SYNTHESIS OF TRITIUM LABELLED THYMIC HUMORAL FACTOR γ2

(THF-γ2, THYMOCTONAN)

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SUMMARY

The synthesis of the title compound labelled with tritium in the proline residue is reported. A five-step sequence starting from the commercially available [^{3}H]proline 1 afforded [^{3}H]THF-γ2 with a radiochemical purity ≥97% and a high specific activity (>3.7 TBq/mmol). The overall radiochemical yield was about 15% from 1.

Key words : THF-γ2, Thymoctonan, FCE 25338, Immunomodulator, Tritium labelled peptide, Tritium labelled THF-γ2.

INTRODUCTION

THF-γ2 (code name FCE 25338; thymoctonan) is a synthetic octapeptide of formula H-Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu-OH, which is identical to the natural thymic humoral factor γ2 originally isolated from fetal calf thymus and characterized by Burstein et al. [1]. The compound has shown to exert an immunorestorative effect in a variety of animal models [2], as well as in pilot clinical trials in patients with various immunodeficiencies conditions [3,4]. In order to perform "in vivo " and "in vitro" studies with this new promising immunomodulating agent, the radiolabelled material was required. Since THF-γ2 is expected to be administered at low dosage, tritium had to be chosen as the

Note: Nomenclature and symbolism for amino acid and protecting groups are according to the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, Biochem. J., 219, 345-373 (1984). Other abbreviations are: [Δ^3Pro] for L-3,4-dehydroproline and H-*Pro-OH for L-[2,3,4,5-^{3}H]proline.

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radioactive isotope to be introduced into the molecule. In the present paper we report the synthetic pathway that was followed to prepare tritium labelled THF-γ2.

RESULTS AND DISCUSSION

Proline-containing peptides are successfully tritiated by catalytic hydrogenation with tritium of the analogs in which the aminoacid proline has been substituted with 3,4-dehydroproline [5,6]. Therefore, [Δ^3Pro^5]THF-γ2 was prepared by Solid Phase Peptide Synthesis (SPPS). The peptide sequence was manually assembled by Fmoc-based SPPS on a Wang resin [7]. All amino acids were N-α-Fmoc protected except the leucine in position 1 that was N-α-protected with Boc. OtBu and Boc were used as side chain carboxy and amino protecting groups, respectively. Attachment of the first amino acid to the resin was carried out using diisopropylcarbodiimide in the presence of 4-dimethylaminopyridine [8]. The coupling of the remaining amino acids was performed using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate [9]. After completion of the synthesis, the liberation of the peptide from the resin and the cleavage of the protecting groups were simultaneously achieved by treatment with a mixture trifluoroacetic acid:water (95:5 by volume). The crude material was purified by reverse phase HPLC. Treatment with tritium of [Δ^3Pro^5]THF-γ2 [10] afforded [^{3}H]THF-γ2 with a specific activity of 1.22 TBq/mmol. The obtained specific activity was almost the maximum achievable by the method and was high enough for "in vivo studies". However, for "in vitro" studies, it was necessary to obtain a higher specific activity. Therefore, a different approach was investigated to prepare the tritiated THF-γ2 labelled in the same aminoacidic residue starting from the easy available high specific activity L-[2,3,4,5-^{3}H]proline. The last steps of an in-house procedure previously developed for hundred grams quantities of THF-γ2 [11,12], seemed to be attractive to prepare the title compound with the required specific activity as shown in the scheme. The reaction conditions reported for the "cold " synthesis were modified to suit the nanomolar scale preparation. In particular our strategy consisted in coupling the more expensive radioactive proline or proline containing peptides with a large excess of suitable protected peptide precursors. The amino group of the commercially available H-*Pro-OH 1 was protected by reaction with an excess of di-tert-butyl dicarbonate in t-butyl alcohol at room temperature [13]. The resulting Boc-*Pro-OH 2 was then condensed with about 100 molar equivalents of the tripeptide 3 according to the mixed anhydride procedure using ethyl chloroformate and N-methylmorpholine in tetrahydrofuran and dimethylformamide. After removal of the Boc group with anhydrous 4N HCl in dioxane, the tritiated peptide 5 was reacted with a 200-fold excess of the tetrapeptide 6 using dicyclohexylcarbodiimide in combination with 1-hydroxybenzotriazole. The deprotection of the obtained octapeptide 7 was accomplished by catalytic transfer hydrogenation [14]. The purification of the resulting crude material by HPLC gave [^{3}H]THF-γ2 as trifluoroacetate. The conversion to the corresponding hydrochloride was carried out by circulation of an aqueous solution of [^{3}H]THF-γ2

trifluoroacetate through a strongly basic anion exchange resin in Cl^- form. According to this method, [^{3}H]THF-γ2 was obtained with a radiochemical purity >97% and a specific activity of 3.81 TBq/mmol. The overall radiochemical yield of about 15% from 1.

SCHEME

H-*Pro-OH

1

↓ $(Boc)_2O$

Boc-*Pro-OH + HCl H-Lys(Z)-Phe-Leu-OBzl

2 3

↓ ECC,NMM

Boc-*Pro-Lys(Z)-Phe-Leu-OBzl

4

↓ HCl/AcOH

HCl.H-*Pro-Lys(Z)-Phe-Leu-OBzl + Z-Leu-Glu(OBzl)-Asp(OBzl)-Gly-OH

5 6

↓ DCC,HOBt,NMM

Z-Leu-Glu(OBzl)-Asp(OBzl)-Gly-*Pro-Lys(Z)-Phe-Leu-OBzl

7

↓ Pd/C, NMM; Diaion SA102M (Cl^- form)

H-Leu-Glu-Asp-Gly-*Pro-Lys-Phe-Leu-OH. 2HCl

[^{3}H]THF-γ2

NMM = N-Methylmorpholine
ECC = Ethylchloroformate
DCC = N,N'-Dicyclohexylcarbodiimide
HOBt = 1-Hydroxybenzotriazole

EXPERIMENTAL

General methods.

L-[2,3,4,5-^{3}H]Proline was purchased from Amersham International p.l.c.. All solvents and reagents were of analytical grade and were used without purification unless otherwise indicated. Radioactivity was measured using a Packard 300C liquid scintillation counter using Rialuma (Lumac System A.G.) as liquid scintillation cocktail. The Diaion SA102M resin was purchased from Mitsubishi, Japan. The peptide intermediates 3 and 6 were prepared in our laboratories according to [12]. High Performance Liquid chromatography (HPLC) was performed at ambient using a Perkin-Elmer pump series 400 equipped with a Perkin-Elmer LC 75 UV/VIS spechtrophotometer (λ=210 nm). Radiochemical purities were determined by HPLC using a Packard Trace model 7125 radioactivity flow detector, under the following conditions : Vydac 218TP54 column (250x4.6 mm I.D.; particle size 5 μm) eluted with i) acetonitrile:0.1% trifluroacetic acid (18:82 by volume) at 1 ml/min or ii) acetonitrile (A):0.1% trifluoroacetic acid (B) (from 50%A to 80%A in 15 minutes then isocratic elution at 80%A for 20 minutes) at 1 ml/min or iii) acetonitrile (A):0.1% trifluoroacetic acid (B) (isocratic elution at 18%A for 10 minutes then 18%A to 80%A in 15 minutes and finally isocratic elution at 80%A for 20 minutes) at 1 ml/min. All labelled materials were identified by chromatographic comparison with the corresponding authentic unlabelled samples.

Boc-*Pro-OH (2)

1N NaOH (60 µl, 600 µmol), t-butylalcohol (100 µl) and a solution of di-tert-butyl dicarbonate (60.92 µmol) in t-butyl alcohol (120 µl) were successively added to H-*Pro-OH 1 (~1 mCi; ~10^{-2} µmol; specific activity = 3.7 TBq/mmol) in water (~200 µl). The reaction mixture was stirred at room temperature for 90 minutes. At the end of the reaction (determined by radio-HPLC; system i) the solution was transferred into a separating funnel, added with water (~3 ml), washed with n-pentane (3×1 ml), acidified up to pH 2 with 1N HCl then extracted with ethyl acetate (3×2 ml). The organic phases were combined, dried (Na_2SO_4) and, after solvent evaporation in vacuo, the intermediate 2 (1.07 mCi; 1.04×10^{-2} µmol) was recovered 95% radiochemically pure (by radio-HPLC; system i: Retention Time (RT)= 10.1 minutes).

Boc-*Pro-Lys(Z)-Phe-Leu-OBzl (4)

The intermediate 2 (1.07 mCi; 1.04×10^{-2} µmol) and N-methylmorpholine (1.3 µmol) in tetrahydrofuran (180 µl) were stirred at -12°C for 10 minutes, then ethylchloroformate (1.3 µmol) in tetrahydrofuran (160 µl) was introduced into the flask. After stirring for 10 minutes at -12°C a cold solution (~ -12°C) of the tripeptide 3 (1.3 µmol) and N-methylmorpholine (1.3 µmol) in tetrahydrofuran (180 µl) were added. The reaction mixture was then stirred for 15 minutes at -12°C and 5 hours at room temperature. At the end of the reaction (determined by radio-HPLC; system ii) the mixture was evaporated to dryness in vacuo. The recovered crude 4, 91% radiochemically pure (determined by radio-HPLC; system ii: RT=21.7 min.), was used without purification in the next step.

HCl·H-*Pro-Lys(Z)-Phe-Leu-OBzl (5)

The intermediate 4 and 4N HCl in dioxane (50 µl, 200 µmol) were stirred at room temperature. After 30 minutes the Boc removal was complete (determined by radio-HPLC; system ii). The reaction mixture was then added with tetrahydrofuran (~1 ml) and evaporated in vacuo to dryness. The obtained compound 5, 89% radiochemically pure (determined by radio-HPLC; system ii: RT=11.1 min.), was used without purification in the next step.

Z-Leu-Glu(OBzl)-Asp(OBzl)-Gly-*Pro-Lys(Z)-Phe-Leu-OBzl (7)

Aqueous (12÷17% of water) 1-hydroxybenzotriazole was dissolved in dimethylformamide (~1 ml) and dried in vacuo. The operation was repeated twice. The recovered anhydrous 1-hydroxybenzotriazole (27.56 µmol), was added with a cold solution (5÷6 °C) of the tetrapeptide 6 (1.94 µmol) in dimethylformamide (100 µl) and, after stirring for 10 minutes at 5÷6 °C, with dicyclohexylcarbodiimide (31 µmol). The above solution was then added to a cooled (5÷6 °C) mixture of the intermediate 5 and N-methylmorpholine (1.9 µmol) in dimethylformamide (100 µl). After 12 hours at this temperature the reaction was complete as shown by radio-HPLC (system ii) and the mixture was evaporated to

dryness in vacuo. The recovered crude compound 7, 71% radiochemically pure (determined by radio-HPLC; system ii: RT=27.3 min.; system iii: RT=37 min.) was used without purification in the next step.

2HCl·H-Leu-Glu-Asp-Gly-*Pro-Lys-Phe-Leu-OH ([^{3}H]THF-γ2)

A solution of the intermediate 8 in acetic acid/methyl alcohol (1:1 v/v; 200 µl) was cooled at 5÷6 °C and added under stirring, in order, with 99% formic acid (1.3 mmol), N-methylmorpholine (0.45 mmol) and 10% palladium on charcoal (~4 mg divided in several portions). After 60 minutes of stirring at 40°C, the removal of all protective groups was complete (determined by radio-HPLC; system ii). The reaction mixture was then filtered through a glass-wool filter and the filtrate evaporated in vacuo to dryness. The recovered crude [^{3}H]THF-γ2 (813 µCi), 42% radiochemically pure (by radio-HPLC; system iii: RT=8.2 min.) was suspended under stirring in acetonitrile/0.1% trifluoroacetic acid in water (18:82 v/v;~1ml). It was then filtered through a glass-wool filter and evaporated to dryness under vacuum. The residue was dissolved in acetonitrile/0.1% trifluoroacetic acid in water (18:82 v/v; 250 µl) and purified by HPLC (system iii). The fractions containing [^{3}H]THF-γ2 were combined and evaporated to dryness in a "vacuum manifold". [^{3}H]THF-γ2 trifluoroacetate (218 µCi; 2.1165 nmol) was obtained with a 98% radiochemical purity (by radio-HPLC; system iii: RT=8.2 minutes). [^{3}H]THF-γ2 trifluoroacetate (218 µCi; 2.1165 nmol) was dissolved in water (~1 ml) and converted to the corresponding hydrochloride by circulation for 15 minutes through a Diaion SA 102M resin (Cl- form) packed in a glass column (~6×1.5 mm I.D.). [^{3}H]THF-γ2 hydrochloride (144 µCi; 1.398 nmol) was recovered >98% radiochemically pure (by radio-HPLC; system i: RT=8.2 minutes) with a specific activity of 3.81 TBq/mmol. The radiochemical yield was ~15% from 1.

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