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Synthesis of Tritium-Labelled β-Amyloid Fragments

Éva C. Gulyás, Katalin Soós, József Varga*,*

Géza Tóth[#] *and Botond Penke**

Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences,

H-6701 Szeged, PO Box 521, Hungary

*Department of Medical Chemistry, Albert Szent-Györgyi Medical University,

H-6720 Szeged, Dóm tér 8, Hungary

Summary

Two fragments of β-amyloid(1–42) peptide, β-amyloid(31–35) and β-amyloid(25–35), were synthesized and labelled with tritium in the Leu^{34} residue. Precursor peptides containing ΔLeu^{34} were prepared by solid phase peptide synthesis using manual Fmoc strategy. Tritium labelling was carried out by catalytic saturation, yielding [^{3}H-Leu^{34}]β-amyloid(31–35) and [^{3}H-Leu^{34}]β-amyloid(25–35) with very high specific activities of around 4.44 TBq/mmol (120 Ci/mmol). The labelled peptides were investigated by RP-HPLC and the distribution of the tritium was determined by acidic hydrolysis followed by RP-HPLC analysis.

Key Words:

β-amyloid fragments, solid phase peptide synthesis, catalytic tritiation, [^{3}H-Leu^{34}]β-amyloid(31–35), [^{3}H-Leu^{34}]β-amyloid(25–35)

Introduction

Alzheimer's disease (AD) is the most common cause of progressive intellectual failure in aged humans. The lesions of the filaments determining AD occur within neurones, in extracellular cerebral

[#] Author for correspondence: geza@everx.szbk.u-szeged.hu

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deposits (amyloid plaques), and in meningeal and cerebral blood vessels. Beta-amyloid [Aβ] peptides are minor fragments of the large transmembrane amyloid precursor protein (APP) and consist of at most 42 amino acid residues. Investigation of AD brain revealed that the full-length Aβ is the predominant peptide in amyloid plaques together with the more soluble Aβ(1–40).

Aβ (25–35) and Aβ(31–35) may exert neurotrophic properties under certain conditions *in vitro* (1). The direct dose-dependent neurotoxicity of Aβ fragments has recently been demonstrated both *in vitro* and *in vivo* (2-5). Various investigations have reported that short fragments of Aβ are capable of producing the whole range of effects (including neurotoxicity) of the full-length peptide (6-8).

The qualitative identity of the biological effects of full-length Aβ and its fragments indicates that they may act on the same target by the same mechanism, which remains unknown so far. The existence of a short active centre of Aβ and physiological activity in a very low concentration (10^{-10}–10^{-9} M) (9) are features suggesting that the peptide hormones might have specific receptors. Cowburn and co-workers recently reported (10) that both Aβ(25–35)-NH_2 and Aβ(25–35)-OH cause statistically significant dose-dependent inhibition of [^{3}H]glutamate and [^{3}H]glycine binding to the agonist recognition sites of the N-methyl-D-aspartic acid (NMDA) receptor.

The present report relates to the syntheses of two novel ligands: [^{3}H]β-amyloid(31–35)-OH (H-Ile-Ile-Gly-[^{3}H]Leu-Met-OH) and [^{3}H]β-amyloid(25–35)-OH (Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-[^{3}H]-Leu-Met-OH). The aim of the work was to obtain a suitable tool in the search for the Aβ receptor, which could highlight the molecular mechanism of neuronal death and impaired cognition in AD.

Experimental

Materials and methods

Protected and unprotected amino acids and resins were purchased from Aldrich Chemical Co. or Bachem Fine Chemicals. Coupling agents were from Richelieu Biotechnologies, or from Fluka. Trifluoroacetic acid (TFA) was from Fisher Scientific.

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$PdO/BaSO_4$ (10% Pd) and Pd/C (10% Pd) catalysts, TLC plates, Fertigplatten Kieselgel 60 F_{254}, and solvents were from Merck.

The following solvent systems were used for TLC analysis: (I): butanol-acetic acid-water (4:1:1); (II): butanol-acetic acid-pyridine-water (13:3:12:10), (III): acetonitrile-methanol-water (4:1:1) and (IV): butanol-acetic acid-water (1:1:1). Ninhydrin, UV light and iodine vapour were employed to detect the peptides and amino acids.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Knauer RP-HPLC system (Dr. Ing. Herbert Knauer GmbH), utilizing a BST (Bio Separation Technologies) SI-100S, C-18 (280×16 mm, 10 μm) semipreparative column for preparative purposes, and a Nucleosil C-18 (280×0.46 mm, 5 μm) Knauer column for analytical purposes.

Quantitative amino acid analyses were performed on an HP 1090 Amino Quant amino acid analyser (Hewlett-Packard), on a Hypersyl ODS C_{18} column (200×2 mm, 5 μm, Shandon Scientific).

Molar masses of peptides were determined by mass spectrometry (Finnigan TSQ 7000).

Tritiation reactions were carried out on a self-designed vacuum manifold described earlier (11). $^3H_2$ gas was purchased from Technobexport, Russia, and contained at least 98% tritium.

The radiochemical purities of the labelled peptides were checked by TLC, with detection with a Berthold Radiochromatogram Tracemaster. Radioactivity was counted in a toluene-Triton X-100 scintillation cocktail with a Searle-Delta-300 liquid scintillation counter (LSC).

Tritium-labelled materials were analysed, and purified on an RP-HPLC (Jasco) instrument, using a Vydac 218TP54 C18 (0.46×25 cm, 5 μm) or a Merck 50943 LiChroCART (125-4 LiChrospher 100 RP-18, 5 μm) column, with detection with UV on a Jasco UV-975 spectrometer and on a Canberra Packard 505 TR Flow Radiochromatography Detector.

Syntheses of peptides

Synthesis of [$^{4,5}\Delta$Leu^{34}]β-amyloid(31–35) — H-Ile-Ile-Gly-[$^{4,5}\Delta$Leu]-Met-OH was prepared by a solid phase technique, utilizing Fmoc chemistry (12). The peptide chain was elongated on Fmoc-Met Sasrin (super acid-sensitive) resin (2-methoxy-4-benzyloxybenzyl alcohol) and the synthesis was

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carried out manually. Fmoc deprotection was achieved by treatment with 20% piperidine in N,N-dimethylformamide (DMF). Coupling reactions were carried out with 2 equiv. of Fmoc-amino acid, 2 equiv. of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 4 equiv. of N,N-diisopropylethylamine (DIEA) and 2 equiv. of N-hydroxybenzotriazole (HOBt) in DMF and dichloromethane (DCM). All the coupling reactions were monitored by the Kaiser test (13). After removal of the N-terminal Fmoc group, the peptide resin was dried in vacuum for 12 h. Cleavage of the peptide from the resin was performed with 1% TFA and 0.5% dimethylsulphide (DMS) in DCM at room temperature for 15 min. After filtration, the resin was treated three more times with the cleavage mixture for 3×15 min, followed by washing with methanol. The combined filtrates were neutralized three times with DIEA (90% of the equiv. amount) and evaporated to dryness in vacuum at room temperature. The crude peptide was redissolved in 10% acetonitrile, 0.1% TFA in water, and purified by RP-HPLC, eluting with the following gradient system: 10–25% solution A during 10 min and 25–35% solution A during 45 min (solution A: 0.1% TFA–80% acetonitrile; solution B: 0.1% TFA–water) at 4 mL/min, with detection at 220 nm. The pure fractions were collected and lyophilized.

Synthesis of β-amyloid(31–35) — H-Ile-Ile-Gly-Leu-Met-OH was synthesized by tertiary-butoxycarbonyl (Boc) chemistry. The synthesis was carried out manually on Boc-Met-Merrifield resin, using HOBt active ester coupling methods with N,N'-dicyclohexylcarbodiimide (DCC). The Boc group was removed after each completed coupling step by treatment with 50% TFA in DCM (for 5 and 30 min). To prevent oxidation of methionine, 0.5% 1,4-dithiothreitol (DTT) was added to the TFA solution. The completion of coupling was monitored by the Kaiser test. The peptide was cleaved from the resin with liquid hydrogen fluoride (HF) with 5% anisole, 1.6% p-cresol, 1.6% p-thiocresol and 1.6% DMS for 1 h at -5 °C, washed with diethyl ether and dissolved in acetic acid. The solution was diluted with distilled water, and lyophilized. The peptide was purified by RP-HPLC, with elution with the gradient 10–40% solution A during 30 min at 4 mL/min, with detection at 220 nm.

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Synthesis of [$^{4,5}\Delta$Leu^{34}]β-amyloid(25–35) — The synthesis was carried out in an analogous way as described above for [$^{4,5}\Delta$Leu^{34}]β-amyloid(31–35), on Fmoc-Met-Sasrin resin (0.71 mmol/g, 0.42 g, 0.3 mmol). The peptide-resin, H-Gly-Ser(Bu^t)-Asn-Lys(Boc)-Gly-Ala-Ile-Ile-Gly-[$^{4,5}\Delta$Leu]-Met-Sasrin, was cleaved with a cleavage cocktail (TFA/DCM/dioxane/anisole/DMS in 5:4:1:0.5:0.5 volumetric ratio) for 5 h at room temperature, and the resin was filtered off. The filtrate was neutralized with an equivalent amount of DIEA and diluted with water, and the volatile components were evaporated off in vacuum at room temperature. The resulting peptide was purified by RP-HPLC with the following gradient system: 10–25% solution A during 10 min and then 25–35% solution A during 45 minutes.

Synthesis of β-amyloid(25–35) — H-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH was synthesized by standard solid phase techniques on an ABI 430A automated peptide synthesizer, using Boc chemistry. The synthesis was carried out on Merrifield resin, using the active ester coupling method (DCC and DCC/HOBt as coupling agents). The peptide was cleaved from the resin with liquid HF, solubilized with 50% acetic acid, filtered and lyophilized. The peptide was purified as the [$^{4,5}\Delta$Leu^{34}]-containing analogue.

Analytical characterization of peptides

Peptide identities were verified by mass spectrometry and amino acid analyses. For the amino acid analyses the peptides were hydrolysed in 6 N HCl at 110 °C for 48 h. Amino acids were identified by using the o-phthalaldehyde (OPA) method. The analyses revealed that the peptides had the correct amino acid compositions.

Results of the MS, HPLC and TLC analyses are summarized in Table 1.

Tritium labelling of peptides

Synthesis of tritium-labelled β-amyloid(31–35) — [^{3}H-Leu^{34}]β-amyloid(31–35) (H-Ile-Ile-Gly-[^{3}H-Leu]-Met-OH) was prepared by catalytic saturation, using a [$^{4,5}\Delta$Leu^{34}]-containing analogue. 2.09 mg of the precursor peptide (TFA•H-Ile-Ile-Gly-[$^{4,5}\Delta$Leu^{34}]-Met-OH) and 2 equiv. (6.34 μmol) of triethylamine (TEA) were dissolved in 1 mL of DMF, and 9.21 mg of $PdO/BaSO_4$ catalyst was

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Peptide	MW_{calc}	$[M+H]^+$	TLC				k'
			I	II	III	IV	
$[^{4,5}\Delta Leu^{34}]\beta$-amyloid(31–35)	543.7	544.6	0.617	0.795	0676	0.805	4.31
β-amyloid(31–35)	545.7	546.7	0.584	0.802	0.664	0.805	4.85
$[^{4,5}\Delta Leu^{34}]\beta$-amyloid(25–35)	1058.2	1058.7	–	0.682	–	0.716	5.25
β-amyloid(25–35)	1060.2	1061.2	–	0.682	–	0.716	5.36

Table 1

HPLC — Column: C_{18} 4.6×250 W-Porex, elution: 20–50% solution A during 30 min, 1.5 mL/min; k' — Capacity factor

added. After a magnetic stirrer had been placed in the reaction vessel, it was connected to the tritiation manifold, cooled with liquid nitrogen and evacuated. Tritium was liberated from uranium tritide by heating, and was expanded into the reaction vessel. The reaction mixture was agitated by means of the magnetic stirrer at room temperature for 45 min. The reaction was terminated by freezing the solution and adsorbing the unreacted tritium on pyrophoric uranium. The catalyst was removed by filtration through Whatman GF/C filters, and was washed several times with ethanol. The residues of labile tritium were removed by repeated evaporation of an ethanol–water (1:1) solution from the radiolabelled product. The total activity of the product was measured by LSC to be 9.31 GBq (251.6 mCi). The crude tritiated peptide was checked by TLC with the solvent systems I, II and III, which showed some impurities.

To purify the labelled peptide, an RP-HPLC (Jasco) instrument was applied, using a Vydac 218TP54 column and the following gradient system: 20–50% solution A during 30 min, (solution A: 0.1% TFA–acetonitrile, solution B: 0.1% TFA–H_2O). An LSC and the HPLC chromatogram of the peptide (using a calibration curve) indicated that the specific radioactivity of the purified labelled peptide was 4.50 TBq/mmol (121.6 Ci/mmol). The purified labelled peptide was dissolved in ethanol and stored in 2 mL aliquots (37 MBq/mL) under liquid nitrogen.

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Synthesis of tritium-labelled β-amyloid(25–35) — 2.9 mg (2 μmol) of the precursor peptide ([$^{4,5}\Delta$Leu^{34}]β-amyloid(25–35)) was dissolved in 1 mL of DMF, and 10 mg of Pd/C catalyst was added to the solution. Catalytic reduction with tritium was carried out for 80 min at RT. After filtration, the labile tritium was removed by triple evaporation of an ethanol–water (1:1) mixture. The crude radioactive peptide was purified by RP-HPLC, on a Vydac 218TP54 column, using the following gradient system: 20–36% solution A during 30 min. The concentration of the labelled peptide was determined by means of a calibration curve and the radioactivity was measured on LSC. The specific activity was 4.41 TBq/mmol (119.2 Ci/mmol). The pure labelled peptide was dissolved in doubly distilled water, and stored in 2 mL aliquots (37 MBq/mL) under liquid nitrogen.

Analysis of tritium-labelled peptides

Acid hydrolysis (14) of [^{3}H-Leu^{34}]β-amyloid(31–35) and [^{3}H-Leu^{34}]β-amyloid(25–35) — 50 μCi of labelled peptide, diluted 10-fold with the inactive one, was dissolved in 1 mL of 6 N HCl, sealed in an ampoule and incubated for 60 h at 110 °C. The hydrolysed peptide was subjected to derivation by fluorenylmethoxycarbonyl (Fmoc) (15) and analysed by TLC and RP-HPLC (0–5 min, 0% solution A; 5–10 min, 0–40% solution A; 10–30 min, 40–80% solution A; with detection with UV and tritium radiomatic detectors.

Results and Discussion

Synthesis of precursors for tritiation

Precursor peptide syntheses involving Boc chemistry failed because of the exceptional lability of the $^{4,5}\Delta$Leu-Met peptide bond. This bond was also broken during the final cleavage and deprotection step with concentrated TFA when the Fmoc synthesis was carried out on Rink acid resin.

To avoid the use of a concentrated acid, [$^{4,5}\Delta$Leu^{34}]β-amyloid(31–35) was synthesized by Fmoc chemistry on Sasrin (super acid-sensitive) resin, which can be cleaved with 1% TFA/DCM, the final product being obtained in good yield. This was possible since the peptide sequence (Ile-Ile-Gly-$^{4,5}\Delta$Leu-Met) needed no side-chain protection. [$^{4,5}\Delta$Leu^{34}]β-amyloid(25–35) was synthesized in a similar way, but in this case a higher concentration of TFA was necessary for the final cleavage and

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deprotection step because of the presence of side-chain protecting groups of trifunctional amino acids: Ser(Bu^t) and Lys(Boc). The literature (14) recommends a special cleavage cocktail containing dioxane for side-chain deprotection, with simultaneous preservation of the acid-sensitive $^{4,5}\Delta$Leu-Met sequence. In our experience, this cleavage cocktail is far from perfect (it slowly cleaves the Bu^t group and hence destroys the $^{4,5}\Delta$Leu-Met sequence), but with an appropriately chosen cleavage time it is feasible to prepare a satisfactory quantity of [$^{4,5}\Delta Leu^{34}$]β-amyloid(25–35).

Tritium labelling and the distribution of tritium

The incorporation of one tritium atom per molecule results in a specific activity of 1.07 TBq/mmol (28.8 Ci/mmol). In both of the above cases, the specific activity was more than 4 TBq/mmol, which means that more than 4 tritium atoms were incorporated per molecule. A number of reports demonstrate very high specific activity in the Leu residue on the labelling of peptides (16-19).

To determine the distribution of the tritium in the peptides, the labelled peptides were hydrolysed with hydrochloric acid (14). This required a long time (60 h) because of the presence of the Ile-Ile bond. The hydrolysed peptides were analysed by TLC, using solvent systems (I) and (II), and after Fmoc derivation (15) by HPLC, with detection with UV and a radiodetector. In both cases, the radiolabelling was found exclusively in the Leu residue.

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