Synthesis of /Tyr-^{3}H/^{4}-angiotensin II and /Phe-^{3}H/^{8}-angiotensin II via halogen-derivatives of angiotensin II.

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Summary

A method is described for the tritium labelling of angiotensin II in two different positions. /Tyr-^{3}H/^{4}-angiotensin II and /Phe-^{3}H/^{8}-angiotensin II of high specific radioactivity have been prepared starting from Dbt^{4}-angiotensin II and Cpa^{8}-angiotensin II, respectively.

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--- 3.5-dibromo-L-tyrosine was abbreviated as Dbt, while 4-chloro--L-phenylalanine was abbreviated as Cpa.Other abbreviations used in the text are in accordance with the IUPAC-IUB Biochemical Nomenclature /1971/.All amino acids are of the L-configuration.

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The wide range of studies on the biological function of polypeptide hormones especially those related to their mechanism of action and metabolism are greatly supported by radioactively labelled polypeptide hormone derivatives. Tritium seems to be a reasonable choice for this purpose because of its advantageous properties. On the other hand it can be introduced into polypeptides without altering their primary structure. For this reason, we have /among other groups/ developed several methods for the tritium-labelling of polypeptide hormones.

In the present publication we report the syntheses of two tritium-labelled derivatives of angiotensin II: Asn^{1},/Tyr-^{3}H/^{4}, Val^{5}--angiotensin II /I/ and Asn^{1},Val^{5}, /Phe-^{3}H/^{8}-angiotensin II. /II/

One of the possibilites for the tritium-labelling of peptides is the incorporation of a corresponding tritium-labelled amino acid into the peptide chain by conventional methods of peptide synthesis. This method is theoretically applicable to any peptide.In an earlier work we have also applied this method for the labelling of angiotensin II /1/. Starting from tritium-labelled tyrosine, we obtained angiotensin II labelled in position 4.

However this method is greatly limited in specific radioactivity especially for reasons of radioisotope safety.

Peptides with higher specific radioactivity can be prepared by the widely applied and general method based on the iodination of the native peptide molecule followed by catalytic tritiation of the iodo-derivative.

In the case of angiotensin II the application of this method has yielded a preparation with high specific radioactivity /2/. In the search for new possibilities of the tritium-labelling of polypeptides, we have developed a general method based on the synthesis of peptides with amino acid derivatives containing groups that can be readily

tritiated. The precursor peptides obtained from the synthesis can be exchanged for or saturated with tritium.For this purpose the most appropriate amino acid derivatives are those containing halogen atoms in their aromatic rings. The first one applied in our laboratory was 3.5-dibromo-L-tyrosine, which we incorporated into polypeptides as reported earlier /3,4/.

For the tritium-labelling of angiotensin II we prepared Asn^{1},Dbt^{4}, Val^{5}-angiotensin II /III/. The preparation of p-chloro-L-phenylalanine /5/ opened the way to label angiotensin II in a different position. We synthesized Asn^{1}, Val^{5}, Cpa^{8}-angiotensin II /IV/ as a precursor for position 8. labelling. The great advantage of this labelling method is its applicability to peptides containing no tyrosine but phenylalanine residues. In this manner tritium labelled bradykinin /6/ and a substance P fragment /7/ could be prepared.

The syntheses of the two halogen-containing precursorpeptides followed conventional routes of peptide synthetic methods,choosing reaction conditions not effecting the halogen atoms. The two synthetic schemes are shown in the following figures. A detailed discussion of these syntheses is given elsewhere /3,8/:

The tritium exchange of the halogen atoms was accomplished by catalytic tritiation. Earlier experiments of tritiation in a special glass vacuum manifold /9/ in the presence of Pd/charcoal catalyst yielded peptides with relatively low, 1-2 Ci/mmole specific radioactivity.Since peptides of much higher radioactivity were being required, the advanced techniques of tritiation developed by Fromageot et al /lo/ were applied for the tritiation of the two halogen derivatives of angiotensin II. The catalytic tritiations

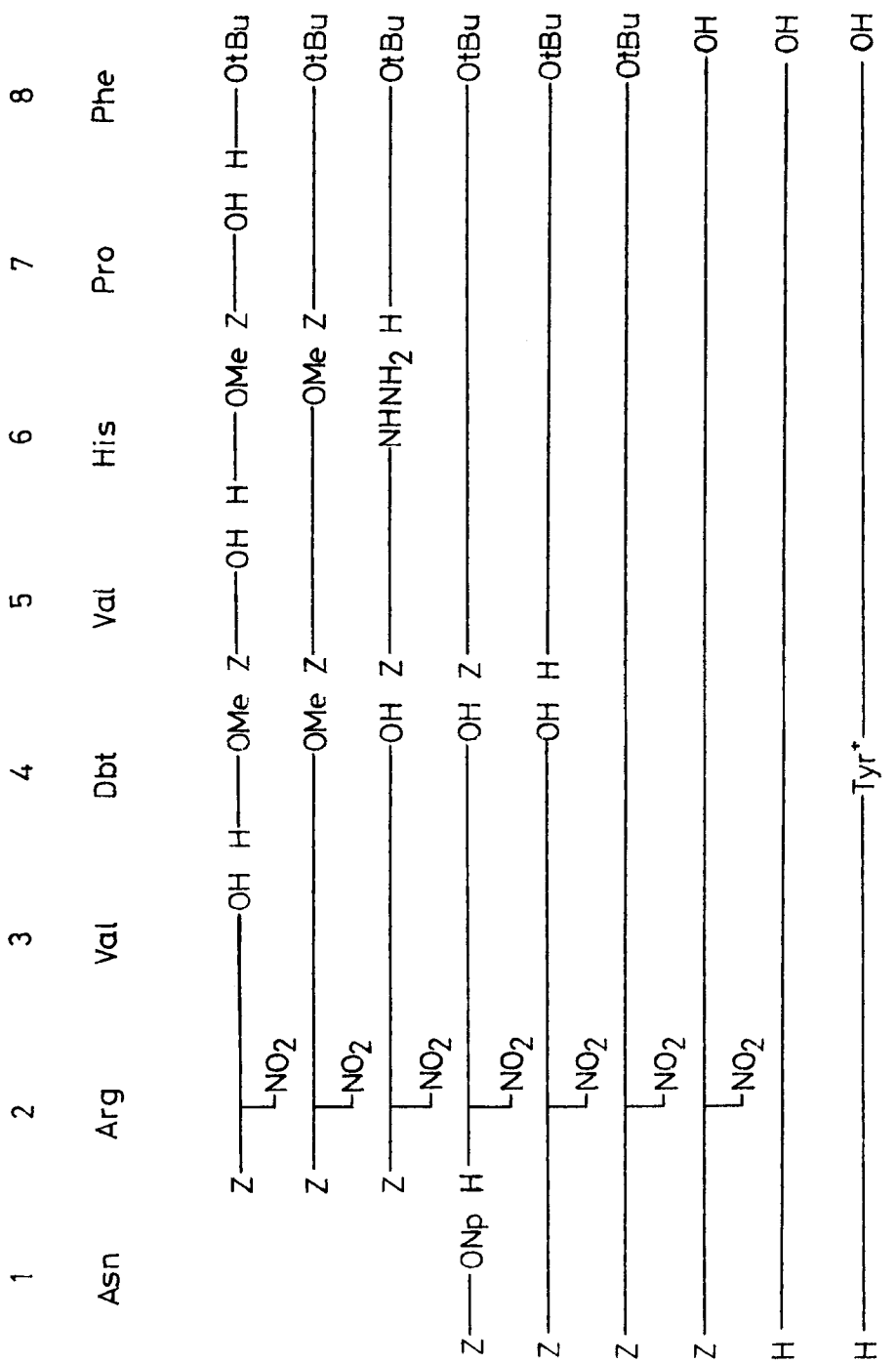

1 2 3 4 5 6 7 8
Asn Arg Val Dbt Val His Pro Phe
Tyr*
OH
OMe
NHNH2
ONp
NO2
OtBu
Z
H

Fig.1.

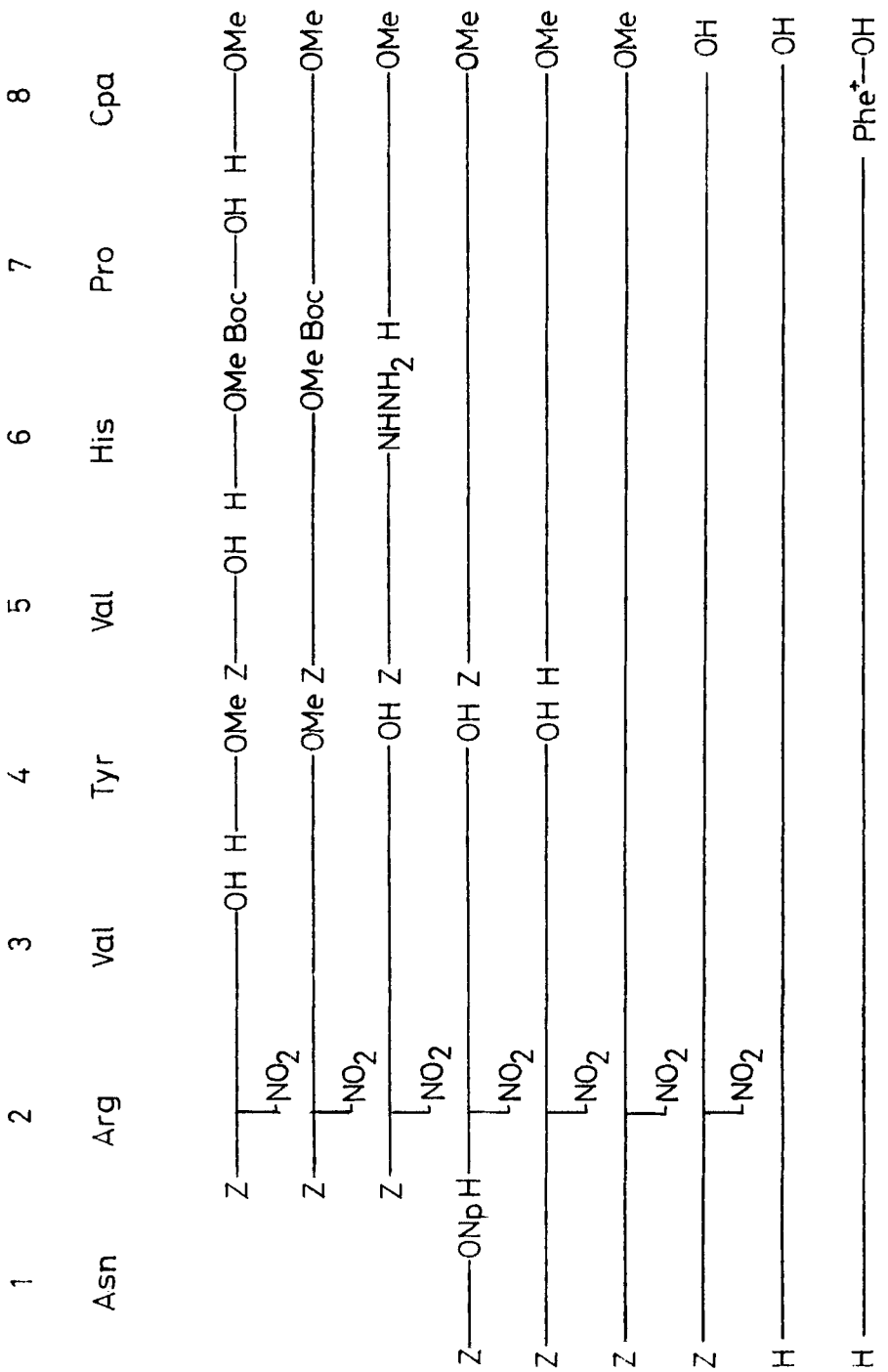

1 2 3 4 5 6 7 8
Asn Arg Val Tyr Val His Pro Cpa
Z NO2 OH H OMe Z OH H OMe Boc OH H OMe
Z NO2 OMe Z OMe Boc OMe
Z NO2 OH Z NHNH2 H OMe
Z ONp H NO2 OH Z OMe
Z NO2 OH H OMe
Z NO2 OMe
Z NO2 OH
H OH
H Phe+ OH

Fig.2.

were carried out in aqueous solutions in the presence of triethylamine and Pd/Al_2O_3 catalyst. The catalyst was saturated with tritium gas before mixing it into the reaction mixture. As a result of the application of this method of tritiation we managed to prepare peptide I with a specific radioactivity of 18-22 Ci/mmole, as well as peptide II with a specific radioactivity of 10-12 Ci/mmol. The tritiation carried out in the special apparatus developed in the laboratories of CEN in Saclay using CEA tritium gas yielded a preparation with especially high 29 Ci/mmole specific radioactivity.

The tritiation of peptide III introduces two tritium atoms into the angiotensin molecule, representing a theoretical specific radioactivity of 58 Ci/mmole. This high value has been reported in the case of tritiations of peptides containing diiodotyrosine /2,12/. In our case the highest specific radioactivity did not come near this value. This difference could be explained by the lower reacitvity of bromine and chlorine bound to aromatic rings as compared to iodine· therefore a longer reaction time is required. As a result of the exchange reactions proceeding parallel to tritiation, the tritium gas is diluted by hydrogen which also contributes to dehalogenation.

The tritium-labelled angiotensin II preparations exhibited full pressor activity against CIBA Hypertensin^R reference material in ganglion-blocked wistar rats.

Experimental Section

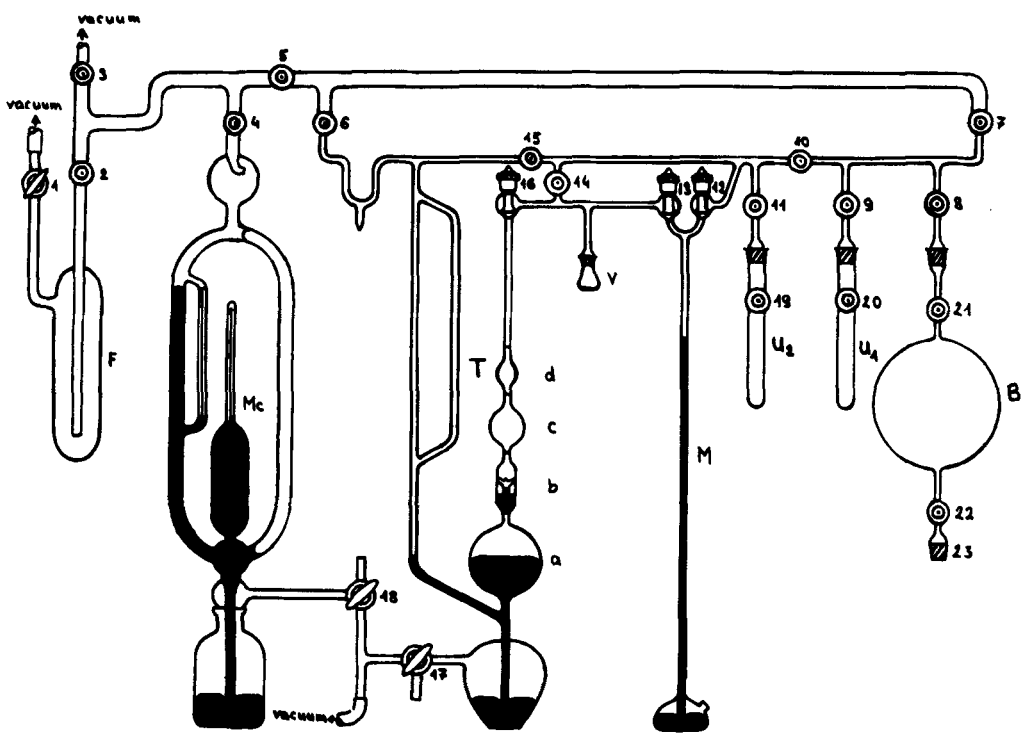
Materials and Methods

Tritium gas was either prepared by the Commissariat a l'Energie Atomique /France/ or purchased from Technabexport /USSR/ and stored in the form of uranium tritide. 10 % Pd/Al_2O_3 catalyst was purchased from Engelhard. /Italy/. Thin layer chromatograms were developed on DC Alufolien, Kieselgel, Merck /Germany/ in ethylacetate: pyridine:acetic acid: water 30:20:6:11 solvent system. Column chromatographic separations were accomplished on Sephadex G-15, Pharmacia /Sweden/ and carboxymethyl cellulose, Reanal /Hungary/ carriers.

The precursor peptides Asn^{1}, Dbt^{4}, Val^{5}-angiotensin II. trihydrofluoride /R_f:0,28 λ_{max} 308/ Asn^{1}, Dbt^{4}, Val^{5}- angiotensin II triacetate /R_f:0,28, λ_{max} 308 nm, λ_{min} 270 nm, $[\alpha]_D$:-31.5° /C 0.888, 0,1 M AcOH/ and Asn^{1},Val^{5},Cpa^{8}-angiotensin II triacetate /R_f:0.20; λ_{max} 275 mm/ were synthesized as described earlier /8/.

Radioactivity measurements were carried out in Intertechnique SL 30 or Beckman LS-355 liquid scintillation counters. Liquid scintillation vials were first saturated with radioinactive angiotensin II or silylated to prevent losses of radioactivity caused by adsorption on glass surfaces. Thin layer radiochromatograms were evaluated in Berthold Scanner II, and LB 2710,respectively. Ultraviolet absorption spectra were taken in Beckman spectrophotometers, models DK 2 A and 25.

Tritiation experiments with tritium absorbed on uranium powder were carried out in the manifold shown on fig.3. /13/. Ampoule U_1 contained uranium tritide, U_2 contained pyrophorous uranium powder to absorb dilute tritium gas.



Asn^1,/Tyr-^3H/^{4};Val^5-angiotensin II. /I./

4.97 mg /1 μmol/ III. 3 HF was dissolved in 0,7 ml water. 0.3 ml 0.1 molar aqueous triethylamine solution /30 μmol/ was added and the solution frozen with liquid nitrogen. Approx.30 mg 10 % Pd/Al_2O_3 catalyst was placed onto the frozen reaction mixture. The vial was connected onto the automatic gas transfer machine /12/ and evacuated to a vacuum of 2×10^{-3} torr while further cooling. 20 Ci of tritium gas from a break sealed ampoule was collected in the cylinder of the automatic gas transfer machine then transferred into the reaction flask. Cooling of the reaction mixture was ceased and the tritium-saturated catalyst was stirred into the melting reaction mixture by magnetic stirring. The starting pressure in the tritiation flask was 0.8 Bar, which lowered to 0,35 Bar in 45 minutes. Then the reaction mixture was frozen by liquid nitrogen and excess tritium was removed. The mixture was filtered through Millex filter; the flask and the filter rinsed with 0,1% acetic acid solution.

The aqueous solution was evaporated in vacuo, and off the residue, 250 ml water was distilled to remove exchangeable tritiums.The material was chromatographed on a column of Sephadex-G-15, pre-equilibrated with 0,05 n formic acid, and eluted with the same solvent. The fractions containing peptide I. were pooled and lyophylized. The material was dissolved in 3 ml water. According to O.D. measurement 2.54 mg peptide I.was obtained which also contained a small amount of peptide III. After lyophylization this material was dissolved in 1 ml 0.01 M ammonium acetate solution and chromatographed on CMC column. The material was eluted by linear gradient formed from 50 ml 0.01 M, pH 5.3 and 50 ml 0.-1 M, pH 5.3 ammonium acetate buffer solutions. The fractions containing peptide I /68-82 ml/ were concentrated to 10 ml. O.D. measurement showed a recovery of 0.8 mg /Tyr-^3H/^{4}-angiotensin II. /I/ which was not contaminated by the precursor peptide. Its molar specific radioactivity was found to be 28.84 Ci/mmole, R_f:0,16.

This tritiation experiment was repeated in another special vacuum manifold /Fig.3./.In this case tritium gas was obtained from uranium tritide by desorption above 450° C. This was transferred into the reaction flask by a Toepler pump. 6.05 mg III. $3CH_3COOH$ was dissolved as described above and frozen. Approx. 40 mg of Pd/Al_2O_3 catalyst was placed onto the frozen mixture. Tritium uptake stopped after 80 minutes. The mixture was filtered as described above, and water was distilled off the material.This was chromatographed on CMC column eluting with a gradient formed from 0.01 M and 0.15 M,pH 5.3 ammonium acetate solutions.The fractions containing peptide I. were pooled and concentrated. According to O.D. measurement, the yield was 2.5 mg /Tyr-$^{3}H/^{4}$-angiotensin II. Molar radioactivity was found to be 20.6 Ci/mmole; R_f 0.16.

Asn^{1},Val^{5}, /Phe-$^{3}H/^{8}$-angiotensin II. /II/

6.15 mg IV. 3 CH_3COOH was dissolved in 0.5 ml water and 0,35 ml 0.1 M triethylamine solution /35 umol/ was added. The mixture was frozen by liquid nitrogen and 40 mg 10 % Pd/Al_2O_3 catalyst was placed onto it. Tritium gas deliberated from uranium tritide was transferred into the reaction flask by a Toepler pump.Tritium uptake stopped after one hour. The reaction mixture was filtered and the filter rinsed with 0.1% acetic acid. The solution was evaporated, water was distilled off the residue, which was finally dissolved in 2 ml 0.01 M ammonium acetate solution and chromatographed on CMC column. Elution was accomplished by a gradient formed from 70 ml each of 0.01 M and 0.15 M, pH 5.3 ammonium acetate buffer solutions. The fractions containing peptide II /120-135 ml/ were pooled and lyohylized. The material was dissolved in 10 ml water and determined for peptide content, which was found to be 2.64 mg with a molar radioactivity of 11.3 Ci/mmole; R_f 0.16.

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