SYNTHESIS OF 6-(N^{α}-ACETYL-LYSYL)-11-NORLEUCINE-SUBSTANCE P-(6-11)-HEXAPEPTIDE LABELLED WITH TRITIUM ON THE PHENYLALANINE RESIDUE IN POSITION 7

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SUMMARY

N^{α}-Ac-Lys-(Phe-4-^{3}H)-Phe-Gly-Leu-Nle-NH_2, a modified Substance P fragment with full activity on smooth muscle has been prepared by catalytic tritiation of the corresponding precursor peptide containing p-chlorophenylalanine in position 7. The precursor peptide has been synthesized by stepwise couplings from the C-terminal end, using t-butyloxycarbonyl-amino acid 2,4,5-trichlorophenyl esters. The specific radioactivity of the tritiated product obtained was 23 Ci/mmole.

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For binding experiments between Substance P peptides (1,2) and subfractions of smooth muscle cells in the physiological concentration range, it was necessary to use a synthetic analogue with high specific radioactivity, possessing at the same time pronounced biological activity.

Recently we have reported that C-terminal hexapeptides and acyl derivatives of similar sequences exhibit high potency in contracting guinea pig ileum as well as other smooth muscle systems (3,4). The possible formation under the experimental conditions of a pyroglutamyl derivative excludes the use of the hexapeptide Gln-Phe-Phe-Gly-Leu-Met-NH_2 (2, Figure 1) for this purpose. As known from our previous work with sequences of eledoisin and physalaemin (4), replacement of glutamine by an N-terminal acetyl-lysyl residue enhances smooth muscle activity and should also increase the resistance against proteolytic degradation.

Tritium labelling can be successfully effected by catalytic exchange of an iodine substituent in tyrosine residues (5,6). Peptides containing 3,5-dibromotyrosine or p-chlorophenylalanine are also useful intermediates (7,8). The successful synthesis of ^{3}H-bradykinin (9) using a precursor containing p-chlorophenylalanine (Phe(Cl)) prompted us to use this method for the labelling of Substance P. Although tritiation of dibromotyrosine^2-α-melanotropin takes place readily (7), catalytic dehalogenation of p-chlorophenylalanine in peptides containing methionine residues may involve difficulties. To avoid possible complications, we substituted methionine in the native sequence by the norleucine residue, on the basis of an observation (10) that norleucine^{11}-Substance P is equipotent with the methionine containing native peptide in the secretion test on rat salivary glands. Replacing methionine by norleucine in the C-terminal

pentapeptide gives compound 3 with a small smooth muscle (guinea pig ileum) activity comparable with that of the pentapeptide having the native sequence. Introduction of the N^{α}-acetyl-lysyl residue increases the activity yielding ED_{50} values similar to that of the unmodified hexapeptide 2 or the intact Substance P undecapeptide 1.

		$ED_{50} \cdot 10^{-8}$ M
1	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	0,5
2	H-Gln-Phe-Phe-Gly-Leu-Met-NH_2	1,0
3	H-Phe-Phe-Gly-Leu-Nle-NH_2	200
4	H-Lys-Phe-Phe-Gly-Leu-Nle-NH_2	2,8
5	Ac-Lys-Phe-Phe-Gly-Leu-Nle-NH_2	1,0
6	Ac-Lys-Phe(Cl)-Phe-Gly-Leu-Nle-NH_2	3,7

Figure 1: Biological activity (isolated guinea pig ileum) of some peptides related to Substance P

Since Substance P does not contain tyrosine residues, but there are two phenylalanines in the C-terminal hexapeptide, a p-chlorophenylalanine analogue as precursor peptide for labelling with tritium seemed to be promising. Synthesis of the $[N^{\alpha}\text{-Ac-Lys}^{6},\text{Phe(Cl)}^{7},\text{Nle}^{11}]$-Substance P-(6-11)-hexapeptide amide was achieved as shown in Figure 2.[x]

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[x] All amino acid derivatives mentioned in this paper are of the L-configuration. Symbols and abbreviations are used according to the tentative rules of the IUPAC IUB Commission on Biochemical Nomenclature, J.Biol.Chem. 247:977 (1972). DMF stands for dimethylformamide.

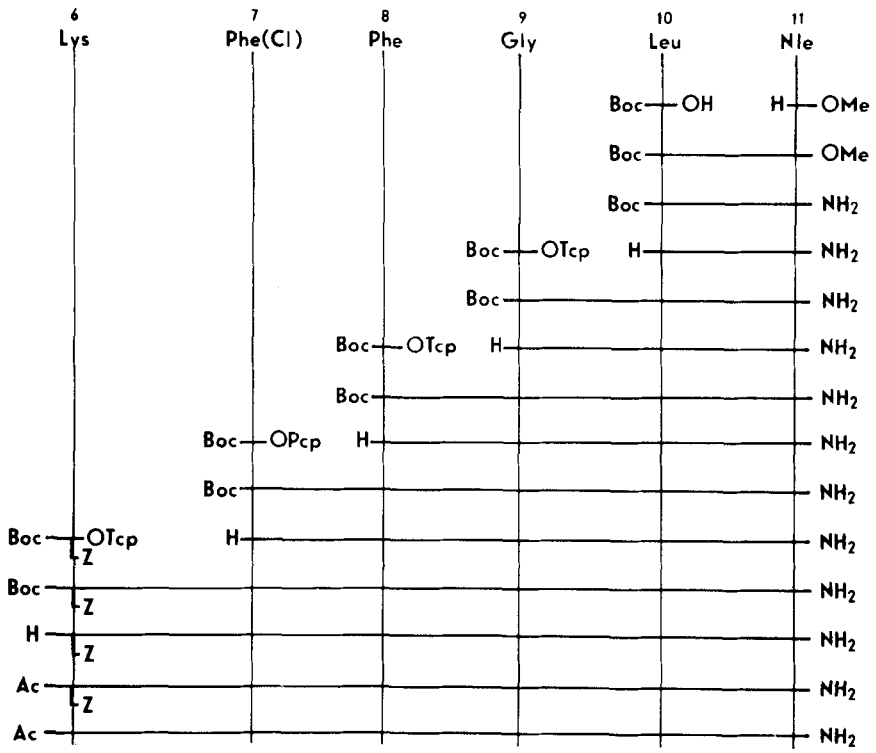

Figure 2: Synthesis of $\left[N^{\alpha}\text{-Ac-Lys}^{6},\text{Phe(Cl)}^{7},\text{Nle}^{11}\right]$-Substance P--(6-11)-hexapeptide amide

Boc-Leu-Nle-NH_2 was obtained by the condensation of Boc-Leu with Nle-OMe using the mixed anhydride procedure, followed by ammonolysis. After deblocking with hydrogen chloride in acetic acid, elongation of the peptide chain was accomplished by acylation with t-butyloxycarbonylamino acid active esters in a stepwise manner. 2,4,5-Trichlorophenyl esters were used in the case of glycine, phenylalanine and lysine, while pentachlorophenyl ester was applied when p-chlorophenylalanine was built into the peptide chain. After the terminal Boc-group had been removed, the acetyl group was introduced by acetylation with p-nitrophenyl acetate. HBr in acetic acid was used for removal of the ε-carbobenzyloxy substituent. The precursor peptide obtained in this way was tritiated according to the procedure described by Girma et al. (6).

Amino acid analysis after total hydrolysis of the labelled compound revealed the absence of p-chlorophenylalanine, and reflected the correct 2:1:1 proportion of Phe:Leu:Nle. The specific radioactivity of the tritiated Substance P fragment analogue was 23 Ci/mmole, calculated from the activity of phenylalanine present in the hydrolysis product. The amino acids Lys, Leu, Gly and Nle contained less than 1% of the total radioactivity.

The yield of the tritiation step was about 40%. The specific radioactivity is comparable with values obtained in the iodine-tritium exchange experiments (5,6), although, taking into consideration the much lower substitution rate of the chlorine atom, a more extensive tritium-hydrogen exchange with the solvent would not have been surprising. These results are in accord with those reported for the tritiation of $Phe(Cl)^5$-bradykinin (9), indicating again the applicability of p-chlorophenylalanine as a

precursor amino acid for stable labelling with tritium.

No radioactive contaminants in the labelled peptide could be detected by thin-layer chromatography, but in paper electrophoresis an impurity migrating to the anode appeared. The latter method was used for the preparative purification of the tritiated compound. The pure material was stored in methanolic solution in a concentration of 10^{-4} M at -20°C; no decomposition was observed after six months storage under these conditions.

The tritiated fragment analogue exhibits the same biological potency on guinea pig ileum as does the unlabelled hexapeptide 5.

EXPERIMENTAL

Thin layer chromatography was carried out on silica gel plates (Kavalier-Works, CSSR) using ninhydrin and chlorine-tolidine reagents and the solvent systems: A = butanol-acetic acid-water (2:1:1), B = hexane-benzene-ethyl acetate (3:3:1), C = ethyl acetate-pyridine-acetic acid-water (60:20:6:11), D = ethyl acetate-pyridine-acetic acid-water (120:20:6:11), E = chloroform--methanol-acetic acid (95:5:3). Thin layer radiograms were carried out on Silica Gel G plates (Merck, GFR) and measured by a scanner from Berthold and Friesecke (GFR).

Electrophoresis was performed on paper type FN 7 (Niederschlag, GDR) in 7% acetic acid for 1 hr at 23V/cm. Radioactive samples were mixed with 10^{-8}M inactive peptide 5 and placed on FN 7 paper, impregnated with 7% acetic acid. For counting the strips were cut and measured in a liquid scintillation counter (Wallac 81000, LKB). For amino acid analysis, samples were hydrolyzed with 6N HCl and analyzed by the AAA881-analyzer (Microtechna, CSSR). Biological tests were performed according to (11).

The peptides 3, 4 and 5 in Figure 1 were synthesized as given for compound 6 in Figure 2: Phe-Phe-Gly-Leu-Nle-NH_2. HCl (3), $C_{32}H_{47}N_6O_5Cl$ (631,2), m.p. 138-144°, $[\alpha]_D^{20}$ = -65° (c = 0.25, DMF); Lys-Phe-Phe-Gly-Leu-Nle-NH_2. 2HCl (4), $C_{38}H_{60}N_8O_6Cl_2$ (795,9), m.p. 171-176°, $[\alpha]_D^{20}$ = -12° (c = 0.5, DMF); (Boc-Lys(Boc)-OTcp (12) was used for the introduction of Lys); Acetyl-Lys-Phe-Phe-Gly-Leu-Nle--NH_2. HCl (5), $C_{40}H_{61}N_8O_7Cl$ (801,4), m.p. 167-177°, $[\alpha]_D^{20}$ = -45° (c = 0.25, DMF), amino acid analysis: Lys 0.98, Phe 2.03, Gly 1.00, Leu 0.99, Nle 1.00, (Z-Lys(Boc)-OTcp (13) was used for the introduction of Lys, followed by hydrogenolysis, acetylation with p-nitrophenyl acetate and treatment with hydrogen chloride in acetic acid).

t-Butyloxycarbonyl-p-chlorophenylalanine 2,4,5-trichlorophenyl ester

To a solution of Boc-Phe(Cl) (8) (1.00 g, 3.33 mmoles) and 2,4,5-trichlorophenol (790 mg, 4.00 mmoles) in 25 ml ethyl acetate dicyclohexylcarbodiimide (760 mg, 3.66 mmoles) was added at -5°C. The solution was stirred at -5°C for 1 hr and at room temperature for 10 hr. After filtration the solvent was removed in vacuo. The crystalline residue was recrystallized from ethyl acetate-hexane to yield 1.37 g (87%) ester, m.p. 143-145°, R_f(B) 0.68, $[\alpha]_D^{20}$ = -40° (c = 1, DMF). Calcd. for $C_{20}H_{19}NO_4Cl_4$ (479,2) : C 50.13, H 4.00, N 2.92, found: C 50.04, H 3.82, N 2.85 %.

t-Butyloxylcarbonyl-p-chlorophenylalanine pentachlorophenyl ester

This compound was prepared as described for the 2,4,5--trichlorophenyl ester. The yield after recrystallisation from ethyl acetate was 80%, m.p. 182-186°, R_f(B) 0.65, $[\alpha]_D^{20}$ = -37° (c = 1, DMF). Calcd. for $C_{20}H_{17}NO_4Cl_6$ (548.1) : C 43.83, H 3.13, N 2.56, found: C 43.62, H 3.03, N 2.27 %.

t-Butyloxycarbonyl-leucyl-norleucine methyl ester

To a solution of Boc-Leu (14) (6.94 g, 30 mmoles) and

N-ethyl-morpholine (3.78 ml, 30 mmoles) in 50 ml tetrahydrofuran isobutylchloroformate (4.12 ml, 30 mmoles) were added at -15°C. After stirring for 5 min a mixture of Nle-OMe (15) (5.45 g, 30 mmoles) and N-ethyl-morpholine (3.78 ml, 30 mmoles) in 50 ml DMF, chilled to -20°C was added. The temperature was kept at -10°C for 30 min. The stirring was continued for 2 hr at room temperature. After evaporation in vacuo the residue was dissolved in ethyl acetate-water. The organic layer was washed with 5% $KHSO_4$-solution, $NaHCO_3$-solution, water and dried over sodium sulfate. After evaporation the residue was crystallized from ethyl acetate-petroleum ether. Yield 7.2 g (67%), m.p. 98-101°, R_f(D) 0,8, $[\alpha]_D^{20} = -28°$ (c = 1, DMF). Calcd. for $C_{18}H_{34}N_2O_5$ (358.5): C 60.31, H 9.56, N 7.82, found: C 59.90, H 9.42, N 7.87 %.

t-Butyloxycarbonyl-leucyl-norleucine amide

Boc-Leu-Nle-OMe (5.30 g, 14.8 mmoles) was dissolved in 160 ml methanol, saturated with ammonia. The solution was allowed to stand at room temperature for three days. After evaporation in vacuo a crystalline residue was obtained, which was recrystallized from ethanol. Yield 4.55 g (90%), m.p. 172-174°, R_f(E) 0.5, $[\alpha]_D^{20} = -31°$ (c = 1, DMF). Calcd. for $C_{17}H_{33}N_3O_4$ (343.5): C 59.45, H 9.68, N 12.23, found: C 59.41, H 9.64, N 11.89 %.

t-Butyloxycarbonyl-glycyl-leucyl-norleucine amide

Boc-Leu-Nle-NH_2 (2.60 g, 7.54 mmoles) was stirred with 15 ml 2N hydrogen chloride/acetic acid for 20 min at room temperature. The deprotected peptide was isolated by addition of 150 ml ether, filtration and drying over KOH pellets in vacuo. The yield was nearly quantitative. M.p. 127-130°, R_f(D) 0.36, $[\alpha]_D^{20} = +28°$ (c = 1, DMF). H-Leu-Nle-NH_2.HCl (2.00 g, 7.14 mmoles) was coupled with Boc-Gly-OTcp (13) (2.78 g, 7,85 mmoles) in the presence of triethylamine (1.08 ml, 7.85 mmoles) in 15 ml DMF. For isolation the reaction product was poured into the mixture of 100 ml N-acetic

acid and 25 ml cyclohexane. The precipitate was filtered off, triturated with N acetic acid, water and ether to give a powder, which was crystallized from acetic acid by dropwise addition of water. Yield 2.06 g (74%), m.p. 169-171°, R_f(A) 0,85, $[\alpha]_D^{20} = -16°$ (c = 1, DMF), calcd. for $C_{19}H_{36}N_4O_5$ (400,5): C 56.98, H 9.06, N 13.99, found: C 56.47, H 8.92, N 13.68 %.

t-Butyloxycarbonyl-phenylalanyl-glycyl-leucyl-norleucine amide

Boc-Gly-Leu-Nle-NH_2 (2.00 g, 5 mmoles) was deprotected as described above, yielding H-Gly-Leu-Nle-NH_2. HCl, m.p. 210-212°, R_f(A) 0.60, $[\alpha]_D^{20} = -31°$ (c = 1, DMF). H-Gly-Leu-Nle-NH_2. HCl (1.30 g, 3.86 mmoles) was coupled with Boc-Phe-OTcp (13), as described for Boc-Gly-Leu-Nle-NH_2 in detail. Yield 1.78 g (85%), m.p. 202-212°, R_f(D) 0.83, $[\alpha]_D^{20} = -28°$ (c = 1, DMF). Calcd. for $C_{28}H_{45}N_5O_6$ (547.7): C 61.40, H 8.28, N 12.79, found: C 61.18, H 8.07, N 12.29 %.

t-Butyloxycarbonyl-p-chlorophenylalanyl-phenylalanyl-glycyl-leucyl--norleucine amide

H-Phe-Gly-Leu-Nle-NH_2. HCl (385 mg, 0.8 mmoles), obtained by treatment of Boc-Phe-Gly-Leu-Nle-NH_2 with hydrogen chloride/acetic acid, m.p. 141-144°, R_f(D) 0.55, $[\alpha]_D^{20} = +11°$ (c = 1, DMF) was converted into the protected pentapeptide using Boc-Phe(Cl)-OPcp (482 mg, 0.88 mmoles) under the conditions mentioned above. Yield 494 mg (85%) amorphous powder, m.p. 229-231°, R_f(E) 0.5, $[\alpha]_D^{20} = -13°$ (c = 0.5, DMF). Calcd. for $C_{37}H_{53}N_6O_7Cl$ (730.3): C 60.85, H 7.31, N 11.51, found: C 60.31, H 7.45, N 11.17 %.

(N^{α}-t-Butyloxycarbonyl-N^{ε}-benzyloxycarbonyl)-lysyl-p-chlorophenyl-alanyl-phenylalanyl-glycyl-leucyl-norleucine amide

Boc-Phe(Cl)-Phe-Gly-Leu-Nle-NH_2 was deprotected by hydrogen chloride/acetic acid, m.p. 184-194°, R_f(D) 0.50, $[\alpha]_D^{20} = -34°$ (c = 0.5, DMF). H-Phe(Cl)-Phe-Gly-Leu-Nle-NH_2.

HCl (167 mg, 0.25 mmoles) was coupled with Boc-Lys(Z)-OTcp (13) (157 mg, 0.28 mmoles), as described above, yielding 220 mg (79%), m.p. 229-234°, R_f(D) 0.74. Calcd. for $C_{51}H_{71}N_8O_{10}Cl$ (991.7): C 61.77, H 7.22, N 11.30, found: C 61.04, H 7.12, N 11.16 %

N^{α}-Acetyl-N^{ε}-benzyloxycarbonyl-lysyl-p-chlorophenylalanyl-phenylalanyl-glycyl-leucyl-norleucine amide

Boc-Lys(Z)-Phe(Cl)-Phe-Gly-Leu-Nle-NH_2 (198 mg, 0.2 mmoles) was treated with hydrogen chloride/acetic acid and isolated in the usual manner, m.p. 202-209°, R_f(D) 0.4. To the solution of this N^{α}-deprotected hexapeptide (104 mg, 0.112 mmoles) in 3 ml DMF triethylamine (0.016 ml, 0.112 mmoles) and p-nitrophenyl acetate (22.3 mg, 0.123 mmoles) were added. After stirring overnight the solvent was removed in vacuo and the residue was triturated with $NaHCO_3$-solution, 1N acetic acid, water and finally with ether. The amorphous product was reprecipitated from DMF-ether. Yield 87 mg (82%), m.p. 218-225°, R_f(D) 0.7, calcd. for $C_{48}H_{65}N_8O_9Cl$ (933.6): C 61.75, H 7.02, N 12.00, found: C 61.12, H 6.81, N 11.56 %.

N^{α}-Acetyl-lysyl-p-chlorophenylalanyl-phenylalanyl-glycyl-leucyl-norleucine amide hydrobromide (6)

Deprotection of Ac-Lys(Z)-Phe(Cl)-Phe-Gly-Leu-Nle-NH_2 (80 mg) was performed by treatment with hydrogen bromide/acetic acid for 30 min. The product was precipitated by addition of ether, and reprecipitated from DMF-ether, yielding 70 mg amorphous powder, m.p. 255-260°C, R_f(A) 0.75, R_f(C) 0.6, R_f(D) 0.2, $[\alpha]_D^{20} = -35°$ (c = 0.5, DMF) amino acid analysis: Lys 0.93, Phe(Cl) 1.00, Phe 1.01, Gly 0.94, Leu 1.07, Nle 1.00, $C_{40}H_{60}N_8O_7ClBr$ (880.4).

N^{α}-Acetyl-lysyl-[phenylalanyl-4-^{3}H]-phenylalanyl-glycyl-leucyl--norleucine amide

N^{α}-Acetyl-Lys-Phe(Cl)-Phe-Gly-Leu-Nle-NH_2. HBr (2.5 mg)

was dissolved in 0.2 ml DMF and 0.8 ml water. The solution was frozen using liquid nitrogen and covered with 10% palladium/aluminium oxide (50 mg; Engelhardt, Rome). The reaction vessel was connected with a tritiation equipment (16). After saturation of the catalyst with tritium gas the solvent was thawed and the mixture stirred for 60 min at 500 mmHg pressure at room temperature. The catalyst was filtered off (Millipore filter, 0.22 /um), washed with 50 ml 0.1 M acetic acid and the solvent was evaporated in vacuo. After repeated evaporations the product was dissolved in a small volume of DMF and diluted with 5 ml methanol. The radiogram in system A shows a single peak (R_f 0.75). In electrophoresis one contamination, migrating to the anode appeared. Purification was performed by electrophoresis of ca. 10^{-7} moles tritiated peptide, followed by elution of the separated spot with 0.1 M acetic acid. Counting of the Phe-eluates obtained after amino acid analysis indicates a specific radioactivity of 23 Ci/mmoles. Other amino acids than Phe contain less than 1% of the total activity. No Phe(Cl) was detectable.

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REFERENCES

1. Lembeck F. and Zetler G. - Int. Encycl. Ther. Sect. 72, Vol. 1, p. 29, Pergamon Press, New York, 1972
2. Leeman S.E. and Mroz E.A. - Life Sci. 15: 2033 (1974)

3. Bergmann J., Bienert M., Niedrich H., Mehlis B. and Oehme P. - Experientia 30: 401 (1974)
4. Niedrich H., Bienert M., Mehlis B., Bergmann J. and Oehme P. - Acta biol. med. germ. 34: 483 (1975)
5. Morgat J.L., Lam Thanh H., Cardinaud R., Fromageot P., Bockaert J., Imbert M. and Morel F. - J. Label. Comp. 6: 276 (1970)
6. Girma J.P., Morgat J.L., Fromageot P., Dubrasquet M., Accary J.P., Vatier J. and Bonfils S. - Biochimie 56: 763 (1974)
7. Nikolics K., Teplán I. and Medzihradszky K. - J. Label. Comp. Radiopharm. 12: 163 (1976)
8. Teplán I., Mező I., Seprődi J. and Kovács J. - Acta Chim. Acad. Sci. Hung., in press
9. Seprődi J., Teplán I. and Medzihradszky K. - 12. Internat. Symposium "Radioaktive Isotope in Klinik und Forschung", Bad Gastein, 1975
10. Van Rietschoten J., Tregear G.W., Leeman S., Powell D., Niall H. and Potts J.T.,Jr. - Proc. 13th European Peptide Symp., Rehovot, 1974, p. 113 (Ed. by Wolman Y.), Wiley, New York, 1975.
11. Oehme P., Bergmann J., Falck M., Reich J.G., Vogt W.E., Niedrich H., Pirrwitz J., Berseck Ch. and Jung F. - Acta biol. med. germ. 28: 109 (1972)
12. Mehlis B., Apelt M., Bergmann J. and Niedrich H. - J. prakt. Chemie 314: 390 (1972)
13. Broadbent W., Morley J.S. and Stone B.E. - J. Chem. Soc. C., 2632 (1967)
14. Schnabel E. - Liebigs Ann. Chem. 702: 188 (1967)
15. Flowers H.M. and Reith W.S. - Biochem. J. 53: 657 (1953)
16. Márton J. and Kovács Á. - Acta Chim. Acad. Sci. Hung. 73: 11 (1972)