202. Synthesis, Characterization, and Biological Properties of Five Enkephalin-like Pentapeptides Containing *p*-Nitrophenylalanine[1])

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Summary

Five pentapeptides with the amino acid sequence L-tyrosyl-D-alanyl-glycyl-L-*p*-nitrophenylalanyl-X, wherein X = L-leucine, L-leucine amide, L-methionine amide, L-l-adamantylalanine amide, and L-neopentylglycine amide were prepared by chemical synthesis in solution and characterized chemically and physically. The effect of the incorporation of the unnatural amino acids adamantylalanine and neopentylglycine, 'fat'-analogues of leucine and methionine, on the inhibition of electrically evoked contractions of the myenteric plexus-longitudinal muscle preparation from guinea pig ileum was studied. Their activities relative to the peptides with X = Leu·OH, Leu-NH_2, and Met-NH_2 (X = Neo-NH_2 is particularly potent) are discussed in terms of hydrophobic and steric factors, and resistance towards enzymic degradation.

In a preliminary communication [1], we reported the high morphine-like potency in the guinea pig ileum assay (inhibition of electrically evoked contractions of the myenteric plexus-longitudinal muscle preparation) and the strong stereospecific affinity to rat brain membranes of the enkephalin-like peptide H-Tyr-ala-Gly-Nip-Leu-NH_2[2]) without giving synthetic details. We now wish to discuss the extension of this work to four other Nip-containing pentapeptides and to give a detailed account of the syntheses. Preliminary measurements of the opioid activity in the guinea pig ileum assay *(P. W.S.)* are also presented.

The five pentapeptides prepared had the general structure H-Tyr-ala-Gly-Nip-X in which X represents a variable amino-acid residue with either a carboxyl or carboxamide function. Continuing our studies on "fat" amino-acids [2], we chose for X, besides the natural leucine or methionine, one of the "fat" residues Ada [3] or Neo

[1]) Part of the Habilitationsschrift of *J. L. F.* submitted to the ETHZ (1981).

[2]) For abbreviations see [6]. Further abbreviations: TFA = trifluoracetic acid, HOBt = 1-hydroxybenztriazole.

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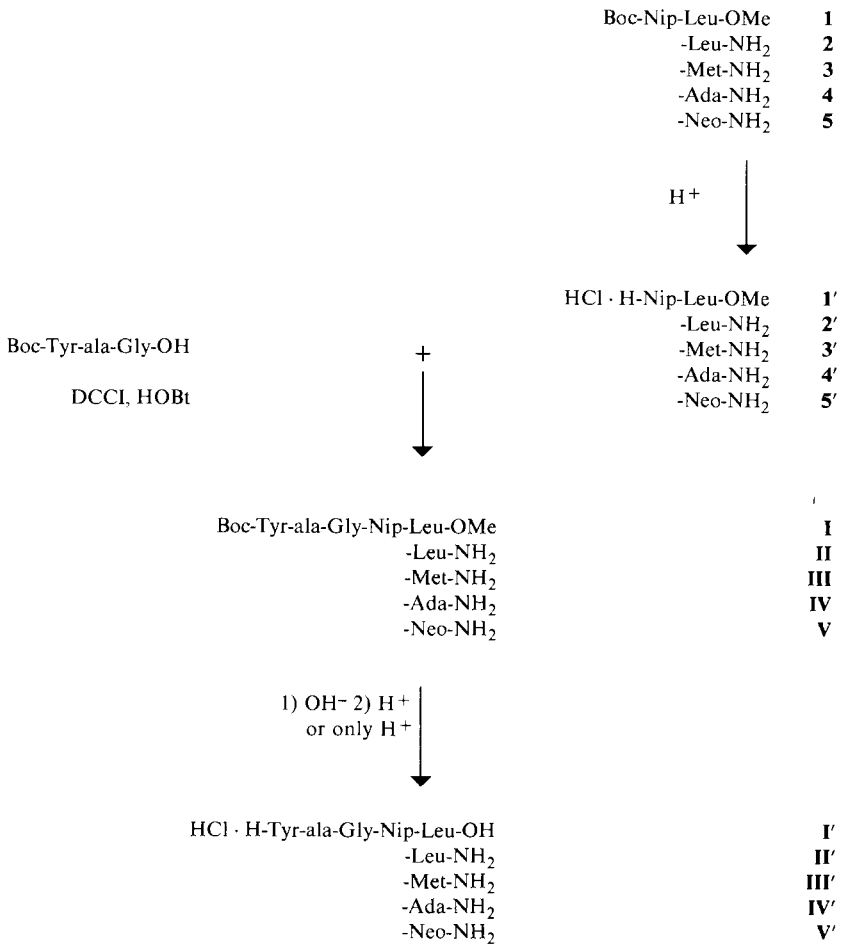
[4]. The purpose of these substitutions was to increase the stability of the peptides against proteolysis and to study the effect of strongly enhanced lipophilicity and increased steric bulk in this position on opiate-like activity.

The peptides were prepared by classical synthesis in solution [5] using the methyl ester or the amide group for *C*-terminal and the Boc group for N^{α}-protection. No side-chain protecting groups were necessary. The procedure is indicated in the *Scheme*. Coupling of the fragment Boc-Tyr-ala-Gly-OH([9], modified preparation, see [6]) with one of the dipeptides **1′** to **5′** was achieved by the procedure of *König & Geiger* [7]. No particular problems were encountered during the synthetic incorporation of Nip, Ada and Neo into the peptide chain. However, because of the presence of Nip, reductive removal of protecting groups was precluded. The products were characterized by elemental analysis both at the dipeptide and the pentapeptide levels. The physical and analytical data are presented in *Table 1*. The results con-

Scheme. *Synthesis of five enkephalin-like peptides*



firmed the feasibility of the introduction of the unnatural residues Nip, Ada and Neo into peptide chains. According to *in vitro* degradation experiments carried out as in [8], the presence of Ada in position 5 (analogue **IV'**) strongly increased the stability of the peptide towards carboxypeptidases as did that of D-alanine in position 2 [10] towards aminopeptidases. Neo had very much the same stabilizing effect (analogue V') as *t*-butylglycine in parent analogues [11].

Table 1. *Physical and analytical data of the dipeptides and pentapeptides prepared*

Compound[a]	$[\alpha]_D^{23}$ $c=1$, MeOH	M.p.[b]	Rf (TLC.)				Elemental analysis (calc./found)		
			B/A/W 72:7:21	C/M/A 95:5:3	C/M 1:1	I/P/W 36:32:32	C	H	N
1	–15.2	97°	0.86	0.82			57.65/57.31	7.14/7.16	9.61/9.42
2		199°	0.61	0.87		0.75			
2'	– 2.1	(229°)	0.47	0.10		0.66	50.21/49.80	6.46/6.40	15.61/15.54[c])
3		190°	0.74	0.46	0.67				
3'	18.3	(210°)	0.40		0.43		44.62/44.49	5.62/5.56	14.87/14.48[d])
4	–24.2	122°	0.78	0.83	0.69	0.75			
4'	– 6.7	(145°)	0.62			0.69	58.59/58.35	6.93/6.77	12.42/11.88
5	–21.3	174°	0.77	0.42	0.74	0.75	57.78/57.65	7.39/7.49	12.84/12.56
I	10.7	218°	0.80		0.87		57.68/57.56	6.64/6.60	11.53/11.49
I'	27.1		0.62		0.56	0.75			
II	13.9	196°	0.71	0.18					
II'	31.3		0.54			0.69	55.80/56.25	6.63/6.75	13.15/13.40
III	11.6	159°	0.66		0.84		52.85/52.53	6.32/6.24	13.07/12.73[e])
III'	47.2		0.48		0.56	0.71	50.23/49.66	5.73/5.41	14.68/14.90[f])
IV	10.3	167°	0.87	0.28					
IV'	18.8	(195°)	0.62			0.73	58.25/57.79	6.52/6.35	13.21/13.10[g])
V	13.9	245°	0.72	0.09	0.74		57.76/57.32	6.79/6.77	13.47/13.29
V'	42.2		0.52		0.48	0.70			

[a]) Compound number is the same as in *Scheme*. [b]) Melting point (m.p.) of non crystalline compound is given parentheses. [c]) Cl 4.78/4.48. [d]) Cl 9.41/9.33. [e]) S 4.28/4.87. [f]) S 5.31/5.12. [g]) Cl 4.77/4.99.

The above syntheses led to neuropeptide analogues with significantly increased potency for multiple receptor binding (to be published) and opiate activity. The inhibitory actions of the enkephalin peptides in the guinea pig ileum assay are compared with that of methionine-enkephalin in *Table 2*. All Nip containing analogues displayed higher potency (lower IC_{50}) than H-Tyr-ala-Gly-Phe-Leu-NH_2. We attribute this increase to favourable electronic features of the side-chain of Nip, when occupying this position [8]. A similar observation was made by *Pless et al.* [9], when they replaced *N*-methylphenylalanine by Nip in FK 33-824. A potentiating steric effect seems to be excluded, since tyrosine[4]-enkephalin derivatives are only weakly active [12]. The most active compound contains, in addition to Nip in position 4, the unnatural residue Neo in position 5. It is approximately 30 times more active than methionine-enkephalin on this tissue. The comparatively low increase in potency observed for the corresponding Ada[5]-analogue (**IV'**) may be due to a pronounced adverse steric effect. Another interesting observation is the activity of the analogue H-Tyr-ala-Gly-Nip-Leu-OH (**I'**) which is lower than that of its amide (**II'**). Given the preponderance of μ-receptors in the guinea pig ileum [13], this result

seems to indicate that while Nip in position 4 notably enhanced the inhibitory potency, it did not modify the specificity of the hormone derivatives for μ- and δ-receptors, this being determined mainly by the *C*-terminal function (carboxylic acid or carboxamide), in agreement with [14].

Table 2. *Relative potency of enkephalin analogues in the guinea pig ileum assay*

Compound		IC50 [nm][a] [b]	Relative potency ([Met^5]enkephalin = 1)
H·Tyr-ala-Gly-Nip-Leu·OH	**I'**	5.58 ± 1.67	8.69 ± 2.21
H·Tyr-ala-Gly-Nip-Leu·NH_2	**II'**	3.13 ± 1.28	18.3 ± 7.5
H·Tyr-ala-Gly-Nip-Met·NH_2	**III'**	2.31 ± 0.77	24.8 ± 8.3
H·Tyr-ala-Gly-Nip-Ada·NH_2	**IV'**	3.81 ± 0.03	15.0 ± 1.0
H·Tyr-ala-Gly-Nip-Neo·NH_2	**V'**	1.89 ± 0.75	30.3 ± 12.0
H·Tyr-ala-Gly-Phe-Leu·NH_2		11.1 ± 3.2	5.15 ± 1.48
[Met^5]enkephalin		57.2 ± 6.0	1

[a]) Mean of three determinations ± SEM. [b]) Normalized values.

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Experimental Part

Bio-assay. The assay based on electrically evoked contractions of the guinea pig ileum was performed as described elsewhere [15]. However, considerable variation in the IC50-values of the standard [Met^5]-enkephalin between individual preparations was observed (range of IC50-values: 3×10^{-8} to 1.2×10^{-7} M). A log-dose/response curve for [Met^5]-enkephalin was therefore determined with each individual ileum preparation and the observed IC50-value of the analogue being tested ($IC50_{obs}$) was normalized as follows:

$$IC50_{norm} = IC50_{obs} \frac{IC50_{std.\ mean}}{IC50_{std.\ obs}}$$

where $IC50_{std.\ mean}$ is the mean IC50 of the standard determined with a large number of ileum preparations and $IC50_{std.\ obs}$ is the IC50 of the standard observed with the ileum preparation used in the actual experiment.

Analytical. Microanalyses were performed in the Laboratorium für organische Chemie, ETHZ *(D. Manser).* Optical rotation was measured on a *Perkin-Elmer* polarimeter 141. TLC. was carried out on *Merck* silica gel plates using the solvent systems indicated in [6].

Preparation of the dipeptides **1**,**2**,**3**,**4** *and* **1'**,**2'**,**3'**,**4'**. – *General procedure.* The solution of 620 mg (2 mmol) of Boc-Nip-OH [16] in 15 ml of DMF is treated with 2 mmol of one of the following four compounds H-Leu-OMe·HCl, H-Leu-NH_2·HCl, H-Met-NH_2·HCl, or H-Ada-NH_2·HCl [3] and neutralized with 0.25 ml (2 mmol) of *N*-ethylmorpholine. Coupling is then achieved with the help of 450 mg (2.2 mmol) of DCCI in the presence of 300 mg HOBt. The solution is stirred for 1 h at 0° and for 16 h at RT. A few drops of acetic acid are added and DCU is eliminated by filtration at 0°. After evaporation of the solvent, the residue is taken up and washed in ethyl acetate with 5% $NaHCO_3$-, 5% $KHSO_4$- (pH2) and satd. NaCl-solutions. Because of the poor solubility of **2** and **3** in ethyl acetate, large volumes of the organic phase have to be chosen in order in minimize losses. The dried product is recrystallized from ethyl alcohol/ether (**2** and **3**) or from ethyl acetate/ether (**1** and **4**). Yield: 70–72% (**2** and **3**), 80–85% (**1** and **4**). The product is taken up in 0.4 N HCl in HCOOH (1.5 mol-equiv.). In the case of **2**, 0.75 ml mercaptoethanol are previously added to the formic acid. The solvents are cautiously evaporated at 30° *in vacuo* and the product suspended in ether and centrifuged three times. Recrystallization from methanol/pentane (**2** and **3**). Reprecipitation by diisopropylether from the solution in 2-propanol. Yield: 90–95%.

Preparation of the protected pentapeptides **II** *and* **III**. Boc-Tyr-ala-Gly-OH (409.4 mg, 1 mmol) is dissolved in DMF (15 ml) and **2'** or **3'** (1 mmol) is added. Coupling is performed after neutralization with 1 mmol of *N*-ethylmorpholine in the presence of 1 mol-equiv. of HOBt and of 1.1 mol-equiv. of DCCI, the latter being added at 0°. The reaction mixture is kept for 1 h at 0° and for 16 h at RT. Then it is treated with a few drops of acetic acid that help the formation of DCU from DCCI. The precipitate is filtered off after and the solvent is evaporated *in vacuo*. As the residue is not soluble in ethyl acetate or cloroform, it is stirred in 50 ml ethyl acetate for 10 min at 40°, and separated from the mother liquor by filtration. HOBt and residues of Boc-Tyr-ala-Gly-OH and of DCU are well removed in this way with practically no loss of **II**. The precipitate is then recrystallized from methanol/ethyl acetate (**II**) or from methanol/chloroform (**III**). Yield: 75–82%.

Preparation of the protected pentapeptides **I** and **IV**. These compounds are obtained by the same coupling procedure form Boc-Tyr-ala-Gly-OH and from **1'** and **4'**, respectively. The crude residue from the DMF evaporation is soluble and can be washed in ethyl acetate successively with 5% $NaHCO_3$-, $KHSO_4$- (pH 2) and satd. NaCl-solutions. The dried crude product is then recrystallized from ethanol/water (**I**) or from methanol/chloroform (**IV**). Yield: 78–86%.

Preparation of the pentapeptides hydrochlorides **II'**, **IV'** and **V'**. Final deprotection is achieved by dissolving 50 mg samples of **II**, or **IV** 0.4 N HCl in HCOOH (0.5 ml) and keeping the solution for 20 min at RT. The product is then precipitated by addition of 10 ml ether and gathered by centrifugation. Suspension in ether and centrifugation is repeated 3-times. The deprotected peptide is dissolved in alcohol and precipitated by addition of ether. Yield: 95%.

Preparation of the pentapeptide hydrochloride **I'**. After dissolving 364 mg (0.5 mmol) of **I** in 4 ml methanol/dioxane 1:1 2 ml 1 N NaOH are added. The solution is kept 15 min at RT, then cooled to 0° and put to pH 2 with 0.1 N HCl. Threefold extraction into 10 volumes of ethyl acetate affords a compound that is pure on TLC.: Rf 0.76 (B/A/W 72:7:21), 0.69 (C/M 1:1). Boc elimination on 50 mg portions of this product is then performed as for **II'** and **IV'**. Overall yield: 64%

Preparation of the pentapeptide hydrochloride **III'**. Because of the presence of methionine, some precautions must be taken for final deprotection. After dissolving 150 mg (0.2 mmol) **III** in 5 ml TFA/mercaptoethanol 20:1 the solution is kept 20 min at RT., then the solvent is evaporated at 0° and the residue dissolved in methanol (2 ml) and treated with 2 N HCl (0.11 ml). The product is precipitated by addition of ether and gathered by centrifugation. It is further purified by column chromatography on *Sephadex G 10* (∅ 1.5 × 50 cm) in water. Light and oxydative conditions are avoided. The pure fractions are lyophilized, redissolved in methanol and reprecipitated by ether. Yield: 58%.

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