SYNTHESIS OF THE TRITIUM LABELLED ß-CASOMORPHINE ANALOGUES ^{3}H-PHE-PRO-GLY-OH AND $^{3}H_2$-TYR-PRO-^{3}H-PHE-PYRROLIDIDE

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Summary

The precursor peptides H-Phe(I)-Pro-Gly-OH (III) and H-Tyr(I_2)-Pro-Phe(I)-pyrrolidide (VIII) were synthesized by stepwise elongation from the C-terminal end and by coupling of Boc-Tyr(I_2)-Pro-OH with H-Phe(I)-pyrrolidide and following deprotection of the Boc-residue respectively. Catalytic dehalotritiation yielded tritated peptides with specific radioactivities of 450 and 1500 GBq/mmol respectively. Cleavage of $^{3}H_2$-Tyr-Pro-^{3}H-Phe-pyrrolidide by dipeptidylpeptidase IV resulted in fragments with specific radioactivities of 950 ($^{3}H_2$-Tyr-Pro) and 590 GBq/mmol (^{3}H-Phe-pyrrolidide).

Key words: ß-Casomorphine analogues, catalytic dehalotritiation

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Nomenclature is in accordance with the JUPAC-JIB rules: Biochem. J. 219, 345 (1984)

Abbreviations: Boc, tert.-butyloxycarbonyl; -ONb, 4-nitrobenzylester; MA, mixed anhydrid method; THF, tetrahydrofuran; AcOH, acetic acid; TEA, triethylamine; NEM, N-ethylmorpholine,; IBCF, isobutyl chlorformate

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Introduction

For investigations of the peptide transport at the intestinal epithelium, which had been prompted by the recent finding of specific binding sites for ß-casomorphins at the intestinal epithelial cell layer /1/, the ß-casomorphine analogues ^{3}H-Phe-Pro-Gly-OH and $^{3}H_2$-Tyr-Pro-^{3}H-Phe-pyrrolidide had to be synthesized. The preparation and dehalotritiation of the corresponding iodinated precursors is described in the present study.
Additional experiments were performed for comparison with the results of analogous earlier peptide tritiations /2,3/ and modeldeuterations /4-6/ and to obtain information about a possible mutual influence of the two halogenated amino acids contained in one of the precursors on the labelling result. Such influence is implied by strongly reduced specific radioactivities obtained after several dehalotritiations of precursorpeptides containing more than one halogenated amino acid /7-10/. On the other hand also the theoretical specific radioactivities were achieved in tritiation experiments of this kind /8, 11-14/.

Results and discussion

For the preparation of III we applied a stepwise elongation (figure 1) by using 4-nitrobenzylester for carboxyl protection. The elongation was accomplished by the mixed anhydride procedure. After alkaline hydrolysis and deprotection of the Boc-group with hydrogen chloride in acetic acid the tripeptide H-Phe(I)-Pro-Gly-OH was isolated as hydrochloride.

The route of synthesis for (VIII) is represented in figure 2. Boc-Tyr(I_2)-OH was condensed with L-proline by the 2,4,5-trichlorophenylester procedure. The resulting Boc-Tyr(I_2)-Pro-OH was coupled with H-Phe-pyrrolidide using the mixed anhydride method. After deprotection with hydrogen chloride in dioxane the tripeptide pyrrolidide was characterized as hydrochloride.

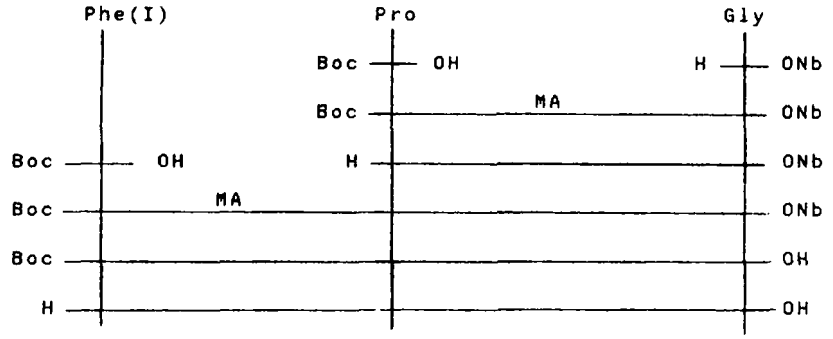

Fig. 1. Synthesis scheme of H-Phe(I)-Pro-Gly-OH

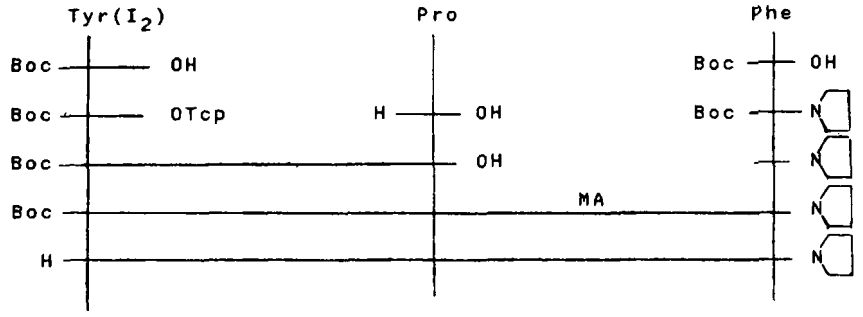

Fig. 2. Synthesis scheme of Tyr(I_2)-Pro-Phe(I)-pyrrolidide

The results of the tritiation experiments are collected in the table. From the total specific radioactivities achieved no influence of the presence of the second halogenated amino acid in VIII on the tritiation rate can be derived.

The cleavage of $^3H_2$-Tyr-Pro-^{3}H-Phe-pyrrolidide by dipeptidylpeptide hydrolase (DPIV) and ascertaining the specific radioacitvites of the fragments revealed different tritiation results for the halogenated Phe- and Tyr-residues related to the number of exchanged iodine atomes (table). This finding corresponds with the results of previous modeldeuterations of 4-I-Phe- and 3,5-I^2-Tyr-derivatives /4,5/. The found specific radioactivities of the fragments are well explainable by the isotopic effects k_H/k_D derived for the deuteration of the Phe- and Tyr-derivatives (about

Table: *Catalytic tritiation of H-Phe(I)-Pro-Gly-OH (III) and H-Tyr(I_2)-Pro-Phe(I)-pyrrolidide (VIII) using 10 % Pd on Al_2O_3 and $^3H_2$ containing 15 % $^1H_2$*

peptide	solvent (/ul)	mg catalyst/ /umol peptide	^{3}H-activity in the reaction solution (in the solvent) (GBq)	specific radioactivity (GBq/mmol) of ^{3}H pept. total	$^3H_2$-Tyr-Pro[a)]	^{3}H-Phe-Pro[a)]
III	H_2O/Et_3N	2.5/1.8	5.7	460		
	500/0.4		(4.9)			
VIII	$DMA/H_2O/Et_3N$		10.0			
	100/400/0.4	2.5/0.6	(9.0)	1540	950	590
VIII	DMA/Et_3N		8.1			
	500/0.4	2.5/0.6	(7.3)	1400	840	560
VIII	DMA/Et_3N	2.5/3.0	13.0	1440	860	580
	500/0.4		(8.5)			

a) obtained after cleavage by DP IV

3 and 6 respectively /4,5/), if these values are modified by the exponential factor of 1.4 in analogy to /15/ and taking into account the hydrogen content of 15% in the used tritium gas.

The difference found between the specific radioactivities of ^{3}H-Phe-Pro-Gly-OH and the ^{3}H-Phe-pyrrolidide fragments (table) should be caused by a varying direct transfer of solvent hydrogen to the substrate during the catalytic tritiation in accordance with results of earlier studies /2-6/. For an explanation on the basis of the commonly preferred indirect mechanism by dilution of the reacting gas with solvent hydrogen, a difference in the enrichment of the tritium gas by solvent hydrogen of about 5% would be needed (taking into account an isotopic effect of 6 as above). But the found solvent radioactivity (table) corresponds in all cases to a total dilution of the tritium gas by solvent hydrogen of only 1%. Based on this interpretation the higher specific radioactivity of the ^{3}H-Phe-Pro-Gly-OH corresponds to a reduced incorporation of solvent hydrogen which should be explainable in analogy to model results /6/ by a stronger competition of VIII with the solvent for the reaction with the activated catalyst. Precondition for such an explanation is a higher affinity of VIII compared to that of III to the activated catalyst, which is implied by strongly different reaction times needed for quantitative dehalodeuterations of both precursor peptides (less than 5 min. for VIII compared to 30 min. for III using the conditions given in the experimental part).

In the same way, an extremely high affinity of VIII to the catalyst would explain also the finding that, in contrast to model deuterations /4-6/ and foregoing peptide tritiations /2,3/, no clear influence of the catalyst-to-substrate ratio and of the type of the solvent on the tritiation result could be observed (table).

Experimental

Materials and methods

TLC was performed on silicagel 60 plates (Merck) preferably using the following solvent systems: (A) n-butanol/acetic acid/water/ethyl acetate 1/1/1/2, (B) n-butanol/acetic acid/water/ethyl acetate /1/1/1/1 (C) sec.-butanol/formic acid/water 75/15/20, (D) n-butanol/pyridine/acetic acid/water 30/20/6/24, (E) n-butanol/pyridine/acetic acid/water 10.5/6/1/7.5, (F) phenol/water (w/w) 3/1, (G) chloroform/methanol 9/1, (H) benzene/acetone/acetic acid 25/10/0.5, (I) ethyl acetate/pyridine/acetic acid/water 90/15/4.5/8.3. Compounds were visualized by UV, ninhydrin and KI/starch.

Dimethylacetamide (Merck,Schuchard) was distilled an stored over molecular sieves 10X and 3A before use. Tritium gas (^{1}H-content about 15%, estimated by means of an ionisation chamber) was purchased from Techsnab-export (SSSR) and stored in the form of uranium tritide.

Palladium on alumina (10%) catalyst (Engelhard, Hannover) was of the same batch as that used in foregoing peptide tritiations /2,3/. Dipeptidylpeptidase IV (26 units/mg) was prepared from porcine kidney according to (20).

Fluorescence measurements were performed with a specol-spectrometer (Carl Zeiss, Jena) equipped with a fluorescence additive.

Analytical HPLC characterization was carried out on a Licrocarb RP 18, 5 µm, column (125 x 4 mm) using the following mobile phases: (1) a linear gradient of A: 0.1% TFA in water and B: 70% acetonitrile in 0.1% TFA; (2) acetonitrile 33.6%/ 0.05 m KH_2PO_4(KOH), pH 7.6, 66.4 %; at a flow rate of 1 ml/min.

MS/FAB analysis was performed on a TSQ 700 spectrometer (Finnigan MAT) using a glycerol matrix and xenon at 8 keV.

Peptide synthesis

L-4-Iodophenylalanyl-L-prolyl-glycine

Boc-Phe(I)-Pro-Gly-ONb (I)

391 mg (1mmol) Boc-Phe(I)-OH /16/ were dissolved in 6 ml THF, cooled to -15 °C treated with 0.13 ml (1mmol) NEM and 0,13 ml (1 mmol) IBCF followed by 344 mg (1 mmol) H-Pro-Gly-ONb·HCl /17/ and 0,13 ml (1mmol) NEM after an activating time of 10 min. After 1 h at -15 °C the mixture was stirred 5 h at room temperature and then concentrated in vacuo. The residue was dissolved in ethyl acetate and washed with brine, 5% $KHSO_4$, brine, saturated $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness. The resulting oil was purified by dissolution in ethyl acetate and precipitation by diisoproyl-petrol ether, followed by colomn chromatography on silicagel using benzene-acetone-acetic acid (25:10:0,5) elution: 310 mg (55%), R_fG 0,65;R_fH 0,32; R_fI 0,84

Boc-Phe(I)-Pro-Gly-OH (II)

A solution of 70 mg (0,1 mmol) I in 5 ml acetone and 0,21 ml 0,5 n NaOH in nitrogen atmosphere was stirred 4 h at room temperature, and then the acetone evaporated. The residue was diluted with saturated $NaHCO_3$ solution, extracted with ethyl acetate, acidified with $KHSO_4$ (pH 3) repeatedly extracted with ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated to dryness. Crystallization from ethyl acetate-petrol ether yielded 44 mg (78%) of the desired product.
R_fG 0,31; R_fH 0,15; R_fI 0,31

H-Phe(I)-Pro-Gly-OH·HCl (III)

The Boc protecting group was removed by treating II (44 mg, 0.08 mmol) with 0.5 ml 1 n HCl/AcOH for 30 min. The product was

precipitated by addition of ether: 35 mg (87%), m.p. 149-152°C, $/\alpha/^{20}_D$ - 7.75° (c=1, AcOH); R_fB 0.61; R_fC 0.46; R_fD 0.60 hplc (1) t_R = 15.4 min (97 %), MS/FAB: 446.0 (MH^+). Calcd. 445.3 (M)

L-3,5-Diiodotyrosyl-L-prolyl-L-4-iodophenylalanin-pyrrolidide

Boc-Tyr(I_2)-Pro-OH (IV)

170 mg (0.24 mmol) Boc-Tyr(I_2)-OTCp /18/, 27 mg (0.2 mmol) 1-hydroxy-benzotriazole and 42 ul (0.3 mmol) L-proline. After stirring for 1 h at 0 °C and 5 h at room temperature, the solvent was evaporated in vacuo. The residue was diluted with $NaHCO_3$ solution. After extraction with ethyl acetate, the aqueous solution was acidified with $KHSO_4$ (pH 3) and then extracted with ethyl acetate again. The organic phase was washed with brine and dried over Na_2SO_4. The solvent was removed and the crude product was crystallized from ethyl acetate-petrolether and afterwards from diisopropylether. Yield 106 mg (70%); m.p. 194-198 °C; R_fG 0,39; R_fH 0.44; R_fI 0.45

Boc-Phe(I)-pyrrolidide (V)

To a mixture of 587 mg (1.5 mmol) Boc-Phe(I)-OH /19/ and 0,19 ml (1.5 mmol) NEM in 10 ml THF, cooled to -15 °C, 0.19 ml (1.5 mmol) IBCF were added, followed by 107 mg (1.5 mmol) pyrrolidine after an activating time of 8 min. After stirring for 1 h at -15 °C and 5 h at room temperature the work-up was performed as described above for I. Crystallization from diisoproylether yielded 400 mg (60 %) of the desired product; m.p. 114-118 °C: R_fG 0.66; R_fH 0.40; R_fI 0.84

H-Phe(I)-pyrrolidide.HCl (VI)

280 mg (0.63 mmol) V were treated with 3 ml 4 n HCl in ethyl acetate. After 30 min the solution was concentrated in vacuo and

the product crystallized from ethyl acetate: 232 mg (97%); m.p. 225-229 °C; R_fB 0.59; R_fC 0.38; R_fD 0.70

Boc-Tyr(I_2)-Pro-Phe(I)-pyrrolidide (VII)

To a stirred solution of 106 mg (0.17 mmol) IV and 21.3 ul (0.17 mmol) NEM in 5 ml THF, cooled to -15 °C, 21.8 µl (0.17 mmol) IBCF and after 10 min 64 mg (0.17 mmol) VI and 21.3 µl (0.17 mmol) NEM were added. After 1 h at -15 °C the mixture was stirred 3 h at room temperature and worked up as described above for I. Crystallization from ethyl acetate-petrol ether yielded 118 mg (74 %); m.p. 112 °C decomp.; R_fG 0.59; R_fH 0.30; R_fI 0.69

H-Tyr(I_2)-Pro-Phe(I)-pyrrolidide·HCl (VIII)

118 mg (0.12 mmol) VII were dissolved in 2 ml 4 n HCl in dioxane. After 30 min at room temperature the product was precipitated by addition of ether. Yield 100 mg (91%); m.p. 156 °C decomp.; $[\alpha]_D^{20}$ -19.3 ° (c=1, AcOH); R_fB 0.64; R_fC 0.35; R_fD 0.58

hplc (2) t_R = 9.0 min (98 %), MS/FAB: 857.0 (MH^+), Calcd. 856.3 (M)

Tritium labelling

For all tritiation experiments the same batch of tritium gas and catalyst was used.

To obtain information about the minimum catalyst-to-substrate ratio needed for quantitative dehalogenation, the halogenated peptides were dissolved in DMA or water according to their solubility and treated with deuterium at normal pressure in the presence of different catalyst amounts. The results were evaluated by TLC (solvent systems A and C). A twofold of the catalyst-to-substrate-ratio, which led to quantitative dehalogenation of Phe(I)-Pro-Gly-OH after about 30 minutes was used for the tritiation of this precursor. For the purpose of comparison, this catalyst-to-substrate-ratio was also used for the tritiation of the other

precursor peptide, although in this case clearly lower catalyst amounts were sufficient to achieve a quantitative dehalogenation.

For the tritiation, the reaction vessel containing the dissolved precursor peptide and the catalyst (see tabel) was connected to the tritiation manifold, cooled with liquid nitrogen and subsequently evacuated (0.1 Pa). After introduction of tritium gas (0.3 mmol) the reaction mixture was agitated by means of a magnetic stirrer for 30 minutes at ambient temperature and a tritium pressure of about 60 kPa.

After stopping the reaction, the catalyst was centrifuged off and washed with 10 ml water. From the combined filtrate a sample was taken for counting the solvent radioactivity, and afterwards the solution was freeze-dried 4 times using water to remove labile tritium. The remaining solids were dissolved in water and then purified by TLC (solvent system A).

The tritiated peptides were eluted from the silicagel using water, and were stored at -20 °C in water/ethanol 1/1 at a radioactive concentration of about 150 MBq/ml. Related to the used amount of the halogenated peptide, yields of about 40% were obtained after the TLC-purification.

The products have been proved to be radiochemically pure to more than 90 % and to be identical with Phe-Pro-Gly-OH and Tyr-Pro-Phe-pyrrolidide, respectively, by TLC using the solvent system A-E and radioscanning.

In order to estimate the specific radioactivity, 5-20 MBq of the purified labelled peptides was dissolved in 2.0 ml of 0.05 m borate buffer pH. To this solution 0.5 ml of a solution of fluram ^{R} in dioxane (2 mg/10 ml) was added under shaking, and the intensity of the fluorescence at 365 nm was measured within 30 minutes. In the same way calibration values were obtained using 5-50 nanomoles of the unlabelled reference peptides.

Cleavage by DP IV (dipeptidylpeptide hydrolase [EC 3.4, 14.5.]) /19,20/

To a solution of about 1.5 MBq of the labelled Peptide and 100 µg of Tyr-Pro-Phe-pyrrolidide · HCl in 120 µl H_2O were added 12 µl of DP IV-solution (0,8 mg/ml 70% $(NH_4)_2SO_4$). After 40 h standing at ambient temperature no starting peptide could be detected and the mixtures were separated by TLC using solvent F. The two resulting radioactive fragments, which have been proved by TLC to be identical with the references Tyr-Pro and Pro-pyrrolidide (R_F-values 0.48 and 0.76 respectively), were eluted from the silicagel using methanol. The radiochemical purity of the eluted fragments was higher than 90 % according to TLC checking. The specific radioactivity was determined by the fluram method as described above. From the ratios of the values obtained for both fragments in connection with the specific acitivity of the labelled starting peptide the specific activities shown in the table were calculated.

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