Synthesis and tritium labeling of the highly potent mast cell-degranulating substance P analog H-Arg-Pro-Lys-Pro-NH-$C_{12}H_{25}$

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Summary

Tritium labeling of the mast cell degranulating substance P analog H-Arg-Pro-Lys(3,4-^{3}H-Pro)-NH-$C_{12}H_{25}$ by catalytic saturation of the dehydroproline (Dhp[1]) double bond is described. Catalytic tritiation in water afforded the radioactive analog with a specific activity of 1.07 TBq/mmol. Tenfold enhancement of the catalyst-to-substrate ratio resulted in a reduced specific activity of 0.74 TBq/mmol.

Introduction

Searching for the structural requirements of substance P-induced histamine release we found that the activity on rat peritoneal mast cells of six investigated analogs increases with the lipophilicity of the C-terminal heptapeptides /1,2/. A positively charged cluster in connection with a hydrophobic domain /3/ seems to be sufficient for mast cell degranulation with no indication for substance P-specific stereochemical requirements. This view was supported by testing analog VI, in which the C-terminal heptapeptide of substance P is replaced by the lipophilic dodecyl residue.

1) Abbreviations: Ac, acetyl; Dhp, L-3,4-dehydroproline; DMA, dimethylacetamide; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; MA, mixed anhydride coupling; NMM, N-methylmorpholine; ONSu, N-hydroxysuccinimide ester; TEA, triethylamine.

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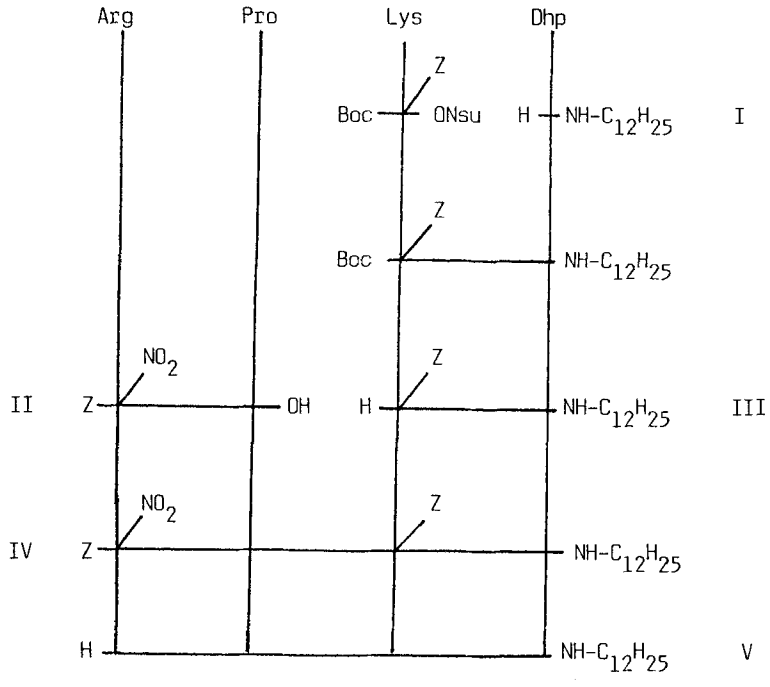
This analog was found to be much more active than substance P on rat peritoneal mast cells /1,2,4/ indicating that the existence of substance P-specific mast cell receptors is unlikely.

On-going studies on the peptide - mast cell interaction required synthesis of the highly potent tetrapeptide dodecylamide in its tritium labeled form. In this paper we describe the synthesis of H-Arg-Pro-Lys-(3,4-^{3}H)-Pro-NH-$C_{12}H_{25}$, VI, via the Dhp-containing precursor V. Dhp was shown to be a suitable precursor amino acid for tritium labeling of various proline containing peptides /5,6/.

Results and dicussion

Synthesis of the precursor peptide V is outlined in Scheme 1. Intermediate I was synthesized via the preformed HOBt ester which was obtained by use of the mixed anhydride procedure. This procedure circumvents formation of urethane by undesired attack at the wrong carbonyl group of the mixed anhydride /7/. Urethane amounts to about 30 % when the dipeptide is synthesized by normal mixed anhydride protocols. In addition lactam formation is negligible under these conditions in contrast to the situation when 2,4-dinitro-phenyl-ester or DCC mediated couplings /8/ are used.

The protected tetrapeptide IV was obtained by the mixed anhydride coupling of III with II. IV was purified by chromatography on silica gel. Deprotection of IV was accomplished by treatment with liquid hydrogen fluoride in the presence of anisole. In model experiments with Ac-Dhp-NH_2 we demonstrated that HF does not attack the double bond of dehydroproline under normal condition (1 h at + 2 °C). Moreover, investigation of the final product V by MS/FAB gave no evidence for addition of hydrogen fluoride to the Dhp-residue. Catalytic tritiation of the dehydroproline-containing peptide V in water at a molar ratio of Pd per mole of substrate of 0.8 gave a reaction product with a specific radioactivity of 1070 GBq/mmol.



Scheme 1: Synthesis of H-Arg-Pro-Lys-Dhp-NH-$C_{12}H_{25}$

In accordance with earlier model deuterations using N-acetyl-D,L-Dhp-amide and 10 % Pd/Al_2O_3 /9/ an increase in the amount of catalyst (mol Pd/mol substrate ratio of 8) caused a decrease in the specific radioactivity (740 GBq/mmol), probably due to a more pronounced catalyst-mediated transfer of solvent-hydrogen to the substrate. The values for tritium incorporation are compatible with assumption of complete saturation of the double bond, taking into account a hydrogen content of 10-20 % in the commercial tritium gas and an isotopic affect k_H/k_T of about 10 /9/.

It is noteworthy, that the tritium incorporation obtained in the Pro^4-residue of V using the above mentioned labeling conditions is significantly higher, than that recently reported for labeling of substance P and substance P (1-7) via Dhp^{2,4}-derivatives, reactions which were performed in ethanol as a solvent /10/.

Surprisingly in an initial labeling experiment of V using a mol Pd/mol substrate ratio of 0.8 and DMA as solvent a very low incorporation of

tritium (37 GBq/mol) was observed, probably due to incomplete saturation of the double bond. This result, when considered in connection with model experiments /9/, points to the requirement for higher catalyst/substrate ratios when the tritiation is carried out in DMA instead of water.

Experimental

Materials and methods

TLC was effected with Silica Gel 60 on precoated glass plates from Merck using as mobile phases the following solvent systems: (1) ethyl acetate/pyridine/acetic acid/water= 120:20:6:11, (2) 60:20:6:11, (3) 240:20:6:11, (4) 30:20:6:11; (5) chloroform/ethanol= 8:2; (6) n-butanol/pyridine/acetic acid/water= 10.5:6:1:7.5, (7) n-hexane/ethyl acetate= 1:1. Detection of TLC spots followed visualization by ninhydrin or Cl_2/benzidine.
Analytical HPLC characterization was carried out using a Shimadzu LC-6A system on Nucleosil C-18, 7 µm, column dimensions 250x4.0 mm using the following mobile phases : (1) Acetonitrile 33 %, 0.01 M NaH_2PO_4+0.15 M $NaClO_4$ pH 2.0 67 %; (2) linear gradient of A, 10 % acetonitrile/90 % 0.05 % TFA in water, and B, 90 % acetonitrile/10 % 0.05 % TFA in water. Electrophoresis was carried out on paper type FN 7 (VEB Papierfabrik Niederschlag, GDR) at 25 V/cm in 0.01 M ammonium acetate pH 7.4.
Tritium gas (tritium content 80-90 %) was purchased from Techsnabexport (USSR) and stored in the form of uranium tritide.
MS/FAB analysis was carried out on a ZABEQ spectrometer (VG Analytical, Manchester) with xenon at 8 kV as the bombarding gas. Fluorescence measurements were performed with a specol-spectrometer (VEB Carl Zeiss, Jena, GDR) equipped with a fluorescence additive.
Boc-Dhp and N-acetyl-Dhp-NH_2 were obtained according to ref. /11/ and /9/, respectively. The synthesis of Z-Arg(NO_2)-Pro-Lys(Z)-Pro-OH /12/ was accomplished as earlier described /13/.

Peptide synthesis

H-Dhp-NH-$C_{12}H_{25}$·HCl (I)

213 mg of Boc-L-Dhp (1 mmol) in 5 ml of THF were treated at -5 °C with 0.112 ml NMM and 0.136 ml isobutylchloroformate and after stirring for 5 min a solution of dodecylamine (200 mg, 1.07 mmol) was added. The solution was stirred at 0 °C for 30 min. After the normal work-up procedure 405 mg of a colorless oil was obtained, $R_f(3)=0.9$. The crude material was deprotected by treatment with 10 ml of 4N HCl/ethyl acetate for 20 min at room temperature. The solution was concentrated in vacuo and the remaining solid was recrystallized from dioxane. Yield 245 mg (77 %), $R_f(4)=0.3$, mp 82-85 °C, $|\alpha|_D^{20} = -148.9$ ° (c= 0.5, methanol), Analysis calcd for $C_{17}H_{32}N_2O$·HCl, C 64,43, H 10.50, N 8.84; Found C 64.13, H 10.54, N 8.64.

MS/FAB 281.3 (MN^+), calcd. for $C_{17}H_{32}N_2O$ 280.3.

H-Lys(Z)-Dhp-NH-$C_{12}H_{25}$·HCl (III)

358 mg of Boc-Lys(Z)-ONsu (0.75 mmol) were added to a solution of 220 mg (0.69 mmol) of I and 100 µl TEA in 5 ml DMF. The solution was stirred for 20 h. After normal work-up procedure the crude material was purified on silica gel 60 (40-63 µ). Elution with n-hexane/ethyl acetate 2:1, 1:1 and 1:2 gave 300 mg (68 %) pure Boc-Lys(Z)-Dhp-NH-$C_{12}H_{25}$ as a colourless resin, $R_f(7)=0.25$. The N -deprotection was accomplished by treatment with 4N HCl/ethyl acetate for 45 min, evaporation of the reagent and precipitation of the hydrochloride VII, dissolved in ethyl acetate, by addition of n-hexane. Yield 252 mg colourless resin, $R_f(1)=0.3$.

Z-Arg(NO_2)-Pro-OH (II)

To a solution of 13.64 g of Z-Arg(NO_2) (38.6 mmol) in 120 ml of DMF 5.35 ml of TEA (38.6 mmol) were added. At -15 °C isobutyl chloroformate (38 mmol) was added within 3 min under vigorous stirring. Subsequently, 5.74 g of HOBt (42.5 mmol), dissolved in 6 ml of DMF, were added and stirring was continued at -5 °C. After 1 h a solution of 6.22 g (54 mmol) L-proline in 70 ml of DMF/water 6:1 was added and the temperature was kept at 0 °C for

2 h. The solvent was removed in vacuo, the residue was dissolved in 100 ml CH_2Cl_2 and the solution was subsequently washed with 5 % aqueous $KHSO_4$ solution and saturated NaCl-solution. The organic layer was dried with $MgSO_4$. Filtration and evaporation of the solvent gave a resin which crystallized when triturated with methanol. The product was recrystallizid from 100 ml of methanol/water 1:1. Yield 12.3 g (70 %), R_f (1) 0.3, hplc (1) t_R 5.6 min (97 %), mp 107-111 °C, $|\alpha|_D^{20}$= -27.8 ° (c= 1, DMF) (ref. /14/: mp 119 °C, $|\alpha|_D^{22}$= -26.5 ° (c=2, DMF)

Analysis calcd for $C_{19}H_{26}O_7N_6 \cdot 1\ H_2O$ (468.4)

C 48.71, H 6.02, N 17.94,

Found: C 48.85, H 6.15, N 17.65

H-Arg-Pro-Lys-Dhp-NH-$C_{12}H_{25}$ (V)

124 mg of II (0.43 mmol) and 250 mg of III (0.43 mmol) were converted into the protected tetrapeptide IV via the mixed anhydride procedure and purified by chromatography on silica gel 60 (40-63 µ) in the solvent system $CHCl_3$/ ethanol 15:1, yielding 302 mg of a white powder, homogeneous in tlc, R_f (5) 0.6. 280 mg of VIII were deprotected by treatment with 5 ml of liquid hydrogen fluoride in the presence of 0.25 ml of anisole for 60 min at 2 °C. The hydrogen fluoride was evaporated and the residue triturated with ether. The product was isolated by rapid filtration as a white, hygroscopic powder which was dissolved in 20 ml of distilled water. The filtered solution was shown to be free of Arg(NO_2) and/or scavanger compounds by uv measurements. Lyophilization gave 165 mg of a white, fluffy powder which was used for tritium labeling experiments without further purification. R_f (6) 0.2, hplc (2) t_R= 20.3 min (94 %) (no separation of V and VI in this system $|\alpha|_D^{20}$= -154.7 ° (c= 0.5, 5 % acetic acid)
MS/FAB: 662.6 (MH^+), Calculated for $C_{34}H_{62}N_9O_4$, 661.5 (M).

H-Arg-Pro-Lys-Pro-NH-$C_{12}H_{25}$ (VI)

203 mg of Z-Arg(NO_2)-Pro-Lys(Z)-Pro-OH /13/ (0.25 mmol) in 5 ml of DMF were activated at -10 °C by addition of 30 µl of NMM and 38 µl of iso-

butyl chloroformate and converted into the amide by addition of a solution of 56 mg of dodecylamine hydrochloride and 30 µl of NMM in 6 ml of DMF. After the normal work-up procedure the crude material was chromatographed on a 2 x 50 cm silica gel (40-63 µ) column yielding 161 mg (66 %) of the protected tetrapeptide amide, R_f (5)= 0.75.

The deprotection was achieved by hydrogenation in the presence of 100 mg of 10 % palladium on charcoal in methanol/acetic acid/water 3:2:1 for 4 h. The completness of the reaction was minitored by uv measurements at 280 nm. The catalyst was removed by filtration, the filtrate evaporated to dryness and the residue dissolved in 2 ml of 0.01 M ammonium acetate buffer pH 6.0. The final purification was performed by chromatography on CM-cellulose (2x17 cm column, linear gradient of 200 ml 0.01 M ammonium acetate pH 6.0 and 200 ml 0.5 M ammonium acetate pH 6.0. Fractions containing pure V were collected, diluted with water and lyophilized (3 times) yielding 84 mg V as a white, hygroscopic powder, R_f (6) 0.2, hplc (2) t_R = 21.3 min (95 %), $|\alpha|_D^{20}$= -78.0 ° (c= 0.5, 5 % acetic acid), MS/FAB: 664.6 (MH^+), Calcd. 663.5 (M).

Tritium labeling

Precursor V and the Pd/Al_2O_3 10 % - catalyst (Engelhard, Hannover; FRG) were added to 0.5 ml of the solvent (mg precursor/mg Pd/Al_2O_3: 2,3/2,5 in demethylacetamide and 3,2/3,7 and 1,8/20 in water). The reaction vessel was connected to the tritiation manifold, cooled by liquid nitrogen and evacuated (p= 0.1 Pa). After tritium gas (0.3 mmol) had been introduced the reaction mixture was agitated by means of a magnetic stirrer at ambient tremperature and a tritium pressure of 60 kPa for 30 minutes. After stopping the reaction the catalyst was centrifuged and washed with 10 ml of water. The combined solutions were freeze-dried 4 times using water to remove labile tritium. The remaining solid was dissolved in water/ethanol 1/1 and purified by paper electrophoresis. The tritiated peptide was eluted from the paper using 30 % acetic acid and stored at -20 °C

in 30 % acetic acid/ethanol 1/1 at a radioactive concentration of about 50 MBq/m 1. It was shown by tlc-radioscanning to have a radioactive purity of about 90 %. In order to estimate the specific radioactivity, 5 - 20 MBq of the purified labeled peptide were dissolved in 2,0 ml 0.05 M borate buffer pH 8. To this solution 0,5 ml of a solution of fluram in dioxane (2 mg/10 ml) was added under shaking and the intensity of the fluorescence at 365 nm was measured within 30 minutes. Data were taken from a calibration curve obtained in the same manner using 5 to 50 nanomole samples of an Arg-Pro-Lys-Pro dodecylamide standard.

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