was processed according to the general procedure. Trituration of the residue with ether yielded a white powder; 0.66 g (78%). mp = 87–90°C; $[\alpha]_D^{20}$ = –7.9 (c 1, DMF). Rf(B) = 0.28. Rf(C) = 0.58. MH^+ = 745.

N-Acetyl-Arg(Z)$_2$-OH ***7***
TFA, H-Arg(Z)$_2$-OH (1.11 g, 2 mmol) dissolved in CH_2Cl_2 (5 ml), DIEA (0.69 ml, 4 mmol) was reacted at room temperature with acetic anhydride (0.19 ml, 2 mmol) 2 h. Completion of the reaction was monitored by TLC. After evaporation the residue was purified by silica gel column chromatography with a mixture of CH_2Cl_2/MeOH 9:1 as eluant, to yield a colorless oil 0.89 g (92%). Rf(C) = 0.62. MH^+ = 485.

N-Acetyl-Arg(Z)$_2$-Phe-Phe-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 ***8***
A solution of *N*-acetyl-Arg(Z)$_2$-OH (0.29 g, 0.6 mmol) in CH_2Cl_2 (20 ml), NMM (0.07 ml, 0.6 mmol) was reacted at – 15°C with IBCF (0.07 ml, 0.6 mmol) for 5 min. A solution of the deprotected compound **6** (0.3 g, 0.4 mmol) in DMF (10 ml) was added, followed by NMM (0.05 ml, 0.4 mmol). The reaction mixture was stirred at – 15°C for 1 h, then at 4°C for 16 h. The solvents were removed under reduced pressure (5 mmHg). Trituration of the residue with ether yielded a white solid which was recrystallized from a mixture of MeOH and ether. Yield: 0.38 g (85%). Rf(D) = 0.28. MH^+ = 1111.

N-Acetyl-Arg-Phe-Phe-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 ***9***
(or [Ac-Arg^6, ^{9}Ψ^{10},CH_2-NH, Met(O_2)^{11}] SP (6–11) ***9***)
The Z protected peptide (0.167 g, 0.15 mmol) was treated with HBr (33% in AcOH, 10 eq) at 0°C for 2 h. After evaporation under reduced pressure the residue was triturated with anhydrous ether to yield a white solid which was washed with ether. The solid was purified by HPLC and a linear gradient was applied, from 0 to 100% of B over 40 min. *k'* = 4.9. Yield: 0.075 g (59%). mp = 155–159°C. $[\alpha]_D^{20}$ = – 7.3 (c 1.1, DMF). Rf(E) = 0.30. MH^+ = 843. ^{1}H NMR (DMSO-d_6) 2 rotamers, δ = 8.18 (m, 2H), 8.1–7.95 (m, 3H), 8.05 (m, 2H), 7.75 (d, 1H), 7.45 (m, 4H), 7.15 (m, 32 H), 4.95 (m, 1H), 4.81 (m, 2H), 4.5 (m, 2H), 4.42 (m, 2H), 4.2 (m, 2H), 3.6–1.15 (59 H), 0.85 (m, 12H).

A first attempt to acetylate the pseudopeptide H-Arg(Z)$_2$-Phe-Phe-Sar-Ψ(CH_2-NH)-Leu-Met(O_2)-NH_2 was unsuccessful because this reaction led also to a partial acetylation of the pseudopeptide methylene amino bond. We therefore preferred the direct coupling of *N*-acetyl-Arg(Z)$_2$-OH with deprotected **6**. This process led to a partial racemization (22%) as checked by the HPLC analysis of the hydrolysate derivatized by the Marfey's reagent [26].

Boc-Gly-N,O-dimethylhydroxamate ***10***
Boc-Gly-OH (3.5 g, 20 mmol) dissolved in CH_2Cl_2 (20 ml) was reacted with IBCF (2.6 ml, 20 mmol) in the presence of NMM (2.2 ml, 20 mmol) at – 15°C for 5 min. A solution of *N-O*-dimethylhydroxylamine hydrochloride (2.15 g, 22 mmol, 20 ml) was added, followed by NMM (2.3 ml, 22 mmol). The reaction mixture was stirred at – 15°C for 1 h, then at 4°C for 16 h. The reaction mixture was treated according to the same method as that used for **2** to yield a white powder. Yield: 3.45 g (79%). mp = 101–102°C. Rf(A) = 0.50. MH^+ = 219. ^{1}H NMR ($CDCl_3$) δ = 1.5 (s, 9H, Boc), 3.25 (s, 3H, -CH_3), 3.80 (s, 3H, – OCH_3), 4.10 (s, 2H, -CH_2), 5.30 (s, 1H, -NH).

Boc-glycinal ***11***
Boc-Gly-*N,O*-dimethylhydroxamate (4.4 g, 20 mmol) was dissolved in anhydrous tetrahydrofuran (150 ml) and the reaction mixture was cooled down to 0°C. $LiAlH_4$ (3.84 g, 100 mmol) was added portionwise over a period of 30 min. After 90 min, the reaction mixture was treated according to the same method as that used for **3**, to yield a colorless oil that was used rapidly for the next reaction. Yield: 2.63 g (82%). Rf(A) = 0.55. MH^+ = 160. ^{1}H NMR ($CDCl_3$) δ = 1.55 (s, 9H, Boc), 4.20 (s, 2H, -CH_2), 5.35 (s, 1H, -NH), 9.80 (s, 1H, -CHO).

Boc-Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2 ***12***
Boc-glycinal **11** (1.92 g, 12 mmol) was dissolved in a mixture of MeOH containing 1% AcOH (45 ml) and HCl, Leu-Nle-NH_2 [27] (2.8 g, 10 mmol). $NaBH_3CN$ (2 g, 32 mmol) was added portionwise at room temperature over a period of 30 min. After an additional 30 min, the reaction mixture was cooled in an ice–H_2O bath and a saturated sodium bicarbonate solution (200 ml) was added under stirring and extracted with EtOAc (4 x 100 ml). The organic layer was collected, washed with H_2O (3 x 20 ml), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with CH_2Cl_2/MeOH 98:2 as solvent system to yield a pure compound that gave a white powder by trituration with ether. Yield: 2.09 g (54%). mp = 91–93°C. $[\alpha]_D^{20}$ = – 1.7 (c 1.0, DMF). Rf(A) = 0.25. Rf(D) = 0.42. MH^+ = 387.

Boc-Val-Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2 ***13***
Boc-Val-OH (1.30 g, 6 mmol) dissolved in CH_2Cl_2 (20 ml) and NMM (0.66 ml, 6 mmol) was reacted at – 15°C with IBCF (0.7 ml, 6 mmol) for 5 min, then with TFA, Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2 (1.80 g, 4.5 mmol), NMM (20 ml) at – 15°C for 1 h, then at 4°C for 16 h. The reaction mixture was treated as described in the general procedure. The residue was purified by silica gel column chromatography with EtOAc as eluant, to yield a pure compound that gave a white powder by trituration with ether: 1.97 g (90%). mp = 154–155°C; $[\alpha]_D^{20}$ = – 0.5 (c 1, AcOH). Rf(B) = 0.5. MH^+ = 486.

Boc-Phe-Val-Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2 ***14***
Boc-Phe-OH (0.93 g, 3.5 mmol) dissolved in CH_2Cl_2 (20 ml), NMM (0.4 ml, 3.5 mmol) was reacted at – 15°C with IBCF (0.4 ml, 3.5 mmol) for 5 min then with TFA, Val-Gly-Ψ(CH_2-

NH)-Leu-Nle-NH_2 (1.49 g, 3 mmol), NMM (0.35 ml, 3 mmol). The reaction mixture was stirred at – 15°C for 1 h, 4°C for 16 h. It was then processed as **13**. The crude product was purified by silica gel column chromatography with CH_2Cl_2/MeOH, 85:15 as solvent system to yield a pure compound: 1.73 g (91%), mp = 198–205°C. $[\alpha]_D^{20} = -0.5$ (c 1, AcOH). Rf(B) = 0.5. $MH^+ = 486$.

Boc-Ser(Bzl)-Phe-Val-Gly-Ψ(CH_2NH)-Leu-Nle-NH_2 ***15***
Boc-Ser(Bzl)-OH (0.88 g, 3 mmol) dissolved in CH_2Cl_2 (10 ml), NMM (0.33 ml, 3 mmol) was reacted at – 15°C with IBCF (0.34 ml, 3 mmol) for 5 min, then with deprotected compound **14** (0.96 g, 1.5 mmol), NMM (0.18 ml, 1.5 mmol). The reaction mixture was stirred at – 15°C for 1 h, at 4°C for 5 h. The solvents were evaporated under reduced pressure (5 mmHg), the residue was dissolved in EtOAc (100 ml), washed with 10% aqueous citric acid solution (3 x 10 ml), H_2O (10 ml), 5% aqueous sodium bicarbonate solution (2 x 10 ml). The organic layer was dried over Na_2SO_4 and the crude product was purified by silica gel column chromatography with CH_2Cl_2/MeOH 98:2 as eluant. Yield: 0.71 g (58%), mp = 187–192°C. $[\alpha]_D^{20} = -5.5$ (c 1, AcOH). Rf(D) = 0.56. $MH^+ = 810$.

Boc-Asp(OBzl)-Ser(Bzl)-Phe-Val-Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2 ***16***
Boc-Asp(OBzl)-OH (0.323 g, 1 mmol) dissolved in CH_2Cl_2 (5 ml), NMM (0.110 ml, 1 mmol) was reacted with IBCF (0.130 ml, 1 mmol) for 5 min, then with deprotected compound **15** (0.6 g, 0.75 mmol). The reaction mixture was stirred at – 15°C for 1 h, at room temperature for 3 h. It was processed according to the general procedure. After evaporation *in vacuo* the residue was reprecipitated from MeOH/ether. Yield: 0.52 g (68%), mp = 191–195°C. $[\alpha]_D^{20} = -9.4$ (c 1, AcOH). Rf(D) = 0.5. $MH^+ = 1015$.

TFA, H-Asp-Ser-Phe-Val-Gly-Ψ(CH_2-NH)-Leu-Nle-NH_2 ***17***
(or [^{8}Ψ^9,CH_2-NH, Nle^{10}] NKA (4–10) ***17****)*
Compound **16** (0.20 g, 0.20 mmol) was dissolved in glacial acetic acid (10 ml), hydrogenated at room temperature and under atmospheric pressure for 16 h in the presence of 10% Pd/C as catalyst (0.1 g). The catalyst was removed by filtration (celite), washed with acetic acid (10 ml), and the filtrate concentrated under reduced pressure. The residue was completely deprotected by TFA according to the usual procedure. After evaporation the residue was triturated with anhydrous ether to yield a white powder which was purified by HPLC; linear gradient from 0 to 80% of B in 30 min, $k' = 4.16$. Yield: 0.09 g (63%), mp = 207–208°C. $[\alpha]_D^{20} = -5.2$ (c 1, AcOH). Rf(E) = 0.28. $MH^+ = 735$. ^{1}H NMR (DMSO–d_6); the chemical shifts are listed in table III. The assignments correspond to experiments performed at 50°C since all resonances, at lower temperature, were very broad. All the amide protons have a coupling constant between 6 and 8 Hz, except the Ser-NH that still shows up as a broad singlet at 50°C.

Boc-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2 ***18***
Boc-Glycinal **11** (0.954 g, 6 mmol) was dissolved in a mixture of MeOH containing 1% AcOH (30 ml) and HCl, Leu-Met-NH_2 (16) (1.985 g, 5 mmol). $NaBH_3CN$ (1 g, 16 mmol) was added portionwise at room temperature over a period of 30 min. After an additional 30 min the reaction mixture was treated as **12**. The crude product was purified by column chromatography on silica gel with CH_2Cl_2/MeOH, 95:5 as eluant to yield a pure compound: 1.49 g (74%), mp = 70–72°C. $[\alpha]_D^{20} = -1.6$ (c 1, DMF). Rf(D) = 0.47. $MH^+ = 405$.

Boc-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2 ***19***
Boc-MePhe-OH (0.67 g, 2.4 mmol) dissolved in CH_2Cl_2 (20 ml), NMM (0.22 ml, 2.4 mmol) was reacted at – 15°C with IBCF (0.28 ml, 2.4 mmol) for 5 min, then with deprotected **18** (0.84 g, 2 mmol), NMM (0.20 ml, 2.2 mmol) at – 15°C for 1 h, at 4°C for 16 h. The reaction was treated as described in the general procedure. After evaporation *in vacuo* the residue was crystallized from EtOAc/hexane. Yield: 1.02 g (90%), mp = 116–124°C. $[\alpha]_D^{20} = -27.0$ (c 1, DMF). Rf(D) = 0.47. $MH^+ = 566$.

Boc-Phe-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2 ***20***
Boc-Phe-OH (0.53 g, 2 mmol) dissolved in DMF (20 ml), NMM (0.22 ml, 2 mmol) was reacted at – 15°C with IBCF (0.24 ml, 2 mmol) for 5 min, then with deprotected compound **19** (0.985 g, 1.7 mmol), NMM (0.22 ml, 2 mmol) at – 15°C for 1 h, then at 4°C for 16 h. The reaction was processed as described in the general procedure. The residue which gave a white powder by trituration with ether was dried *in vacuo* over KOH. Yield: 0.86 g (71%), mp = 125–130°C. $[\alpha]_D^{20} = -35.9$ (c 1, DMF). Rf(D) = 0.34. $MH^+ = 713$.

Boc-Phe-Phe-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2 ***21***
Boc-Phe-OH (0.53 g, 2 mmol) dissolved in DMF (20 ml), NMM (0.22 ml, 2 mmol) was reacted at – 15°C with IBCF (0.24 ml, 2 mmol) for 5 min then with deprotected compound **20** (0.86 g, 1.2 mmol), NMM (0.14 ml, 1.2 mmol) at – 15°C

Table III. Assignments of the protons of **17** in DMSO–d_6 at 50°C.

Residue	*NH*	*α*	*β*	*γ*	*δ*
Asp	–	4.35	3.6	–	–
Ser	7.79	3.25	3.20/2.55	–	–
Phe	8.11	4.6	3.15/2.85	7.19 (arom)	–
Val	7.79	4.08	1.97	0.8	–
Gly	7.15	2.5	2.5	–	–
Leu	–	3.23	1.35/1.31	1.6	0.8
Nle	8.05	4.28	1.6	1.2	0.8
NH_2	7.3/6.9	–	–	—	–

for 1 h, at 4°C for 16 h. The reaction was processed as described in the general procedure. The residue was dissolved in MeOH and precipitated with ether to give a white powder, which was rinsed several times with ether, and dried *in vacuo* over KOH. Yield: 0.97 g (94%). Rf(D) = 0.47. MH^+ = 860.

Boc-Asp(-Phe-Phe-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2)-OBzl ***22***

Deprotected **21** (0.4 g, 0.46 mmol) was dissolved in DMF (10 ml) in the presence of Boc-Asp(OSu)-OBzl (0.25 g, 0.6 mmol) [28] and DIEA (0.17 ml, 1 mmol). The reaction mixture was stirred for 5 h at room temperature. The solvent was concentrated under reduced pressure. The residue was dissolved in EtOAc, washed with a saturated sodium bicarbonate solution, H_2O, 10% aqueous citric acid solution and H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography with CH_2Cl_2/MeOH 99:1 as eluant. Yield: 0.25 g (52%), mp = 94–102°C. $[\alpha]_D^{20}$ = – 49.8 (c 1, DMF). Rf(B) = 0.25. MH^+ = 1065.

H-Asp(-Phe-Phe-MePhe-Gly-Ψ(CH_2-NH)-Leu-Met-NH_2)-OH ***23***

(or [β-Asp[4], MePhe[7], [8]Ψ[9], CH_2-NH] NKB (4–10)) ***23***

The benzyl ester protective group of **22** was removed by catalytic transfer hydrogenation [29]. The substrate (0.210 g, 0.2 mmol) was dissolved in 4 ml of absolute ethanol and 10% aqueous acetic acid solution was added. A gentle stream of argon was passed through the reaction mixture. Palladium black (20 mg) was added, followed by the addition of 1,4-cyclohexadiene (0.2 ml, 2 mmol). The reaction mixture was stirred at room temperature for 48 h. Completion of the reaction was monitored by TLC. The mixture was filtered through a column of celite, rinsed with EtOH/AcOH 9:1 and evaporated under reduced pressure. The crude product obtained was completely deprotected by TFA according to the usual procedure. After evaporation the residue was triturated with anhydrous ether and the solid was purified by isocratic HPLC. Eluants: (CH_3CN + H_2O 40:60) + 0.1% TFA. k' = 1.31. Yield: 0.12 g (61%). mp = 152–159°C. $[\alpha]_D^{20}$ = – 68.3 (c 1, DMF). Rf(E) = 0.25. MH^+ = 875. ^1H NMR (DMSO–d_6) 2 rotamers, δ = 9.0 (bs, 1H), 8.68 (bs, 1H), 8.5 (m, 3H), 7.95 (s, H), 7.65 (s, 1H), 7.45 (m, 2 H), 7.47–7.0 (m, 24H), 6.82 (m, 3H), 4.95 (m, H), 4.6 (m, 2H), 4.55 (m, 2H), 4.5–4.3 (m, 6H), 3.95–0.7 (77H).

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