TRITIUM-LABELLING IN TWO PHENYLALANINE RESIDUES OF NORLEUCINE^{11}-SUBSTANCE P

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SUMMARY

Norleucine^{11}-substance P and 4-chlorophenylalanine $^{7.8}$-Norleucine^{11}-substance P were synthesized by fragment condensation. Phe/^{3}H/$^{7.8}$-norleucine^{11} -substance P was obtained by catalytic tritiation of the precursor peptide with a specific radioactivity of 27 Ci/mmole and full biological activity.

Recently we reported the tritium labelling of the modified partial sequence of substance P, acetyl-Lys-Phe/^{3}H/-Phe-Gly-Leu-NleNH$_2$*, by catalytic tritiation of the corresponding synthetic Cpa-containing precursor peptide /1/. The labelled peptide was purified using preparative paper electrophoresis and possessed a specific radioactivity of 23 Ci/mmole /1/ - a surprisingly high labelling rate for the tritiation of a 4-chlorophenylalanine peptide. The labelled compound had high biological activity in vivo but in receptor studies no specific binding

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* Symbols and abbreviations are used according to the rules of the IUPAC IUB Commission on Biochemical Nomenclature, J.Biol.Chem. 247, 977 /1972/; Cpa=4-clorophenylalanine, Dit=3.5-diiodotyrosine. All amino acid derivatives are of the L-configuration.

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was found /2/.It seemed necessary to conduct further binding studies with the tritiated substance P undecapeptide. Additionally this highly labelled undecapeptide is expected be useful in studying the metabolism of substance P, as well.

$$\underset{\substack{1\quad 2\quad 3\quad 4\quad 5\quad 6\quad 7\quad 8\quad 9\quad 10\quad 11\\ \text{substance P}}}{\text{Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met}NH_2}$$

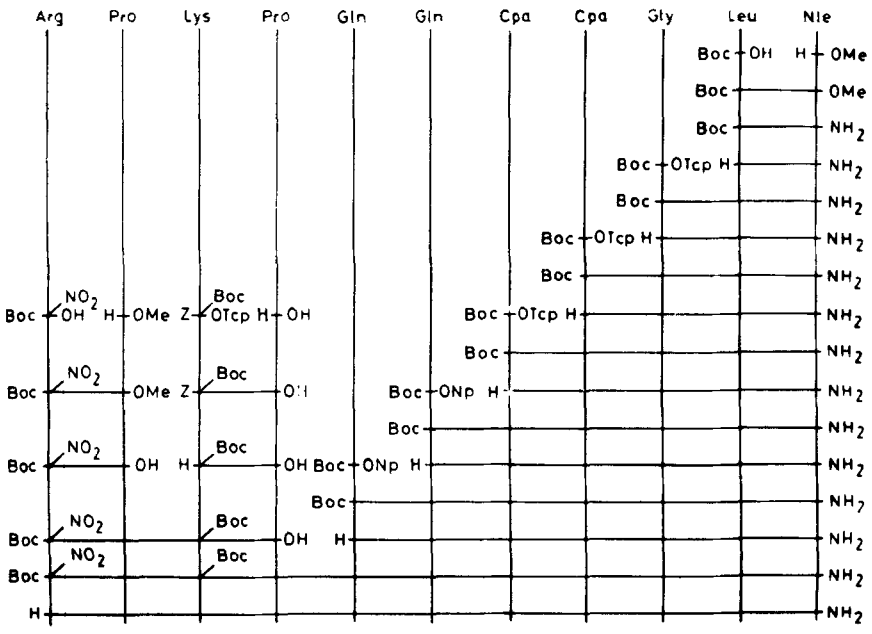
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-NleNH_2 I

Arg-Pro-Lys-Pro-Gln-Gln-Cpa-Cpa-Gly-Leu-NleNH_2 II

Arg-Pro-Lys-Pro-Gln-Gln-Phe/^{3}H/-Phe/^{3}H/-Gly-Leu-NleN_2 III

The successful synthesis of the tritium labelled hexapeptide prompted us to use a 4-chlorophenylalanine precursor for labelling the undecapeptide, as well. Catalytic dehalogenation of 4-chlorophenylalanine in peptides containing methionine residues may involve difficulties. To avoid possible complications we substituted methionine in substance P by norleucine. The synthesis of Nle^{11}-substance P /I/ and Cpa$^{7.8}$-Nle^{11}-substance P /II/ was achieved by condensation of the fragments Boc--Arg/NO_2/-Pro-Lys/Boc/-Pro-OH /3/ with H-Gln-Gln-Phe-Phe-Gly-Leu-NleNH_2 or H-Gln-Gln-Cpa-Cpa-Gly-Leu-NleNH_2 as shown in figure 1 for the synthesis of compound II.

Figure 1



Synthesis of Cpa$^{7.8}$-Nle^{11}-substance P, II

The synthesis of the heptapeptides was accomplished starting from H-Gly-Leu-NleNH$_2$.HCl /1/ by stepwise chain elongation according to the synthesis of native substance P as described earlier /3/ /4/. Phenylalanine and 4-chlorophenylalanine were coupled as Boc-aminoacid--2.4.5-trichlorophenyl esters. The pentapeptide Boc-Cpa-Cpa-Gly-Leu--NleNH$_2$ was synthesized alternatively via mixed anhydride coupling of Boc-Cpa-Cpa-Gly-OH with H-Leu-NleNH$_2$.HCl. The introduction of Boc--glutamine giving the hexa- and heptapeptides was achieved by the mixed anhydride method or by using Boc glutaminyl-p-nitrophenyl ester. The protected tetra- and heptapeptides were condensed by the mixed anhydride method with a high yield. After treatment with hydrogen fluoride, the undecapeptides I and II have been purified by partition chromatography. All protected intermediates which have been synthesized are summarized in table 1 /see experimental section/.

The prepared Nle^{11}-substance P/I/when tested for its smooth muscle activity /guinea pig ileum/ was comparable with the methionine containing sequence, in agreement with the observation of VAN RIETSCHOTEN et al. /5/

The precursor peptide Cpa $^{7.8}$-Nle^{11}-substance P /II/ was dehalogenated with tritium gas in the presence of palladium/alumina catalyst as described earlier /6/. The amino acid analysis after total hydrolysis of a sample reflected the correct ratio of amino acids and the absence of 4-chlorophenylalanine. By both thin layer chromatography and electrophoresis about 90% of the total radioactivity migrated with the expected undecapeptide III. Purification of the labelled peptide for use in biological experiments was achieved by subsequent paper electrophoresis in aliquots. To calculate the specific radioactivity, the concentration of the peptide in solution was determined using both amino acid analysis and fluorescence measurement after reaction with fluorescamine /7/. The specific radioactivity calculated by these two methods was with good correlation found to be 27 Ci/mmole. The labelled peptide was stored in aqueous ethanol /1:1/ in a concentration of 10^{-4}M at -25°C.

Whereas the hexapeptide acetyl-Lys-Phe/^{3}H/-Gly-Leu-NleNH$_2$ was relatively stable when stored in methanolic solution at -20°C /1/, the undecapeptide showed considerable radiolysis under these conditions. By electrophoretic evaluation the radioactivity corresponding to III decreased to 70, 30 and 17% after one, seven and ten months of storage respectively and one slower moving second peak appeared in increasing amounts. The separation of III from the product of radiolytic

decomposition was performed by preparative paper electrophoresis.

No difference has been found in guinea pig ileum contraction between the labelled compound III and the unlabelled I. Results of binding studies and the investigation of the enzymatic degradation will be published elsewhere /8/.

EXPERIMENTAL SECTION

Materials and methods

Thin layer chormatography was carried out on silica gel plates /Kavalier, Czechoslovakia/ using ninhydrine and chlorine-tolidine reagents and the solvent systems:

A n-Butanol-acetic acid-water 4-1-1
B Chloroform-methanol-water 40-15-5
C Ethyl-acetate-pyridine-acetic acid-water 120-20-6-11
D n-Butanol-pyridine-acetic acid-water 21-12-2-14
E Chloroform-methanol-acetic acid 95- 5 3

Silicagel G plates /Merck, GFR/ were used for thin layer radiograms, which were measured by a scanner from Berthold and Friesecke /GFR/. Electrophoresis was performed on paper type FN 7 /VEB Papierwerk Niederschlag, GDR/ in 7% acetic acid at 23 V/cm for 1 hr. In analytical experiments the radioactive samples were mixed with 10^{-8} mole unlabelled peptide I-dissolved in 20 µl 0.1 M acetic acid - and placed on FN 7 paper, impregnated with 7 % acetic acid.After development the paper strips were cut into 0.5 cm wide sections and counted in a liquid scintillation counter /Wallac 81 000, LKB, Sweden/. For amino acid analysis, samples were hydrolyzed with 6 n hydrochloric acid and analysed by an Amino Acid Analyzer AAA 881 /Microtechna,Praha,CSSR/. For the detection of 4-chlorophenylalanine, sodium citrate pH 6.45, 0.6n sodium chloride was used as third buffer /in a one column system/ at a temperature of 85°C. Under these conditions 4-chlorophenylalanine is eluted after lysine.In the case of the labelled peptide III the eluate was collected /3.5 ml fractions/ after passing the photo cell and aliquots were counted. To determine the concentration of the radioactive peptide III in solution, the reaction with fluorescamine /Fluram,La Roche, USA, 15 mg/100 ml acetone/ was used according to /7/. The concentration dependence of the fluorescence was measured with peptide I /dissolved in 0.1n borate buffer pH7.8/ in the range of 0.13 to 13 nmole and was found to be linear. The fluorescence

measurements were carried out using a"Spekol" /VEB Carl Zeiss,Jena,GDR/ spectrophotometer fitted with the fluorescence equipment FK /365 nm/. Ultraviolet absorption spectra were taken in a "SPECORD-UV-VIS" /VEB Carl Zeiss Jena, GDR/ Scanning spectrophotometer.

Tritium gas was purchased from Technabexport /USSR/ and stored in the form of uranium tritide. 10 % palladium/alumina catalyst was a product of ENGELHARD /Italy/.

Peptide synthesis

The synthesis of the protected N-terminal tetrapeptide Boc--Arg/NO_2/-Pro-Lys /Boc/-Pro-OH is described elsewhere /3/. Boc-Cpa-OTcp, H-Leu-Nle-NH_2, H-Gly-Leu-NleNH$_2$, H-Phe-Gly-Leu-NleNH$_2$ and H-Phe-Phe-Gly-Leu-NleNH$_2$ were prepared earlier /1/. The preparation of the hexa- and heptapeptides, the fragment condensations, deprotection and purification by ion exchange-or partition chromatography in the system n-butanol/pyridine/ 1 % acetic acid=5:3:11 of the undecapeptides was achieved according to the syntheses of native substance P /3/. Some data of the peptides I and II and all intermediates not described earlier are summarized in table 1.

Table 1 Synthesized substance P-undecapeptides and intermediates

Compound /Formula/	M_p /°C/	α_D^{24}	R_f A[a]	B	C	D	E
Boc-Cpa-Gly-Leu-Nle-NH_2 /$C_{28}H_{44}N_5O_6Cl$/	211-213 /decomp./	-20,8 c=0,5, AcOH	0,86	0,88	0,76		
Boc-Cpa-Gly-OMe /$C_{17}H_{23}N_2O_5Cl$/	108-110	-8,5 c=1,0 DMF	0,80	0,90	0,89		
Boc-Cpa-Cpa-Gly-OMe /$C_{26}H_{31}N_3O_6Cl_2$/	211-214 /decomp./	-14,0 c=0,5, DMF	0,88	0,94	0,91		
Boc-Cpa-Cpa-Gly-OH /$C_{25}H_{29}N_3O_6Cl_2$/	192-198	-13,5 c=1,0 DMF	0,89	0,92	0,68		
Boc-Cpa-Cpa-Gly-Leu-Nle-NH_2 /$C_{37}H_{52}N_6O_7Cl_2$/	235-243 /decomp./	-33,3 c=0,5, DMF	0,87	0,92	0,79		
Boc-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 /$C_{42}H_{62}N_8O_9$/	235-238	-36,6 c=0,5, DMF	0,81	0,87	0,47		0,18
Boc-Gln-Cpa-Cpa-Gly-Leu-Nle-NH_2 /$C_{42}H_{60}N_8O_9Cl_2$/	247-253 /decomp./	-41,6 c=0,5, DMF	0,83		0,57	0,9	0,20
Boc-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 /$C_{47}H_{70}N_{10}O_{11}$/	260-265 /decomp./	-35,8 c=0,5, AcOH	0,73	0,52	0,19		
Boc-Gln-Gln-Cpa-Cpa-Gly-Leu-Nle-NH_2 /$C_{47}H_{68}N_{10}O_{11}Cl_2$/	260-270 /decomp./	-26,0 c=0,5, AcOH	0,76	0,55	0,23	0,95	
Boc-Arg/NO_2/-Pro-Lys/Boc/-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 /$C_{74}H_{115}N_{19}O_{19}$/	260-262 /decomp./	-41,6 c=0,25, AcOH	0,40	0,51	0,12		
Boc-Arg/NO_2/-Pro-Lys/Boc/-Pro-Gln-Gln-Cpa-Cpa-Gly-Leu-Nle-NH_2 /$C_{74}H_{113}N_{19}O_{19}Cl_2$/			0,43				
H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH_2 b/ /$C_{64}H_{100}N_{18}O_{13}$/	amorph.	-53,0 c=0,25, AcOH				0,32	
H-Arg-Pro-Lys-Pro-Gln-Gln-Cpa-Cpa-Gly-Leu-Nle-NH_2 b/ /$C_{64}H_{98}N_{18}O_{13}Cl_2$/	amorph.	-48,0 c=0,5, AcOH				0,34	

a/ Solvent systems see experimental section

b/ Correct amino acid analysis was obtained

Arg-Pro-Lys-Pro-Gln-Gln-Phe/^{3}H/-Phe/^{3}H/-Gly-Leu-NleNH$_2$,III

3.2 mg /ca 2 μmole/ lyophylized II were dissolved in 500 μl water and 2 μmole triethylamine were added. The solution was frozen using liquid nitrogen and the catalyst /50 mg/ was placed on to the solid. The reaction vessel was connected to the tritiation manifold /6/. After saturating the catalyst with tritium gas the frozen mixture was melted and stirred at room temperature for 50 min. The catalyst was removed by filtration /Millipore filter, 0.22 μm/ and washed several times with 1 % acetic acid. Exchangeable tritium was removed by repeated rotary evaporations. The remaining solid was dissolved in 5 ml ethanol/water 1:1 and stored at -25°C.

Rf/A/ 0.05; the radiogram shows a single peak, corresponding to the spot dedectable with ninhydrine. Rf/D/ 0.32 /Merck plate/; the radiogram shows a strong peak/Rf 0.32/ and two additional weak peaks with Rf 0.25 and Rf 0.40. Rf/D/ 0.41 /Silufol plate/. Electrophoresis: Using ninhydrine reagent a strong spot - migrating 8 cm cathodally - and a weak spot /5.5 cm/, representing 5 % of the radioactivity of the major peak were detected. The amount of the minor component increased up to 50 % of the total radioactivity due to radiolytic decomposition within ten months.

In all systems used compounds I and III were shown to be identical. Amino acid analysis: Arg 0.88 /1/, Lys 1.15 /1/1, Pro 2.00 /2/, Glu 2.20 /2/, Phe 1.70 /2/, Gly 1.20 /1/, Leu 1.06 /1/, Nle 1.15 /1/; Cpa was not present. After counting the phenylalanine eluates /38.2 nmole/ a specific radioactivity of 28 Ci/mmole was calculated. By means of the fluram method the peptide content of the stock solution was determined to be 0.27 μmole/ml and a specific radioactivity of 26 Ci/mmole was calculated. For purification 200 μl of the stock solution /ca.50 nmole/ were subjected to electrophoresis; the paper strip containing III was eluted with 2 ml ethanol/water 1:1.

Biological evaluation of the material in the guinea pig ileum test found it to be identical with compound I.

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