84. Synthesis and Antagonistic Activity of Two Covalently Linked Dimers of Adrenocorticotropin-(11-24)-tetradecapeptide

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The chemical synthesis of two dimers of the sequence 11 to 24 of adrenocorticotropin is described. The two monomers were covalently linked through their N- or C-termini, respectively, using *N*-acetylglutamic acid or lysine amide as spacers. Compared to the monomer, the first dimer showed moderately, the second strongly increased inhibitory potency upon steroidogenesis in isolated adrenal cells.

Introduction. – The organization of the hormonal information in the ACTH (adrenocorticotropic hormone)[1]) molecule is well understood [1]. While the segment ACTH (5–10) bears the central message, the sequence ACTH (11–24) is the major binding element of the hormone. The fragments ACTH (1–4) and ACTH (25–39) have mainly potentiating and protective functions, respectively. Experimental work has confirmed that ACTH (11–24) is a competitive antagonist of ACTH (1–10) and of ACTH (1–39) [2] and that when the tetradecapeptide is complemented at its N-terminus with at least the fragments of the message sequence Glu-His-Phe-Arg-Trp-Gly for cAMP accumulation, steroidogenesis, and lipolysis; His-Phe-Arg-Trp-Gly for steroidogenesis without cAMP accumulation; and Phe-Arg-Trp-Gly for adenylate cyclase stimulation, it turns into an agonist [3] [4]. Shorter fragments at the N-terminus have *inhibitory* properties in the same tests. In addition, the corticotropin inhibiting peptide (CIP) which is believed to exist in circulation as a free natural ACTH inhibitor [5], has the sequence ACTH (7–38). Unfortunately, these antagonist, as well as those produced by synthesis so far [6–8], are not very potent.

To enhance the inhibitory potency of these ACTH conjugates, using the concept of covalent dimerization, two conjugates of ACTH-(11–24)-tetradecapeptide were prepared. The present work describes in detail the synthesis of the conjugates and the preliminary biological evaluation of the new antagonists.

Strategy. – The chemical structure of the dimers is given in the *Figure*. In the first conjugate, the N-terminal amino groups of two ACTH fragments are covalently linked to the carboxyl functions of *N*-acetylglutamic acid. In the second, the C-terminal carboxyl groups of the ACTH fragments are covalently attached to the amino functions of lysine amide. These structures were designed in order to allow: *1.* their unequivocal preparation by classical methods of peptide synthesis; *2.* their easy characterization by amino-acid

[1]) Abbreviations for hormones, amino acids and protected derivatives according to the IUPAC-IUB recommendations [20]. Further abbreviations: DMF = *N,N'*-dimethylformamide, TFA = trifluoroacetic acid. DCCI = *N,N'*-dicyclohexylcarbodiimide, MEIC = morpholinoethyl isocyanide.

Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH
Ac-Glu-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH · 10 HCl

1

H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro
H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Lys-NH_2 · 14 HCl

2

Fig. *Chemical structure of the synthetic dimeric conjugates*

analysis after total hydrolysis; *3.* the comparison of the effect on biological activity of the attachment of the spacer to the N- or to the C-termini; *4.* the production by degradation *in vivo* of only non-toxic compounds (amino acids). This strategy can in principle be extended to the preparation of longer spacers (*e.g.* by one or several glycines) or longer peptide fragments.

Synthesis. – *Schemes 1* and *2* show that the syntheses were conducted so as to produce a well-defined product in each step and to avoid any coupling at two reaction centers

Scheme 1. *Synthesis of the Dimeric Conjugate* **1** *of ACTH (11–24)*

Boc Boc Boc H_2^+ H_2^+ Boc
H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OtBu, $2Cl^-$

3

↓ + Ac-Glu(OSu)-OBzl

Boc Boc Boc H_2^+ H_2^+ Boc
Ac-NH–CH–$(CH_2)_2$–CO-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OtBu, $2Cl^-$
COO-Bzl

4

↓ + H_2(Pd)

Boc Boc Boc H_2^+ H_2^+ Boc
Ac-NH–CH–$(CH_2)_2$–CO-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OtBu, $2Cl^-$
COOH

5

↓ + **3** (DCCI)

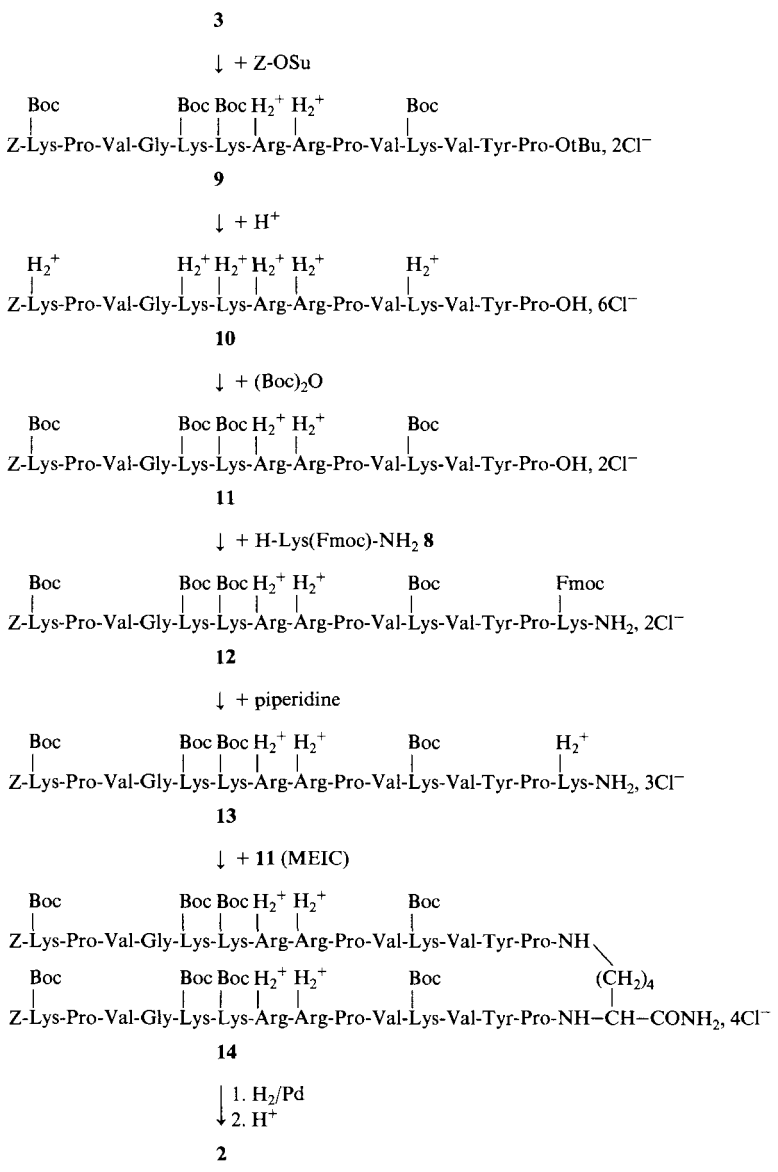
Boc Boc Boc H_2^+ H_2^+ Boc
CH_2–CO-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OtBu
CH_2 Boc Boc Boc H_2^+ H_2^+ Boc
Ac-NH–CH–CO-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OtBu, $4Cl^-$

6

↓ + H^+

1

Scheme 2. *Synthesis of the Dimeric Conjugate* **2** *of ACTH (11–24)*



simultaneously. This was achieved by the design of two differentially protected spacer molecules, Ac-Glu-OBzl and H-Lys(Fmoc)-NH_2, which allowed after a first coupling, selective deprotection before the second coupling could occur. Starting material for the syntheses was the early described [9] and now industrially prepared tetradecapeptide dihydrochloride **3**. The first dimer **1** was obtained in four steps *(Scheme 1)* by coupling of **3** with commercially available Ac-Glu(OSu)-OBzl, followed by elimination of the BzlO group through catalytic hydrogenation, by coupling with a second fragment **3** and by

final acidic deprotection. The synthesis of the second dimer **2** was considerably more complex *(Scheme 2)*, the combination of the protecting groups in **3** having to be modified before coupling. Compound **3** was first benzyloxycarbonylated at its N-terminus, then liberated from its Boc and *t*-BuO groups, finally protected again by Boc, leaving the C-terminal function free for coupling to the spacer (compound **11**). Reaction of **11** with H-Lys(Fmoc)-NH_2 in the presence of MEIC, followed by selective alkaline Fmoc-splitting and coupling with a second fragment **11** afforded the protected dimeric conjugate, which was converted to a free peptide by catalytic hydrogenation and acidic treatment. The introduction of the Z-group in fragment **3**, although not compulsory for the present synthesis, was performed in view of the preparation of N_α-elongated dimers. No major difficulties were encountered during synthesis, except for the coupling of **11** with **13** *(Scheme 2)* which could not be brought to completion and occurred with correspondingly low yield. As expected, gel filtration was found to be very efficient to separate dimeric from monomeric products, both in their protected and free forms. Due to the covalent binding of the hormonal segments to the spacer as well as to the spacer protection throughout *via* amide bonds, total hydrolysis of the products led consistently to free amino acids. The ratio of glutamic acid in conjugate **1** or of lysine in conjugate **2** to the other amino acids was a convenient means to prove covalent dimerization. We found in amino-acid analysis of homogeneous **1**, ratios of 0.99/2.06 and 0.99/1.99 for glutamic acid and glycine, and glutamic acid and tyrosine, respectively *(Table)*. Similarly, the ratios of lysine to glycine and tyrosine in homogeneous **2** were 9.37/2.06 and 9.37/1.85, respectively, in all cases within the experimental error limits.

Biological Activity. – In preliminary experiments [10], log dose/response curves were measured for the stimulation of steroidogenesis by ACTH (1–24) in adrenal fasciculata cells, in the presence of either the monomer ACTH (11–24) (deprotected **3**) or of one of the dimers **1** or **2**, at a constant concentration (10^{-5} M). While the monomer only slightly increased the effective dosis of the agonist for half-maximal stimulation ($ED_{50} = 3.5 \times 10^{-13}$ M) to $ED_{50} = 7.0 \times 10^{-13}$ M (factor 2), the conjugates **1** and **2** strongly displaced the log dose/response curves to the right. The measured ED_{50} were 2.63×10^{-10} M (factor 750), and 1.58×10^{-9} M (factor 4514) for **1** and **2**, respectively. These

Table. *Physical and Analytical Data of the Products and of the Key Intermediates*

Compound	$[\alpha]_D^{23}$ [a] ($c = 1$, MeOH)	R_f (TLC) [b]				Amino-acid analysis [c]) or elemental analysis [d])						
		BAW	IPW	CMWA	BPFW	Arg	Glu	Gly	Lys	Pro	Tyr	Val
1	− 75.0				0.33	4.07(4)	0.99(1)	2.06(2)	7.85(8)	6.33(6)	1.99(2)	6.08(6)
2	− 72.2				0.25	4.35(4)	–	2.06(2)	9.37(9)	5.78(6)	1.85(2)	6.75(6)
6	− 70.0	0.27	0.74	0.12		4.09(4)	0.99(1)	2.04(2)	7.87(8)	6.46(6)	2.05(2)	6.05(6)
11	− 63.3	0.30	0.75	0.27		2.17(2)	–	1.0(1)	4.01(4)	2.85(3)	0.87(1)	3.08(3)
13	− 53.3	0.08	0.68	0.21		2.35(2)	–	1.0(1)	4.88(5)	2.55(3)	0.86(1)	3.00(3)
14	− 66.8	0.23	0.77	0.33		3.88(4)	–	2.0(2)	8.68(9)	5.68(6)	1.55(2)	6.01(6)
7	+ 2.4	0.70	0.78	0.84		C 66.79/66.65			H 7.11/7.16		N 8.99/8.85	
8	+ 6.0	0.38	0.63	0.46		C 62.45/62.21			H 6.49/6.49		N 10.40/10.20	

[a]) Specific rotation. [b]) R_f values on silica-gel plates in the solvent systems BuOH/AcOH/H_2O (BAW) 72:7:21; *i*-PrOH/pyridine/H_2O (IPW) 36:32:32; $CHCl_3$/MeOH/H_2O/AcOH (CMWA) 700:250:47:3; or on cellulose plates in BuOH/pyridine/HCOOH/H_2O (BPFW) 40:24:6:30. [c]) Theoretical value in parentheses. [d]) Calc./found.

results confirm the weak antagonistic potency of ACTH (11–24) [2] and reflect a significant potentiation of the inhibitory effect due to covalent dimerization. This potentiation will have to be evaluated more closely in terms of receptor microaggregation [11] and of favorable entropy change due to receptor bridging [12]. The difference of potency observed between the two conjugates is indicative of a relatively stronger binding by the residues near the N-terminus of ACTH (11–24). It can also be expected that optimalization of the spacer length and N_α-elongation of the hormonal fragment to be dimerized will further enhance the observed potentiating effect.

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Experimental Part

Materials. Ac-Glu(OSu)-OBzl and Boc-Lys(Z)-OH, dicyclohexylammonium salt, were purchased from *Bachem AG*, CH–4416 Bubendorf. The protected precursor H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH · 2HCl was a generous gift of *Ciba-Geigy AG*, Basel (Dr. *B. Riniker*).

Methods. Elemental analysis was performed in the microanalytical laboratory of the ETHZ *(D. Manser)*. For amino-acid analysis, peptides were hydrolyzed with 6N HCl in sealed, evacuated tubes at 120° for 20 h, and analyzed according to *Moore* and *Stein* [13] with a *BIOTRONIK LC 6001* apparatus (courtesy of Prof. *B. Gutte*). Optical rotation was measured with a *Perkin-Elmer 141* polarimeter. TLC was performed on *Merck* F_{254} precoated plates of silica gel or of cellulose in the solvent systems given in the *Table*. Plates were examined under UV_{254} light and after spraying with ninhydrin, TFA/ninhydrin, and the *Reindel-Hoppe* reagent [14]. The peptides were assembled in soln. using differential protection, selective deprotection and specifically directed condensation of the fragments [15]. The key intermediates were isolated as pure compounds *(Table)*, mainly by the efficient flash chromatography [16] or gel filtration on *Sephadex LH 60 (Pharmacia)*, column size ∅ 2.3 × 170 cm, or on G 50 in H_2O (∅ 2.3 × 50 cm). The inhibitory activity of the fully deprotected conjugates during ACTH-stimulated steroidogenesis in adrenal fasciculata cells was measured according to the procedure in [17] in the laboratory of Prof. *M. Vallotton*, Division of Endocrinology, University Hospital of Geneva.

Ac-Glu-{α,γ-bis[Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OtBu]} · 4HCl (**6**). Ac-Glu(OSu)-OBzl (140 mg, 0.37 mmol) was added to the soln. of 545 mg (0.25 mmol) **3** in DMF (20 ml) and the mixture kept for 16 h at r.t. The solvent was then evaporated *in vacuo* and the residue triturated in AcOEt (30 ml) and separated by decantation. TLC showed completion of the reaction and elimination of the active ester. However, the product was filtered on *Sephadex LH 60* in MeOH and the pure fractions evaporated. Yield: 490 mg **4** (80%). TLC: R_f 0.36 (BAW), 0.70 (IPW), 0.23 (CMWA), (for abbreviations of solvent systems, see the *Table*). Crude **4** (366 mg, 0.15 mmol) was hydrogenated in MeOH (30 ml) in the presence of a few drops of AcOH and of Pd/C (300 mg) for 8 h at 760 Torr. The catalyst was then removed by filtration, the solvent evaporated and the crude product washed with AcOEt. Yield: 317 mg of **5** (90%). TLC: R_f 0.25 (BAW), 0.73 (IPW), 0.14 (CMWA). Compound **5** (235 mg, 0.1 mmol) was coupled in DMF (25 ml) to **3** (218 mg, 0.1 mmol) using 1-hydroxybenztriazole (14 mg) and DCCI (40 mg). The mixture was stirred at r.t. for 16 h, and the formation of **6** followed chromatographically. TLC R_f 0.27 (BAW), 0.74 (IPW), ninhydrin negative, TFA/ninhydrin positive spots, distinct from monomers **3** and **5**. After evaporation of the solvent, the residue was triturated and decanted twice in AcOEt and finally purified by gel filtration on *Sephadex LH 60* in MeOH. Compound **6** appeared first, as expected, followed by a small amount of **5** and **3**. Yield: 210 mg (47%).

Ac-Glu-{α,γ-bis[Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH]} · 10 HCl (**1**). For deprotection, portions of 70 mg of **6** were treated separately with 5 ml of 0.4N HCl in HCOOH for 20 min. at r.t. The soln. was then poured into 50 ml of Et_2O and the precipitate gathered by centrifugation. Suspension in Et_2O and centrifugation were repeated twice. The residue was subjected to chromatography on *Sephadex LH 60* in MeOH. Elution of **1** as a symmetrical peak (A_{280}), evaporation of the solvent and re-precipitation from MeOH/Et_2O afforded a TLC-pure compound (single ninhydrin positive spot, distinct from monomers **3** and **5** after deprotection under the same conditions). Yield: 42 mg (55%).

Boc-Lys(Fmoc)-NH_2 (**7**). Boc-Lys(Z)-NH_2 (1.9 g, 5 mmol) prepared [18] from Boc-Lys(Z)-OH *via* Boc-Lys-(Z)-ONp [19], was dissolved in MeOH (40 ml) and treated with 300 mg of Pd on activated charcoal under N_2. The reaction vessel was then evacuated and connected to a reservoir containing H_2. After 8 h reaction under shaking, the catalyst was eliminated by filtration, the filtrate brought to pH 4 with AcOH and the solvent evaporated. The TLC-pure acetate (R_f 0.27 (BAW), starting material 0.72) was dissolved in a mixture of 10 ml of dioxane and 15 ml of aq. Na_2CO_3 (10%) and treated under cooling with 1.29 g (5 mmol) of Fmoc-Cl *(Fluka)* previously dissolved in dioxane (15 ml). After 1 h at 0° and 16 h at r.t., the reaction was complete. Extraction into AcOEt following evaporation of dioxane, and recrystallization twice from AcOEt/Et_2O yielded a TLC-pure product (1.64 g, 70%).

H-Lys(Fmoc)-NH_2 · HCl (**8**). The soln. of **7** (467 mg, 1 mmol) in AcOEt (5 ml) was treated with 4.5N HCl (10 ml) in the same solvent at r.t. Precipitation of **8** occurred slowly and was brought to completion by partial evaporation of the solvent. Yield: 380 mg (94%).

Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OH · 2 HCl (**11**). Compound **3** (2.18 g, 1 mmol) was thoroughly dissolved in 15 ml of dried and degased DMF and reacted with Z-OSu *(Bachem)* (274 mg, 1.1 mmol) for 16 h at r.t. After evaporation of the solvent, the solid residue was triturated in AcEOt, filtered and precipitated from its soln. in MeOH by $(i\text{-Pr})_2O$. Yield: 2.1 g of **9** (91%). TLC: R_f 0.38 (BAW), 0.48 (CMWA). Intermediate product **9** was treated by TFA (8 ml) at 20° during 1 h after which the soln. was poured into Et_2O (200 ml). The precipitate was separated by filtration, washed and dried. Yield: 1.91 g of **10** (85%). TLC: R_f (cellulose) 0.45 (BPFW). Compound **10** was dissolved in dioxane/H_2O 1:1 in the presence of 1.7 g (8 mmol, 10 equiv.) of di(*tert*-butyl)dicarbonate *(Fluka)* and the pH adjusted to 10.5 with 0.1N NaOH. Reaction proceeded for 1 h at 0°, then for 16 h at r.t. The pH was re-adjusted twice and did not vary after the first 3 h. Acidification to pH 3 with 1N HCl was performed under cooling. Evaporation of dioxane led to precipitation of crude **11** which could be filtered. Further purification was obtained by flash chromatography on silica gel [16] (column ∅ 4.2 × 25 cm) at 0.85 bar pressure (flow 1 ml/sec) in increasingly polar mixtures of $CHCl_3$/MeOH/H_2O/AcOH. The pure fractions were evaporated, the residue re-precipitated from MeOH/AcOEt and characterized analytically. Yield: 1.24 g (65%).

Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Lys-NH_2 · 3 HCl (**13**). The soln. of **11** (1.09 h, 0.5 mmol) and **8** (227 mg, 0.55 mmol) in DMF (25 ml) was neutralized with pyridine (2 ml) and treated with 1-hydroxybenztriazole (81 mg) and, at 0°, with 139 µl (1 mmol) of MEIC *(Fluka)*. Addition of the latter coupling reagent (139 µl) was repeated after 16 h at r.t. After 2 days, the solvent was evaporated and the solid residue triturated in AcOEt and filtered. The crude material was purified by chromatography on *Sephadex LH 60* with MeOH and the pure fractions collected and evaporated. Yield of TLC-pure **12**: 1.0 g (80%), R_f 0.35 (BAW), 0.50 (CMWA). The splitting of the Fmoc group was achieved by dissolving **12** in DMF (15 ml) and adding of re-destillated piperidine (15 ml). The soln. was kept for 30 min at r.t. The solvents were then evaporated under reduced pressure and the crude product dissolved in MeOH, neutralized with 1N AcOH (0.5 ml) and precipitated by AcOEt. Elimination of the residual impurities was achieved by gel filtration as above on *Sephadex LH60*. Yield: 810 mg (87%).

N_α,N_ε-*Bis[Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro]-Lys-$$NH_2$ · 4 HCl* (**14**). The condensation of **11** (218 mg, 0.1 mmol) with **13** (237 mg, 0.1 mmol) was performed in 30 ml of DMF/dioxane (1:1) in the presence of *N*-ethylmorpholine (13 µl), 1-hydroxybenztriazole (30 mg), and DCCI (26 mg). A new addition of DCCI (26 mg) was made after 16 h at r.t. Coupling could not be brought to completion, even after 2 days stirring at 40°. Isolation of a pure fraction of **14** (112 mg, 25%) was achieved after filtration, evaporation of the filtrate under reduced pressure and washing of the solid residue in AcOEt, by gel filtration on *Sephadex LH 60* on MeOH. Only minor amounts (18 mg) of **14** could be recovered by re-chromatography of the residual fractions which contained significant quantities of the starting materials **11** and **13**.

N_α,N_ε-*Bis[H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro]-Lys-$$NH_2$ · 14 HCl* (**2**). The protected dimer **14** (105 mg, 23 µmol) was treated in MeOH (15 ml + 0.15 ml AcOH) with 50 mg Pd catalyst on activated charcoal. The reaction flask was connected to an H_2 reservoir and reduction occurred during 4 h at 760 Torr. The catalyst was then removed by filtration, replaced by the same amount of fresh one, and hydrogenation repeated for another 4 h. The solid residue obtained after removal of the catalyst, evaporation of the solvent and washing in AcOEt, was used without further purification. Yield: 85 mg (81%). The crude product was divided in two portions and treated separately with 0.4N HCl (1 ml) in HCOOH (10 equiv. /Boc group) for 30 min at r.t. in a centrifuge tube. The reaction was stopped by addition of 15 ml of Et_2O and the product gathered by centrifugation. Resuspension twice in Et_2O and centrifugation led to the recovery of 82 mg (95%) of crude **2** (sum of the two fractions). The free peptide was then dissolved in H_2O (2.5 ml) and passed through a *Sephadex G 50* column and eluted with 0.2M aq. ammonium acetate (pH 6.8). The fractions were then lyophilized at 45°. Finally, the product was dissolved in MeOH/0.01N aq. HCl 1:1 and the soln. evaporated and lyophilized to give the pure hydrochloride **2**. Yield: 34 mg (50%).

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