105. Synthesis and Opiate Activity *in vitro* of Five New *p*-Nitrophenylalanine[4]-Enkephalin-like Peptides

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Summary

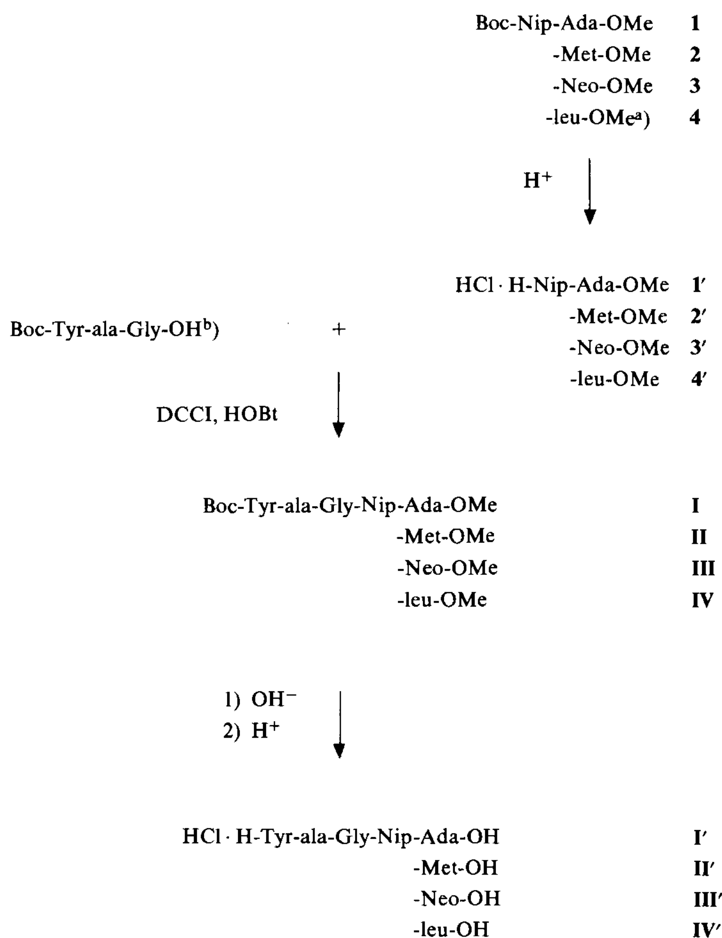
Converging data obtained in biochemical and pharmacological bioassays for opiate activity are presented for five new enkephalin-like peptides. The main structural features of the analogues, the synthesis of which is described in detail, were the presence of D-alanine or D-serine in position 2, *p*-nitrophenylalanine in position 4 and of a free *C*-terminal carboxylic function. Confirming our previous observations [1], the substitution of phenylalanine by *p*-nitrophenylalanine enhanced the overall opiate activity but decreased the selectivity towards μ/δ-receptor sites. Tyrosyl-D-alanyl-glycyl-*p*-nitrophenylalanyl-adamantylalanine was particularly potent in all assays while tyrosyl-D-seryl-glycyl-*p*-nitrophenylalanyl-leucyl-threonine was very selective in the bioassays on isolated tissues and moderately selective in the binding assay. These results will have to be taken into account in future photoaffinity labelling and quantitative structure-activity relationships (QSAR) studies.

Enkephalin-like peptides containing *p*-nitrophenylalanine (Nip)[1]) in position 4 have proved to be more potent as opiate ligands in binding assays and as opiate agonists in isolated tissue assays, than the parent phenylalanine[4] compounds [1]. A recent quantitative structure-activity relationships (QSAR) study of 12 analogues showed that this is mainly due to the favorable electronic properties of Nip, as measured by the difference in pK_a of the corresponding *p*-nitrobenzoic and benzoic acids [2]. In terms of selectivity towards μ- and δ-opiate receptors [3], no definitive

[1]) Abbreviations for amino acids and protected derivatives according to the IUPAC-IUB recommendations [23]. Further abbreviations: Nip = *p*-Nitrophenylalanine, Ada = adamantylalanine, Neo = neopentylglycine, DADL = Tyr-D-Ala-Gly-Phe-D-Leu, DAGO = Tyr-D-Ala-Gly-MePhe-Gly-ol, DMF = *N,N'*-dimethylformamide, TFA = trifluoroacetic acid, TLC. = thin layer chromatography, r.t. = room temperature, GPI = guinea-pig ileum, MVD = mouse vas deferens, Tris = tris(hydroxymethyl)aminomethane.

conclusion could be drawn from our previous studies. The substitution of Phe by Nip in position 4 seemed however to enhance potency without significant modification of the selectivity. The purpose of this work was to investigate the effect of the introduction of Nip into two enkephalin analogues with clear-cut preferences for δ-receptor sites. We chose the derivatives Tyr-D-Ala-Gly-Phe-D-Leu (DADL) [4] [5], and Tyr-D-Ser-Gly-Phe-Leu-Thr [5] [6], and prepared their Nip^4-analogues. In addition, three new Nip^4-analogues of the enkephalins containing adamantylalanine [7], methionine or neopentylglycine [8], respectively, in position 5 were prepared and investigated pharmacologically. Since the corresponding *C*-terminal amides showed only poor μ/δ-selectivity, the *C*-terminal acidic function was kept unsubstituted in these derivatives to enhance the δ-selectivity. We describe in the

Scheme. *Synthesis in solution of four enkephalin-like peptides*



a) leu = D-leucine.
b) ala = D-alanine.

Table 1. *Physical and analytical data of the peptide derivatives prepared*

Compound	$[\alpha]_D^{23}$ [a]) (c = 1, MeOH)	M.p. [b])	Rf (TLC.) [c]) B/A/W 72:7:21	C/M 1:1	I/P/W 36:32:32	Elemental analysis (calc./found) C	H	N	
1	– 12.9	148°	0.80	0.79		63.50/62.18	7.42/7.28	7.93/7.78	
2	– 11.7	129°	0.73	0.82		52.73/52.64	6.42/6.27	9.22/9.20	S: 7.04/6.84
3	– 8.86	147°	0.79	0.79		58.52/58.44	7.37/7.20	9.31/9.22	
4	26.3	115°	0.78	0.78		57.65/57.63	7.14/7.02	9.61/9.69	
I	11.3	165°	0.81	0.75		61.45/61.24	6.88/6.79	10.24/10.69	
I'·$2H_2O$	25.1	186°	0.64			55.49/55.17	6.60/6.37	10.78/10.54	
II	7.65	115°	0.74	0.76		54.68/54.69	6.21/6.20	11.25/11.23	S: 4.29/4.33
II'·$2H_2O$	47.3	185°	0.45	0.42		47.69/47.33	5.86/5.75	11.91/12.27	S: 4.55/4.26 Cl: 5.02/4.80
III	12.2	214°	0.73	0.80	0.73	59.33/59.19	6.92/6.86	9.61/9.33	
III'·$2H_2O$	27.1	175°	0.62	0.49		51.39/51.24	6.47/6.22	11.98/11.85	Cl: 5.06/5.09
IV	15.8	127°	0.69	0.74		57.68/57.50	6.64/6.64	11.53/11.36	
IV'	21.1	165°	0.52	0.39		53.50/53.63	6.04/6.23	12.91/12.58	Cl: 5.44/5.66
5-acetate	– 17.9	176°	0.25		0.67	49.68/49.57	6.41/6.38	13.37/13.20	
V'·$2H_2O$	20.7	181°	0.55		0.70	49.28/48.98	6.26/6.00	12.19/12.19	Cl: 4.40/4.61

[a]) Specific rotation. [b]) Melting point, uncorrected. [c]) Rf-values in the solvent systems BuOH/acetic acid/water (B/A/W), $CHCl_3$/MeOH (C/M) and 2-propanol/pyridine/water (I/P/W).

following the synthesis and characterization of the five analogues and evaluate their binding affinity, their opiate activity (potency to inhibit the contractions of two isolated tissues, GPI and MVD) and their selectivity towards morphine-like (μ) or enkephalin-like (δ) binding and receptor sites.

Synthesis. The pentapeptides Tyr-D-Ala-Gly-Nip-Ada (**I'**), Tyr-D-Ala-Gly-Nip-Met (**II'**), Tyr-D-Ala-Gly-Nip-Neo (**III'**) and Tyr-D-Ala-Gly-Nip-D-Leu (**IV'**) were obtained by classical peptide synthesis in solution [1] (see the *Scheme*). The products were characterized by elemental analysis both at the dipeptide and pentapeptide level *(Table 1).* In contrast, the hexapeptide Tyr-D-Ser-Gly-Nip-Leu-Thr (**V'**) was prepared from its *C*-terminal-pentapeptide fragment which was assembled on a solid support according to the general procedure of *Barany & Merrifield* [9] and

Table 2. *Inhibition of the binding of [tyrosyl-3,5-^{3}H]Tyr-D-Ala-Gly-MePhe-Gly-ol* (DAGO, K_d 2.8 nM) *and of [tyrosyl-3,5-^{3}H]Tyr-D-Ala-Gly-Phe-D-Leu* (DADL, K_d 1.8 nM) *to guinea-pig brain membranes by enkephalin analogues*

Peptide	DAGO K_I (nM) [a])	DADL K_I (nM) [a])	DADL/DAGO [b])
Tyr-D-Ala-Gly-Nip-Ada (**I'**)	0.84 ± 0.25	0.35 ± 0.14	0.41
Tyr-D-Ala-Gly-Nip-Met (**II'**)	3.60 ± 1.40	1.03 ± 0.34	0.28
Tyr-D-Ala-Gly-Nip-Neo (**III'**)	5.60 ± 2.10	1.71 ± 0.54	0.30
Tyr-D-Ala-Gly-Nip-D-Leu (**IV'**)	2.60 ± 0.70	0.87 ± 0.23	0.33
Tyr-D-Ser-Gly-Nip-Leu-Thr (**V'**)	9.10 ± 3.10	2.25 ± 1.01	0.25
leucine-enkephalin (**VI'**)	82.0 ± 10.1	8.05 ± 0.96	0.10
Tyr-D-Ala-Gly-Phe-D-Leu (**VII'**)	9.0 ± 4.3	1.60 ± 0.20	0.18
Tyr-D-Ser-Gly-Phe-Leu-Thr (**VIII'**)	27.2 ± 4.8	1.62 ± 1.22	0.06

[a]) Dissociation constant $K_I \pm$ s.e.m. of the non-radioactive analogue. [b]) Ratio of K_I for DADL- to K_I for DAGO-sites.

after detachment, reacted with the active ester Boc-Tyr-OSu in solution. This strategy has the advantage – not used in this study – to allow the introduction of 3,5-diiodo-tyrosine for labelling purposes in the last step. Although the synthesis on solid phase could be carried out without major problems, it required large amounts of starting materials (and of solvents), which prevented us to apply it for the preparation of the pentapeptides, especially those containing the not yet commercially available residues adamantylalanine and neopentylglycine.

Binding studies (see *Table 2*). All peptide derivatives were appreciably more active than leucine-enkephalin at displacing the preferentially μ-oriented ligand DAGO from its binding sites. The analogue with the highest affinity (compound **I'**) was approximately 100 fold more active than leucine-enkephalin. The affinity of the Nip^4-analogues towards the binding sites of DADL was also generally increased, compared to their phenylalanine^4-parent compounds, although in a more modest manner (2 to 10 fold). In one case (hexapeptide **V'**), there was even a slight loss of affinity. The introduction of Nip into the preferentially δ-oriented peptides **VII'** and **VIII'** led to fourfold and twofold loss of δ-selectivity, respectively. When compared to their corresponding amides [10], compounds **I'**, **II'** and **III'** with free *C*-terminal acidic function showed, as expected, increased δ-selectivity (2 to 10 fold). In summary, the substitution of Phe by Nip in position 4 enhanced the overall μ- and δ-affinity, decreased however the δ-selectivity. The latter effect was more than compensated by the presence of a free carboxylic terminal group, in the place of an amide.

Pharmacological assays. When tested for their inhibitory potency on the GPI, the five new enkephalin-like peptides were considerably more active than natural leucine-enkephalin *(Table 3)*. Particularly active was compound **I'** in which the conjunction of the favorable electronic features of Nip with the steric bulk and high lipophilicity of Ada (*cf.* [11]) led to a 700 fold potency increase compared to leucine-enkephalin. The Nip^4-analogue of DADL (**VII'**) was about 170 times more active than leucine-enkephalin on the GPI.

The substitution of Phe by Nip in position 4 also generally led to an increase in potency on the MVD. However, the increase was less pronounced than on the

Table 3. *Inhibitory potencies* (IC50, nM) *and relative potencies to leucine-enkephalin in the guinea-pig ileum* (GPI) *and mouse vas deferens* (MVD) *bioassays*

Compound	GPI		MVD		IC50 (GPI)[a]) / IC50 (MVD)
	IC50 (nM)	Rel. potency	IC50 (nM)	Rel. potency	
Tyr-D-Ala-Gly-Nip-Ada (**I'**)	0.353±0.104	696±204	0.458±0.092	24.9±5.0	0.77
Tyr-D-Ala-Gly-Nip-Met (**II'**)	7.03±1.20	35.0±6.0	0.223±0.013	51.2±3.1	31.5
Tyr-D-Ala-Gly-Nip-Neo (**III'**)	19.5±6.4	12.6±4.1	1.16±0.30	9.8±2.5	16.8
Tyr-D-Ala-Gly-Nip-D-Leu (**IV'**)	1.41±0.41	174±50	1.09±0.66	10.5±6.4	1.29
Tyr-D-Ser-Gly-Nip-Leu-Thr (**V'**)	44.6±7.0	5.51±0.86	0.139±0.030	82.1±17.7	321
leucine-enkephalin (**VI'**)	246±39	**1**	11.4±1.1	**1**	21.6
Tyr-D-Ala-Gly-Phe-D-Leu (**VII'**)	22.8±1.5	10.8±0.7	0.564±0.017	20.2±0.6	40.4
Tyr-D-Ser-Gly-Phe-Leu-Thr (**VIII'**)	205±56	1.20±0.33	0.260±0.003	43.8±0.5	788

[a]) Ratio of inhibitory potencies as a measure of the selectivity towards GPI-(μ) and MVD-(δ) receptors.

GPI. The Nip[4]-analogue of DADL is even one exception being twice less active than the parent compound. This fact seems to be related to the D-configuration of leucine, since the replacement of Phe by Nip in Tyr-D-Ala-Gly-Phe-Leu has already been shown to enhance the potency on the MVD by a factor of 6 [10] [12]. The most potent derivatives on this tissue were the peptides **V'** and **II'** which were about 80 and 50 times as active as leucine-enkephalin, respectively.

In terms of selectivity, two of the new derivatives, **I'** and **II'** could be compared to their corresponding amides [10]. While the selectivity ratio of **I'** remained unexpectedly unchanged (ratio of amide 1.29), the δ-selectivity of **II'** was 10 times higher than for the amide (ratio 3.12). The replacement of Phe by Nip in the two reference compounds **VII'** and **VIII'** caused an increase in potency by a factor of 4 and 16, respectively, in the GPI, and by a factor of 2 in the MVD. These uneven increases led to a loss of selectivity compared to the parent compounds. However, the analogue **V'** is still a remarkably selective δ-agonist with a ratio of inhibitory potencies amounting to 321. The latter findings may be important for photoaffinity-labelling experiments designed to use the side chain of Nip as the photoreactive moiety [13]. While compound **V'** is likely to preferentially label the δ-binding sites, this will not be the case for the Nip[4]-derivative of DADL, although DADL has been for long considered as the representative δ-ligand.

On the whole, the present results confirm a general increase of the biological activity in all assay systems (with the exception of the Nip[4]-analogue of DADL on the MVD) when Phe is replaced by Nip in the parent compound. The binding affinities compare well with the pharmacological potencies, confirming the relevance of the guinea-pig brain homogenate as test system. However, there is no doubt that the introduction of the very selective δ-ligands Tyr-D-Ser-Gly-Phe-Leu-Thr [5] or of the not yet commercially available Tyr-D-Thr-Gly-Phe-Leu-Thr (tritiated form prepared by *B. Roques & J. L. Morgat*, personal communication), instead of DADL, as primary δ-ligand, will improve the biochemical characterization of the opiate binding sites.

The results are presently being analyzed in a more quantitative manner (QSAR study) and compared with those obtained with a series of enkephalin amides [2] to get clues as to the role of electronic, steric and hydrophobic factors on the activity and selectivity of these opioid peptides.

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Experimental Part

Binding studies. The reference peptides leucine-enkephalin and DADL were products of *Bachem AG*, CH-4416 Bubendorf, while Tyr-D-Ser-Gly-Phe-Leu-Thr (**VIII'**) was purchased from the *Peninsula Laboratories Inc.*, San Carlos, CA 94070, USA. The primary ligands [tyrosyl-3,5-^{3}H][D-Ala[2], MePhe[4], Gly[5]]enkephalin-ol (33 Ci/mmol), called tritiated DAGO, and [tyrosyl-3,5-^{3}H][D-Ala[2], D-Leu[5]]-enkephalin (42 Ci/mmol), tritiated DADL, were purchased from the *Radiochemical Center*, Amersham,

U.K. The binding experiments were conducted as in [14]. Briefly, guinea-pig brain was homogenized in ice-cold 50 mM Tris-HCl at pH 7.4 and centrifugated at 17000 g for 20 min. The pellet was incubated in the same buffer for 45 min at 37° to allow dissociation of endogenous opioids, recentrifugated and washed twice in Tris by resuspension and centrifugation. For binding assays, incubations of 1 g brain (wet weight) for 1 h at 23° in the same buffer with ^{3}H-labelled ligand in a final volume of 2 ml were performed in triplicate. Samples cooled in ice were the filtered through *Whatman GF/C* filters that had been washed with water saturated with 1,1-dimethyl-1-propanol. After washing (3×) with cold buffer, scintillation counting of the filters occurred. Specific binding was calculated as the difference between binding of ^{3}H-labelled ligand in the absence and presence of 1 µM levallorphan. The dissociation constants (K_d) and binding capacities (B_{max}) were estimated from complete saturation curves with the primary ligands, using a computer-fitting procedure [15]. For competition studies, unlabelled ligands previously dissolved in the buffer, were added 20 min prior the primary ligand, the latter being dissolved to a 1 nM final concentration. Slopes of the competition curves were determined by least-squares methods on log-logit plots. From the IC50 obtained, the equilibrium dissociation constant of the non-radioactive ligand was calculated using the classical equation $K_I = IC50/(1 + L/K_d)$, where L is the concentration of the labelled ligand and K_d its equilibrium dissociation constant [16]. All competition experiments were performed on three different preparations.

Bioassays (performed in the laboratory of *P.W.S.*). The assay based on the inhibition of electrically induced contractions of the myenteric plexus-longitudinal muscle preparation from the GPI was carried out as reported in detail elsewhere [17]. The similar assay on the MVD was also performed according to a published procedure [18]. A log-dose response curve was determined for leucine-enkephalin in each ileum or vas-deferens preparation and served as standard for the normalization of the IC50-values of the enkephalin analogues [19].

Chemistry. TLC. was carried out on *Merck* silica-gel plates using the solvent systems indicated in *Table 1*. Optical rotation was measured on a *Perkin-Elmer* polarimeter *141*. Elemental analyses were performed in the Laboratorium für organische Chemie, ETHZ *(D. Manser)*. Amino-acid analyses were carried out with a *Biotronik BT 6110* analyzer and a *Hewlett-Packard 3388A* automatic integrator (Prof. *H. Zuber*).

Dipeptides **1**, **2**, **3**, **4** *and* **1'**, **2'**, **3'**, **4'**. They were prepared according to the general procedure described in [1]. The yields are also within the same range: coupling 75–85%, deprotonation 85–95%.

Pentapeptides **I**, **II**, **III**, **IV**. They were also prepared following the general procedure described in [1]. Due to the presence of the *C*-terminal methyl-ester group, all compounds were soluble and could be washed in EtOAc successively with 5% $NaHCO_3$-, $KHSO_4/K_2SO_4$- (pH 2) and sat. NaCl-solutions. The dried crude products were then recrystallized (**I**, **II**, **III**) or precipitated (**IV**) from $MeOH/CHCl_3$ mixtures. Yields: 71–81%.

Pentapeptide hydrochlorides **I'**, **III'**, **IV'**. Each one of the protected pentapeptides **I**, **III** or **IV** (0.05 mmol of each) were dissolved in 4 ml MeOH/dioxane 1:1 and 2 ml 1N NaOH was added. The solution was kept for 15 min at r.t., then cooled to 0° and acidified to pH 2 with 0.1N HCl. Threefold extraction with 10 volumes of EtOAc followed by evaporation of the solvent afforded a crude compound. In two cases, this was pure on TLC., while for the derivative of **I**, the alkaline treatment had to be repeated once. For final deprotection, portions of about 50 mg sample were dissolved in 0.5 ml 0.4N HCl in HCOOH and kept for 20 min at r.t. The product was precipitated by addition of 10 ml ether and gathered by centrifugation. Precipitation from MeOH/ether yielded pure products (yield 72–82%).

*Tyr-*D*-Ala-Gly-Nip-Met, HCl, 2 H_2O* (**II'**). Pentapeptide **II** (150 mg, 0.2 mmol) was dissolved in 5 ml dioxane/water 2:1 and the solution kept under N_2 during the whole procedure; 0.5 ml 1N NaOH was then added. After 15 min, the org. solvent was evaporated, the solution acidified to pH 2 at 0°, the product extracted with EtOAc and precipitated from this solution with hexane. TLC. showed completion of the splitting of the methyl-ester group (Rf 0.65, B/A/W 72:7:21, *Table 1*). The residue was then treated with 1 ml 0.4N HCl in HCOOH, in the presence of 50 µl mercaptoethanol, for 20 min at r.t. The solvents were then evaporated, MeOH repeatedly added and evaporated, and the last residue of mercaptoethanol extracted with ether. The insoluble product was then precipitated from MeOH by addition of ether. Further purification was obtained on a silica-gel column (∅ 0.8 × 8.5 cm) in $CHCl_3$/MeOH 2:1. After evaporation of the pure fractions, the water soluble part of the residue was recrystallized from MeOH/ether. Overall yield 37%.

D-*Ser-Gly-Nip-Leu-Thr* · CH_3COOH (**5**). Copoly(styrene-divinyl benzene) (10 g; 0.7 meq./g, *Fluka AG*, CH-9470 Buchs) was reacted with 3.1 g (7 mmol) Boc-Thr(Bzl)-OCs prepared as a solid cesium salt [20], for 16 h at 50°. The mixture was filtered and washed several times with DMF, MeOH and CH_2Cl_2. The dry resin contained approximately 0.24 mmol threonine pro g according to the test of *Gisin* [21]. Splitting of the *N*-terminal Boc-group was achieved in each cycle by treating the resin with 50% TFA in CH_2Cl_2 and followed by neutralization with 5% triethylamine in CH_2Cl_2. The symmetrical anhydride of one of the following protected residues Boc-Leu-OH, Boc-Nip-OH, Boc-Gly-OH or Boc-D-Ser(Bzl)-OH (20 mmol) was then prepared in CH_2Cl_2/DMF 9:1 (1 h reaction with 1.1 equiv. DCCI followed by filtration) and poured into the vessel containing the resin. After 3 h reaction under shaking at r.t., the solid material was separated by filtration and washed alternatively twice by MeOH and CH_2Cl_2/DMF 9:1. Coupling was then repeated with the same amount of the same anhydride. After the filtration and the washing steps, a new cycle was started. Half of the substituted resin (8 g) was then treated as a suspension in 60 ml TFA with gaseous HBr for 90 min at r.t. After filtration, washings (2×20 ml TFA) and evaporation under reduced pressure, the residue was taken up into alcohol and re-evaporated until no more HBr could be detected. The product was extracted with 0.1N acetic acid and the solid residue was discarded. Purification was achieved by gel filtration on *Sephadex G10* (column ⌀ 5 × 85 cm) in 0.5N acetic acid, then on *Sephadex G25* (same column size) equilibrated with the aq. layer of the two-phase system BuOH/acetic acid/water 4:1:5, the elution being achieved with the org. phase. The fractions containing a TLC.-pure product were collected, evaporated and analyzed. Peptide **5** obtained in this way as monoacetate (360 mg, half of total material) gave correct elemental *(Table 1)* and amino-acid analyses: (hydrolysis in 6N HCl for 24 h at 110°) ser 0.95 (1), Gly 1.05 (1), Nip 1.00 (1), Leu 1.06 (1), Thr 0.97 (1).

Tyr-D-*Ser-Gly-Nip-Leu-Thr* · *HCl* · 2 H_2O (**V'**). Peptide **5** (100 mg, 0.16 mmol) was dissolved in 20 ml DMF and treated with 90.8 mg (0.24 mmol) of Boc-Tyr-OSu [22] and 23 µl (0.16 mmol) triethylamine. The solution was kept for 18 h at r.t. The solvent was then evaporated and the product extracted from a cold aq. solution at pH 2 with EtOAc. After evaporation of the solvent, the product could no longer be completely dissolved in EtOAc. TLC. showed that the insoluble residue had been partially purified by this procedure; 50 mg of this material was then subjected to a gel filtration on *Sephadex LH20* (column ⌀ 1.8×80 cm) with MeOH, recovery: 35 mg TLC.-pure material (Rf 0.59, B/A/W, 0.48 C/M, *Table 1*). Recrystallization: from EtOH/ether. For deprotection, the product was treated with 2 ml 0.4N HCl in HCOOH for 20 min at r.t. The peptide was poured into 20 ml ether and repeatedly from ether suspensions. Further purification was obtained on *Sephadex G10* (column ⌀ 2×85 cm) in 0.1N acetic acid. The pure fractions evaporated and reprecipitated from MeOH/EtOAc amounted to 24 mg. Aminoacid analysis: (hydrolysis in 6N HCl for 24 h at 110°) Tyr 0.98 (1), ser 0.96 (1), Gly 1.03 (1), Nip 1.00 (1), Leu 0.99 (1), Thr 0.94 (1).

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