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A new somatostatin analog with optimized ring size inhibits neointima formation induced by balloon injury in rats without altering growth hormone release

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Summary — We report on the synthesis and pharmacological properties of a new series of somatostatin analogs. Two lower homologs of lysine, 2,3-diaminopropanoic acid and 2,4-diaminobutyric acid, were prepared and used for cyclization *via* amide formation with the side chain of aspartic or glutamic acid in place of natural cystine present in many somatostatin analogs. One resulting compound, although having low binding affinities for somatostatin receptors, displayed a strong potency in inhibiting neointima formation induced by balloon injury in rats at the dose of 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$. This dissociation of the antiproliferative effect from the endocrine effect seems to indicate that myointimal growth is not related to a change in growth hormone secretion.

somatostatin analog / somatostatin receptor / antiproliferative activity / growth hormone / peptide synthesis

Introduction

Somatostatin and somatostatin analogs have been shown to have a wide range of hormone regulatory effects, such as growth hormone suppression and antiproliferative properties [1, 2]. Their use to prevent myointimal hyperplasia in restenosis after angioplasty as well as accelerated atherosclerosis of transplanted organs has been demonstrated [3, 4]. A therapeutic response to these analogs has also been obtained in metastatic prostatic cancer [5] and in several other neuroendocrine neoplasms in man [6].

This multiplicity of effects probably reflects interactions with a number of distinct receptors and there is a need for new selective somatostatin agonists which could be related to well-defined biological functions. In efforts to improve receptor selectivity, various conformationally restricted somatostatin peptide analogs have been synthesized [7]. In particular, an octapeptide amide molecule (now called angiopeptin), which contains the non-coded amino acid D-β-2-naphthylalanine in position 1, D-tryptophan in position 4, and which is cyclized *via* a disulfide bridge between the two cysteines (table I), displays high antiproliferative properties *in vivo* [8].

In the present study, we decided to investigate the effects of ring size on the pharmacological potency and selectivity of the resulting analogs. For this purpose, we chose to replace the disulfide bridge of angiopeptin by an amide bond, which enabled us to use the side chains of the available analogs of lysine and glutamic acid for cyclization.

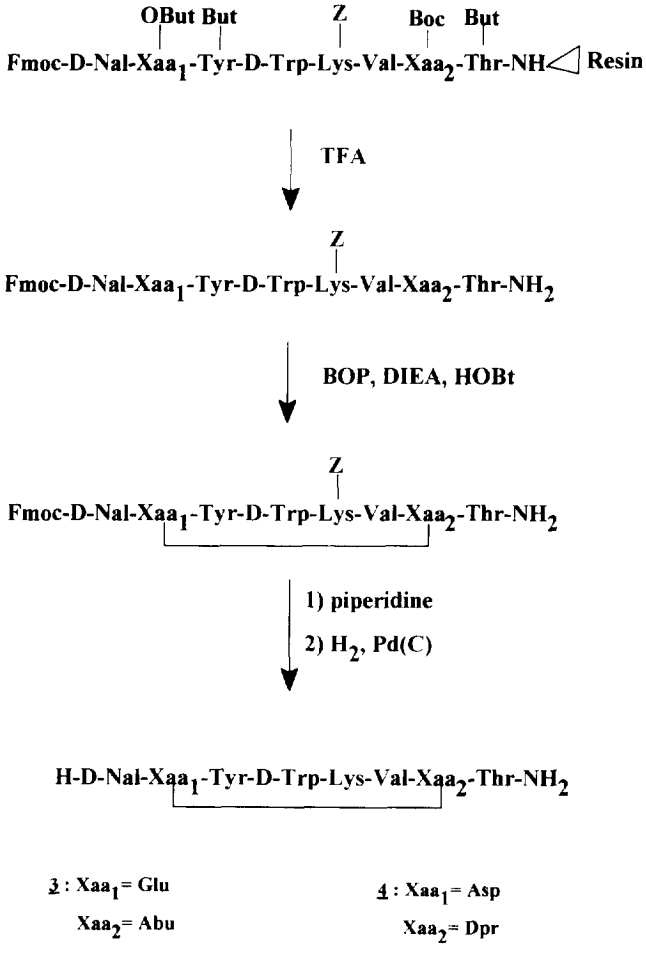
We report here the design, synthesis, binding affinity and pharmacological potency of three new amide analogs of somatostatin. One of them strongly inhibits neointima formation induced by balloon injury in rats without altering growth hormone release.

Abbreviations: The symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biomedical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides (1984) *Biochem J* 219, 345–373. The following further abbreviations were used: AAA, amino-acid analysis; All, allyl; Alloc, allyloxycarbonyl; Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)-tris-(dimethylaminophosphonium)hexafluorophosphate; Dab, 2,4-diaminobutyric acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; Dpr, 2,3-diaminopropanoic acid; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine; SRIF, somatotropin release inhibiting factor, 1-14 or 1-28; TFA, trifluoroacetic acid; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

Table I. Structures of somatostatin analogs.

Peptide	*Structure*	*n*[a]
Angiopeptin[b]	H-D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	20
3	H-D-Nal-Glu-Tyr-D-Trp-Lys-Val-Dab-Thr-NH_2	22
4	H-D-Nal-Asp-Tyr-D-Trp-Lys-Val-Dpr-Thr-NH_2	20
5	H-D-Nal-Glu-Tyr-D-Trp-Lys-Val-Lys-Thr-NH_2	24

[a]Number of atoms engaged in the cycle. [b]Nal = β-2-Naphthylalanine.



Scheme 1. Synthesis of compounds **3** and **4**.

Chemistry

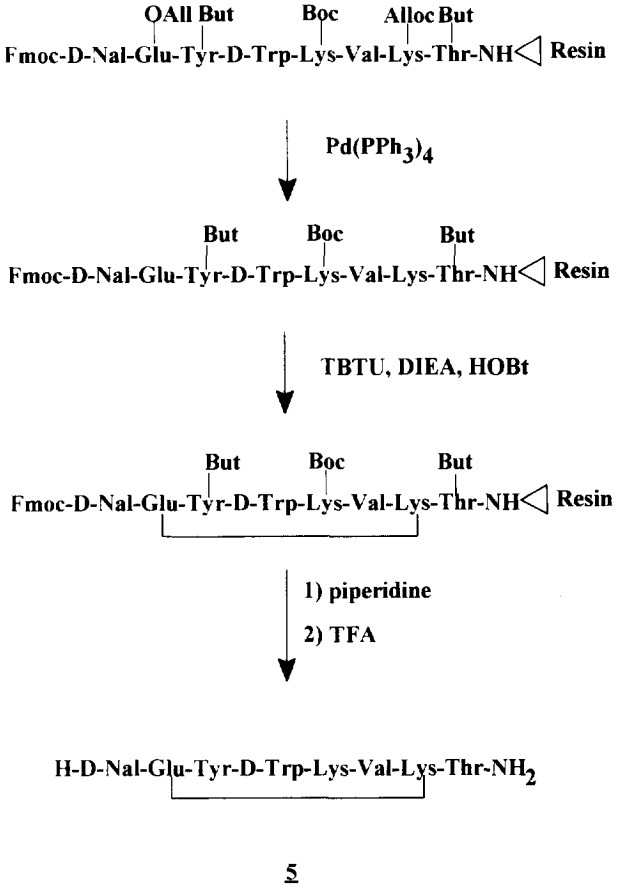
The conformation of the partially cyclic analogs of angiopeptin was varied by both replacing the disulfide by an amide bond and modulating the number of atoms engaged in the ring (table I). Starting from Asn and Gln, respectively, a one-step synthesis of L-2,3-diaminopropanoic acid **1** and L-2,4-diaminobutyric acid **2** was originally described by Waki *et al* [9]. We have developed the method to synthesize residues **1** and **2** with suitable protecting groups for their incorporation into peptides *via* standard solid phase methods using Nα-Fmoc protection [10].

The linear precursors of peptides **3–5** were prepared by the solid phase methodology [11] using DCC/HOBt [12] for activation in the coupling steps. Differential protection and selective deprotection were used in order to permit cyclization to occur unequivocally between the side chains of residues in positions 2 and 7 (scheme 1). To this end, the protecting groups (Boc and OBut) of the side chains of lysine and glutamic acids or their short-chain analogs had the same stability level as the peptide-resin bond, while Fmoc was used for peptide elongation at the N-terminus, Z for protection of lysine-5, and But for tyrosine and threonine. Partial deprotection and cleavage from the resin of the octapeptide amide was obtained by TFA treatment and amide cyclization was achieved using BOP [13] as the condensation agent. After a two-step final deprotection, the analogs were purified by preparative HPLC and characterized by analytical HPLC, amino-acid analysis and FAB-mass spectrometry. Lactone formation between residues Glu^2, Asp^2 and residues Tyr^3 or Thr^8 during the cyclization process of compounds **3** and **4**, respectively, can be excluded on the basis of NMR and mass spectral data analysis. A typical NOE effect was observed between the amine lateral chain of residues Dpr or Dab and the CH_2 of the aspartic or glutamic residues for compounds **4** and

3, respectively. In addition, mass spectral analysis showed a fragmentation pattern in total accordance with the proposed structures of compounds 3–5.

The synthesis of the two final compounds 3 and 4 succeeded in reasonable yields by the strategy outlined in scheme 1, despite some undesired Z-removal from the side chain of lysine during cleavage of the peptide from the resin. On the contrary, an alternative synthesis showed that the dichloro derivative of Z on lysine-5 [14] was a too stable protection to be removed after cyclization by an agent other than liquid HF.

For the synthesis of compound 5, an alternative protection scheme was devised in which the recently described allyl group was used [15], so that elongation and cyclization were directly performed on the solid support as described in scheme 2.



5

Scheme 2. Solid phase synthesis of compound **5** using the allyl ester and the allyloxycarbonyl protecting groups.

Biochemical and pharmacological assays

Binding

The apparent affinities of the synthesized compounds for somatostatin binding sites were determined by displacement of ^{125}I[Tyr]SRIF14 from rat adrenal membranes, brain cortex membranes and pancreatic homogenates [16].

Bioassays

The effects of compound 3, angiopeptin (100 $\mu g \cdot kg^{-1} \cdot d^{-1}$, sc) and compound 4 (100 and 200 $\mu g \cdot kg^{-1} \cdot d^{-1}$, sc) *versus* their vehicles (distilled water, 1 $ml \cdot kg^{-1} \cdot d^{-1}$, sc) were studied on [^{3}H]thymidine uptake of rat aorta after balloon injury. After 2 d of pretreatment, rats were anesthesized and endothelium of aortas were removed as described by Clowes *et al* [17]. Animals were kept under the same treatment during the 3 following days. Three days after endothelial denudation, rats were sacrificed and the aorta was removed for the determination of the [^{3}H]thymidine uptake according to the method described by Capron and Bruneval [18]. Total DNA assay was performed as described previously by Brunk *et al* [19]. The ratio DNA radioactivity/total DNA content was calculated for each aorta. This ratio was used as the proliferation index of the vascular smooth muscle cells.

To determine the activity on myointimal hyperplasia induced by endothelial injury, rats were treated 2 d prior the endothelial denudation with either compound 3, angiopeptin (100 $\mu g \cdot kg^{-1} \cdot d^{-1}$, sc), or their respective vehicles (distilled water, 1 $ml \cdot kg^{-1} \cdot d^{-1}$, sc) infused *via* osmotic minipumps. The left carotid artery was injured and chronic treatments were continued for the subsequent 5 d post injury. On day 14, rats were sacrificed and the left carotid was processed for histomorphometric analyses. Another set of experiments was performed to evaluate the effects of compound 5 (100 $\mu g \cdot kg^{-1} \cdot d^{-1}$, sc) or its vehicle on neointima formation.

Serum growth hormone levels

The effects of compound 3 and angiopeptin (100 $\mu g \cdot kg^{-1} \cdot d^{-1}$, sc) *versus* vehicle (distilled water, 1 $ml \cdot kg^{-1} \cdot d^{-1}$, sc) on serum growth hormone levels was measured after 6 d of chronic treatment. Drugs were continuously infused *via* osmotic minipumps. Blood samples were collected under anesthesia between 10 and 11 am to limit the fluctuation of concentration due to the circadian rhythm. The serum growth hormone level was determined by radioimmunoassay.

Table II. Potency of somatostatin analogs in inhibiting [^{125}I][Tyr]SRIF14 binding to three rat tissue types.

Peptide	*K_i (nM)*		
	Pancreas	*Adrenal cortex*	*Cerebral cortex*
SRIF 14	2.06 ± 0.14	0.58 ± 0.01	0.59 ± 0.1
Angiopeptin	16 ± 1	5.20 ± 1.5	1.41 ± 0.25
3	3.62 ± 2.08	128 ± 26	1646 ± 51
4	0.48 ± 0.16	> 10 000	> 10 000
5	> 10 000	> 1000	> 10 000

Results

Binding

Displacement studies of iodinated somatostatin from its binding sites on rat membrane preparations from adrenal, brain and pancreatic cortices revealed no detectable affinities for compound **5** and a strong binding of compound **4** (K_i = 0.48 x 10^{-9} M) selectively on pancreatic membranes (table II). The K_i value of compound **3** was 1.6 x 10^{-6} M on cerebral cortex membranes while it was 12-fold lower on adrenal cortex preparation (K_i = 1.28 x 10^{-7} M). Like for compound **4**, the lower K_i value for compound **3** was obtained on pancreas cortex membranes (K_i = 3.62 x 10^{-9} M). These two latter affinities are in the same range as those corresponding to the K_i values for SRIF14 (2.06 x 10^{-9} M) and angiopeptin (16 x 10^{-9} M) obtained on pancreatic cortex, but are considerably higher than those obtained for SRIF14 (0.59 x 10^{-9} M) and angiopeptin (1.41 x 10^{-9} M) on cerebral cortex. In contrast to a work published previously [7], we noted only a three-fold difference in affinity for angiopeptin on adrenal and brain receptors.

Table III. Effects of the somatostatin analogs on [^{3}H]-thymidine uptake 3 d after balloon injury of rat aorta at 100 µg·kg^{-1}·d^{-1}.

Treatment	*n*[a]	*[^{3}H]-Thymidine uptake*[b]	*% Inhibition*[c]
Vehicle	29	8570 ± 893	
Angiopeptin	15	6262 ± 669	26.7*
Vehicle	17	10 116 ± 1172	
3	17	7568 ± 608	25.2*
Vehicle	23	9351 ± 1336	
4	17	8406 ± 476	10.1 (ns)

[a]Number of independent experiments; [b]expressed as dpm·µg^{-1} DNA; [c]*$p < 0.05$; ns: non-significant.

Table IV. Effects of the somatostatin analogs on neointima formation induced by balloon injury in rats at the dose of 100 µg·kg^{-1}·d^{-1}.

Treatment	*n*[a]	*I/M*[b] *(%)*	*Neointima (x 1000 µm²)*	*Media (x 1000 µm²)*
Vehicle	11	133 ± 13	160 ± 18	120 ± 4
Angiopeptin	7	87 ± 23	104 ± 26	120 ± 4
Compound **3**	6	66 ± 10	75 ± 11	114 ± 4
Vehicle	4	116 ± 16	162 ± 25	139 ± 3
Compound **5**	6	120 ± 15	151 ± 25	124 ± 7

[a]Number of independent experiments; [b]intima/media ratio, expressed as a percentage.

[^{3}H]Thymidine uptake

On day 3, DNA synthesis, expressed as [^{3}H]thymidine uptake, was markedly enhanced in the media of injured aorta (table III). Compound **3** and angiopeptin inhibited significantly and to the same extent [^{3}H]-thymidine uptake (–25.2 and –26.7%, respectively). In contrast, compound **4** administered at 100 and 200 µg·kg^{-1}·d^{-1}, sc, did not alter DNA synthesis. The same results were observed with compound **5** (data not shown).

Histomorphometric studies

The results are summarized in table IV. Fourteen days after endothelial injury, a massive proliferation, determined by the intima/media ratio, was observed in carotids of the vehicle-treated groups. The neointima area and the intima/media ratio were significantly reduced by treatment with compound **3** or angiopeptin (–35 and –50%, respectively). In contrast, compounds **4** or **5** did not suppress neointimal thickening, since the neointima area and intima/media ratio were identical to those observed in the vehicle-treated group.

Growth hormone level

The effects of compound **3** and angiopeptin (100 µg·kg^{-1}·d^{-1}, sc) *versus* vehicle are summarized in figure 1. After 6 d of chronic treatment, compound **3** did not alter significantly the serum growth hormone level (median: 143 *vs* 119 ng·ml^{-1}, respectively), while the latter was diminished significantly by angiopeptin (median: 143 *vs* 25 ng·ml^{-1}, respectively).

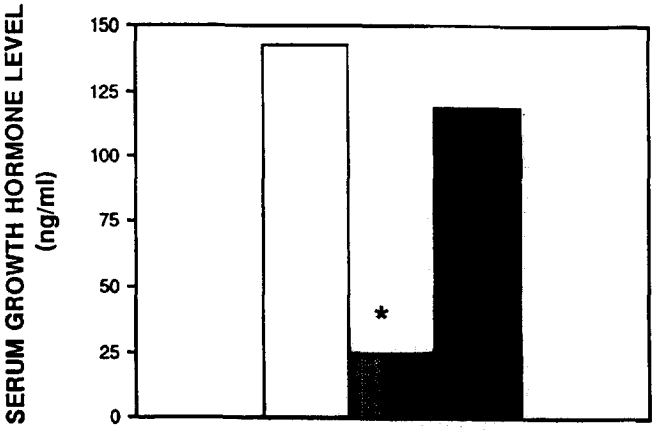


Fig 1. Effects of compound **3** (■) and angiopeptin (□) on growth hormone serum level at the dose of 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$ in the rats (vehicle □).

Discussion

In this study, we have described the synthesis and pharmacological properties of three new octapeptides derived from the somatostatin analog angiopeptin. This compound is known to exert strong antiproliferative properties *in vivo* [8]. In particular, it was found to inhibit myointimal hyperplasia and cell proliferation after balloon injury of both the male and female rabbit aorta [20–23].

In the design of the new analogs, we have focused our structural modifications on the disulfide bridge, which was replaced by an amide bond. We decided to investigate the effects of ring size on the pharmacological selectivity of the molecules. For this purpose, we chose to use the side chains of the available analogs of lysine and glutamic acid, instead of cystine for cyclization. From the chemical point of view, the use of stable Alloc for the protection of Lys^5 in compound **3** instead of the benzyloxycarbonyl (Z) group did not improve significantly the overall yield of **3** (10 compared to 8.6%). This can be explained by the extreme insolubility of the crude product bearing the Alloc group just after the cleavage from the resin. On the contrary, differential protection of Glu^2 by allyl and Lys^7 by Alloc, was a successful and convenient strategy for the synthesis of compound **5**.

Compared with angiopeptin, all three lactam analogs had greatly reduced (200- to 16 000-fold) affinity for somatostatin receptors on rat adrenal cortical and cerebral cortical membranes. Two analogs (n = 20- and 22-atom ring) have slightly improved (4–32-fold) affinity for rat pancreatic somatostatin receptors. Of these two analogs the optimized length was found to be a 22-atom ring, compared to 20 in angiopeptin, since the analogs with a 20- or 24-atom ring were both inactive in inhibiting myointimal proliferation.

The active analog, compound **3**, was effective in preventing neointima formation following balloon catheterization in rats. In rats treated 2 d before and 5 d after injury, compound **3** significantly reduced myointimal proliferation compared to control and angiopeptin, whereas the inhibition of [^{3}H]thymidine uptake in the media of the injured vessel appeared to be the same for the two compounds.

The most striking differences between the two molecules came from the control of growth hormone release.

Chronic treatment of rats with compound **3**, at a dose effective on myointimal growth inhibition, did not change the serum level of growth hormone, whereas angiopeptin significantly reduced this level. These results seem to indicate that the inhibitory effect of compound **3** on myointimal proliferation in response to vascular injury is not related to a reduction in growth hormone release and that this peptide may have a direct or indirect effect on the vessel by a mechanism that is still unknown. The dissociation of growth hormone suppression from antiproliferative activity is interesting and will need further studies to define biological events responsible for antiproliferative activity.

In conclusion, we have produced a series of new somatostatin analogs derived from angiopeptin. A convenient synthesis of stable disulfide bridge surrogates was developed and these modifications conferred high activity on one of the analogs. Compound **3**, which has a strong activity in preventing myointimal thickening after injury, may represent the lead of a new class of agents acting on smooth muscle cell proliferation without interfering with growth hormone secretion.

Experimental protocols

General methods

All peptides were synthesized by the solid phase method of Merrifield [11] using standard procedures on a Milligen 9050 or a Labortec SP650 peptide synthesizer. Analytical data of the synthetic peptides are given in table V. The Fmoc-NH-benzhydrylamine resin [24], Nα-protected (Fmoc) amino acids [25] and reference peptide angiopeptin were purchased from Bachem, except for SRIF14 and SRIF28, which were purchased from Peninsula. Peptides were cleaved from the resin by TFA (10 mL/g of the resin) containing 30% of a dichloromethane/anisole/ethane dithiol (2:1:1, in mL/g of the resin) mixture. Reverse-phase HPLC was performed on a Waters 625 LC system equipped with a photodiode array UV detector, utilizing a DeltaPak C18 (spherical 5 μm) column (3.6 x 150 mm). Retention times are given for gradient elution at 1 mL/min in the binary solvent system 0.1% TFA in water/

Table V. Analytical data of synthetic peptides.

Analytical data	*Compound*		
	3	***4***	***5***
Nal[a]	0.95 (1)	1.01 (1)	1.09 (1)
Asp	–	1.07 (1)	–
Glu	1.04 (1)	–	1.03 (1)
Lys	1.06 (1)	1.08 (1)	1.87 (2)
Tyr	1.02 (1)	1.04 (1)	1.06 (1)
Val	0.97 (1)	1.00 (1)	1.06 (1)
Thr	1.02 (1)	0.94 (1)	1.04 (1)
Dpr/Dab[b]	0.98 (1)	0.96 (1)	–
% Peptide content	80	78	77
$[M + H]^+$	1103	1075	1131
HPLC t_R[d]	17[e]	13[f]	17[g]

[a]D-β(2)-Naphthylalanine; [b]diaminopropanoic or diaminobutyric acid; [c]one single peak in HPLC, difference to 100%: trifluoroacetate counter-ion; [d]gradient CH_3CN per 30 min 0.1%TFA aqueous buffer at 1.0 mL/min, t_o = 1.5 min; [e]10–48% CH_3CN; [f]10–70% CH_3CN; [g]10–40% CH_3CN.

0.1% TFA in acetonitrile. Preparative HPLC was routinely performed on a Waters Prep LC 3000 system equipped with a Waters 490E multiwavelength detector on a PrePak cartridge (47 x 300 mm) filled with a C18-silica (300 Å, 15 µm) phase. The operating flow rate was 60 mL/min.

For amino-acid analyses, peptides were hydrolysed in 6 N HCl (0.3 mL) for 20 h at 110°C in sealed tubes. Hydrolysates were analyzed with a Varian LC90 Star system. The whole procedure including liquid transfer, mixing, Fmoc-derivatization, pentane extraction, and separation on a Aminotag C18 (5 µm) column (4.6 x 150 mm) is completed within 40 min. Molecular weights of peptides were determined by FAB mass spectrometry on a Normag R10-10C apparatus. The samples were dissolved in a glycerol/thioglycerol matrix (1:1), and ionization was effected by a beam of krypton atoms accelerated through 6–8 keV. ^{1}H-NMR spectra were recorded on Bruker spectrophotometers at 200 or 400 MHz as indicated, with Me_4Si as external standard.

Fmoc-Dpr(Boc)-OH 1

A solution of 2 g (5.6 mmol) of Fmoc-Asn-OH in 120 ml acetonitrile/water (3:1) was treated at 60°C with 2.6 g (6.2 mmol) bis(trifluoroacetoxy)iodobenzene, and then with 0.9 ml (11.3 mmol) pyridine. The yellow solution was kept for 5 h at 60°C under stirring and then concentrated in a vacuum at 45°C. The resulting watery gel was isolated by filtration and washed on the filter with water, ethyl acetate and ether. The dry residue (1.28 g, 69%) was used in the next step without further purification. An analytical sample (0.8 g) purified by HPLC confirmed the expected structure of Fmoc-Dpr-OH, Tfa. HPLC: t_R 11 min (gradient 30 to 40% CH_3CN in 30 min). ^{1}H-NMR (300 MHz), [$^2H_6$]DMSO: d 3.0–3.6 (2H, 2dd), 4.2–4.55 (4H, m), 7.2–7.6 (4H,m), 7.7 (2H, d), 7.9 (2H, d).

The crude Fmoc-Dpr-OH (1.2 g, 3.6 mmol) was dissolved in 40 ml acetonitrile/water (3:1) and the solution treated first with 4 ml of a 1 M solution of $NaHCO_3$, then dropwise with 800 mg (3.6 mmol) di-*tert*-butyl-dicarbonate dissolved in acetonitrile (20 ml). After 6 h reaction, the organic solvent was removed by evaporation and the aqueous phase washed with ethyl acetate, then adjusted to pH 2. From this solution, the product was extracted with diethylether and the ether solution washed with saturated NaCl, dried over $MgSO_4$ and evaporated to dryness. A white powder was obtained after trituration in pentane. The crude material was further purified by chromatography on silica gel in ethyl acetate/methanol (7:3): 885 mg (57%). TLC: R_f 0.65 $CHCl_3$/MeOH/H_2O/AcOH (75:27:5:0.5). HPLC t_R 15 min (gradient 40 to 100 in CH_3CN in 30 min). $[\alpha]_D^{23}$ –5.4, (c1.2, MeOH). $[M-H]^-$ 425. ^{1}H NMR (300 MHz), [$^2H_6$]DMSO: δ 1.4 (9H, s), 3.25 (2H, m), 4.1 (1H, q), 4.15–4.4 (2H, m), 6.85 (1H, t), 7.45 (1H, d), 7.25–7.5 (4H, m), 7.7 (2H, d), 7.9 (2H, d), 12.7 (1H, broad s, exchangeable). IR (nujol): 3340 (νNH), 2200–3200 (νOH of COOH), 1695 (broad, νCO of carbamate), 1525 (amide II). Anal $C_{23}H_{26}N_2O_6$ (426.5): C, H, N.

Fmoc-Dab(Boc)-OH 2

This compound was obtained by the same procedure as **1**, starting with Fmoc-Gln-OH (2 g, 5.4 mmol). Overall yield: 705 mg (31%). TLC R_f 0.7 $CHCl_3$/MeOH/H_2O/AcOH (75:27:5:0.5). HPLC t_R 12.5 min (gradient 40 to 100 in CH_3CN in 30 min). $[\alpha]_D^{23}$ –10.8 (c 0.6, MeOH). $[MH]^+$ 441. ^{1}H NMR (300 MHz), [$^2H_6$]DMSO: δ 1.4 (9H, s), 1.7–1.9 (2H, m), 4.0 (1H, m), 3.0 (2H, m), 6.85 (1H, t), 4.3 (3H, m), 4.3 (1H, exchangeable), 7.3–7.5 (4H, m), 7.7 (2H, d), 7.9 (2H, d). IR (nujol) 2350–3350 (νNH, νOH), 1714 (νCO) 1525 (amide II). Anal $C_{24}H_{28}N_2O_6$ (440.4): C, H, N, H_2O.

H-D-Nal-c(Glu-Tyr-D-Trp-Lys-Val-Dab)-Thr-NH_2 3

The protected peptide Fmoc-D-Nal-Glu(OBut)-Tyr(But)-D-Trp-Lys(Z)-Val-Dab(Boc)-Thr(But)-NH-resin was assembled starting with Fmoc-NH-benzhydrylamine resin (2 g, 1 mmol) and using 20% piperidine in DMF (2 x 15 min) for Nα-deprotection and HOBt/DCC for coupling (90 min). The usual sequence of 1 min washings of the substituted resin with isopropyl alcohol, methylene chloride and DMF was applied. The protected aminoacids (3 mmol, 3 equivalents) were introduced in the following order: Fmoc-Thr(But)-OH, Fmoc-Dab(Boc)-OH, Fmoc-Val-OH, Fmoc-Lys(Z)-OH, Fmoc-D-Trp-OH, Fmoc-Tyr(But)-OH, Fmoc-Glu(OBut)- OH, Fmoc-D-Nal-OH. Cleavage from the resin and removal of the *tert*-butyl-type protecting groups was achieved in the mixture of TFA (24 mL), CH_2Cl_2 (6 mL), anisole (1 mL) and ethane dithiol (1 mL) for 90 min at room temperature. The oily residue obtained from the filtrate after evaporation in vacuum was then triturated in ether and gathered as a powder: 705 mg (45% yield, trifluoroacetate). However, analytical HPLC revealed the presence of a major side product (about 35% of the main product), which could be shown to have lost the protection by Z of the lysine side chain. The mixture was then separated by preparative HPLC which gave 475 mg of the lysine-protected pure component. For the cyclization, the product (460 mg) was dissolved in DMF to a concentration of 2.5 x 10^{-4} mol/L, treated with

650 µL diisopropylethylamine and 570 mg BOP and kept for 8 h at room temperature under stirring. Chromatography of the residue obtained by evaporation on Sephadex LH20 in DMF allowed the pure fractions to be isolated as a solid residue: 325 mg. The cyclic analog was then treated with piperidine (35 mL) for 15 min, the solvent evaporated and the product washed on the filter with ether. The solid residue was immediately subjected to a hydrogenation on palladium/charcoal (60 mg) in MeOH/H_2O 3:1 (to which 0.3 mL 1 M HCl was added) for 4 h at room temperature. Filtration of the catalyst and preparative HPLC of the concentrated solution afforded 95 mg **3** (0.086 mmol, overall yield from Fmoc-NH-resin 8.6%). The final product was > 99% pure according to analytical HPLC. MS: *m/e* MH^+ 1103.

H-D-Nal-c(Asp-Tyr-D-Trp-Lys-Val-Dpr)-Thr-NH_2 **4**

This compound was synthesized according to the procedure described for **3**. The protected peptide Fmoc-D-Nal-Asp (OBut)-Tyr(But)-D-Trp-Lys(Z)-Val-Dpr(Boc)-Thr(But)-NH-resin was assembled starting with Fmoc-NH-benzhydrylamine resin (2 g, 1 mmol). After cyclization and purification, 31 mg peptide (overall yield 2.8%) was obtained as a lyophilisate. MS: *m/e* MH^+ 1075.

H-D-Nal-c(Glu-Tyr-D-Trp-Lys-Val-Lys)-Thr-NH_2 **5**

The protected peptide Boc-D-Nal-Glu(OAll)-Tyr(But)-D-Trp-Lys(Boc)-Val-Lys(Alloc)-Thr(But)-NH-resin was assembled starting with Fmoc-NH-benzhydrylamine resin (2 g, 1.2 mmol). The allyl side-chain protection was removed using a procedure similar to that described by Albericio *et al* [15]. Briefly, the resin (2 g) was suspended in $CHCl_3$/AcOH/NMM (80:4:2, in mL/g of the resin), $Pd(0)(PPh_3)_4$ (2 g) was added to the media and the reaction was allowed to stand at room temperature for 2 h. The resin was then washed with DMF and CH_2Cl_2, and cyclization was carried out using TBTU/HOBt/DIEA (1:1:2), (5 equivalents to resin substitution) in 40 mL DMF under stirring. Ninhydrin test showed that no free amino groups were present after 8 h. Cleavage of the peptide from the resin and removal of the *tert*-butyl-type protecting groups was achieved as previously described for **3**. After evaporation, the residue was purified by preparative HPLC on reverse phase and lyophilized; 106 mg of pure **5** was obtained (overall yield = 7.7%). MS: *m/e* MH^+ 1131.

Binding assays

[^{125}I][Tyr]SRIF14 (specific activity 81.4 TBq/mmol) was purchased from New England Nuclear (Du Pont de Nemours, France). Semi-purified membrane preparations of adrenal glands and cerebral cortices were obtained as previously described (Krantic *et al*, 1992) [16]. Pancreas devoid of connective tissue and fat were homogenized in 0.3 M sucrose with a glass-glass homogenizer by 30 hand strokes.

Homogenates were filtered over four layers of cotton-cheese. Protein concentration of the filtrate was adjusted to 5–10 mg per mL with 0.3 M sucrose. These filtrates were then frozen and kept at –80°C until use. Different tissue preparations (protein equivalent: 50–100 µg per 50 µL, protein concentration estimated according to Lowry *et al* [26]) were incubated for 60 min at 25°C with 25 µL of [^{125}I][Tyr]SRIF14 in 150 µL of 50 mM Tris-HCl buffer (pH 7.5) containing non-radioactive competitors. Non-specific binding was determined in the presence of 1 µM SRIF14. Incubations were terminated by the addition of 1 ml of ice-cold buffer and filtration through Whatman GF/C glass fiber filters precoated with incubation buffer. The filters were rinsed with 3 x 5 mL of ice-cold buffer and dried, and the radioactivity was counted using a LKB Rackgamma counter. Drug competition binding data were analyzed by iterative curve fitting using the McPherson modified ligand program (1983) [27]. Differences between data were statistically assessed using one-way analysis of variance (Anova) and considered significant at $p < 0.05$.

Measurement of [^{3}H]thymidine uptake

Male Wistar rats (Charles River, France) weighing 300–320 g were used. The method of endothelial denudation was similar to that described by Clowes *et al* [17]. Under methohexital anesthesia (Brietal, Lilly, France, 60 mg/kg, ip), a 2F balloon embolectomy catheter (Baxter, France) was introduced through the left common carotid into the thoracic aorta. At the level of the renal arteries, the catheter was inflated and slowly withdrawn under hard friction. This procedure was repeated three times. This technique is well documented to completely remove the endothelial cells of arteries. The left common carotid was then ligated and the incision sutured. Three days after the endothelial denudation, the thoracic aorta was removed under pentobarbital anesthesia (60 mg/kg, ip, Sanofi, France) and cleaned of fat adventitial tissues. [^{3}H]thymidine uptake was determined according to the method described by Capron and Bruneval [18]. The aorta was preincubated for 1 h in Krebs–Heinseleit buffer (NaCl 120 mM, KCl 4.8 mM, $CaCl_2$ 1.8 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, glucose 5 mM, pH 7.4 and bovine serum albumin 10 g·L^{-1} (BSA, cohn fraction V, Sigma) gassed with carbogen (95% O_2/5% CO_2) and kept at 37°C. The aorta was then incubated for 1 h in Krebs–Heinseleit buffer containing [methyl-^{3}H]thymidine (specific activity: 47.8 Ci/mmol, Dositek, France; buffer concentration: 2 µCi/ml). This incubation was followed by a post incubation of 1 h in [methyl-^{3}H]thymidine Krebs–Heinseleit buffer. After washing in Tris-EDTA buffer (Tris 10 mM, EDTA 10 mM, NaCl 100 mM, pH 7.0), the aorta was stored at –30°C until further biochemical analysis. After soaking the aorta in Tris-EDTA buffer, the intima-media was dissected from the adventicia according to the Wolinsky and Daly method [28]. The intima-media was homogenized in the same buffer, and incubated with pronase (type B, nuclease free, 4 mg/mL, Calbiochem, France) and sodium dodecyl sulfate (0.6255 mg/ml; Sigma) for 30 min at 37°C. The homogenate was centrifugated (2250 *g*, 10°C, 10 min) and the supernatant divided into 2 aliquots: one to determine [^{3}H]DNA radioactivity, and the other to measure total DNA content. DNA was coprecipitated by the addition of 13.3 ml of BSA (3 mg·ml^{-1} of Tris-EDTA) and 1 mL trichloroacetic acid (20%) per mL of supernatant. The mixture was kept at 4°C for 30 min and then filtrated on nitrocellulose filter (45 µm, Millipore, France). The filter was placed in a scintillation vial, 0.5 mL HCl 0.5 N were added and the medium heated at 100°C for 20 min. After cooling, 1 mL ethyl acetate and 10 mL of a scintillation solution (Lumagel) were added. DNA radioactivity was counted in a liquid scintillation spectrometer (Beckman).

Histomorphometric studies

Male Wistar rats (Charles River, France) weighing 420–450 g were anesthesized by a mixture of ketamine (100 mg·kg^{-1}, ip) and acepromazine (5 mg·kg^{-1}, ip). A 2F balloon embolectomy catheter was introduced through the external branch of the left carotid into the aortic arch. The intimal injury was performed as described previously [16] and for the measurement of [^{3}H]-

thymidine uptake. Fourteen days after endothelial denudation, rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg·kg^{-1}). Evans' blue (0.5 mL of 0.5% saline solution) was administered intravenously 20 min before the sacrifice of animals to evaluate the level of endothelium regeneration. The carotids were perfused under a pressure of 90 mmHg with 10% formaldehyde for 20 min. Carotids were then carefully dissected and left in the same fixative until further processed. The mid-portion of each carotid were used for histological studies because this region did not show any evidence of endothelium regeneration (positive staining after Evans' blue injection). The portion was divided in 4 segments (2.5 mm length) and embedded in paraffin. Cross-sections (5 µm) were prepared and stained with orcein. The gap between two cross-sections was 100 µm. Medial and intimal cross-sectional areas were measured using a Biocom computerized image analysis system (Histo software, Biocom, France). Lumen, internal elastic lamina and external elastic lamina were used as border to define medial and intimal area. For each carotid, the intimal/medial ratio was determined from the histomorphometric analysis performed on 12 cross-sections (three cross-sections per segment).

Statistical analysis

[^{3}H]Thymidine uptake and histomorphometric studies were analyzed by one-way analysis of variance and student's *t* test, respectively. The posthoc test used was the Newman–Keuls test. For the effect on growth hormone serum level, statistical comparison was performed by Kruskal–Wallis analysis.

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