Synthesis of tritium labelled CCK_4 (Trp-Met-Asp-[^{3}H]Phe-NH_2).

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SUMMARY

CCK_4 (CCK_{30-33}) is the shortest fragment of cholecystokinin which retains a high affinity (nanomolar range) for brain CCK receptors. In order to determine wether CCK_4 interacts with binding sites distinct from those of CCK_8, [^{3}H]CCK_4 was synthesized. This peptide was prepared in liquid phase using L-3(3',4',5'-tribromophenyl)alanine as the phenylalanine precursor. The reductive tritiation of H-Trp-Met-Asp-Phe(Br)$_3$-NH_2 was performed using PdO as the catalyst to yield [^{3}H]CCK_4 with a specific activity of 35 Ci/mmol (1295 GBq/mmol).

Key words : Cholecystokinin, [^{3}H]CCK_4, CCK receptor.

INTRODUCTION

Cholecystokinin, a peptide originally found in the gastrointestinal tract is present in high concentrations in mammalian brains as its CCK_8, unsulfated CCK_8 (CCK_8-NS) and CCK_4 forms (1). As shown by extensive studies, central and peripheral CCK receptors are probably different biochemical entities (2). CCK_8, CCK_8-NS and CCK_4 exhibit affinities in the nanomolar range for brain binding

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sites (3,4). In contrast, CCK_8 interacts with pancreatic binding sites in the nanomolar range whereas CCK_8-NS and CCK_4 have affinities for these receptors in the micromolar range (3,4). CCK_4 is the shortest fragment which retains a high affinity for brain receptors. Moreover, in various biochemical (5), pharmacological (6,7) and electrophysiological experiments (8), CCK_4 was found to induce effects distinct from those of CCK_8. An hypothesis which could account for these findings is that CCK_8 and CCK_4 interact with different binding sites in the central nervous system. [^{3}H]CCK_4 therefore appeared to be a suitable ligand for characterization of CCK binding sites as well as for studies on possible receptor heterogeneity.

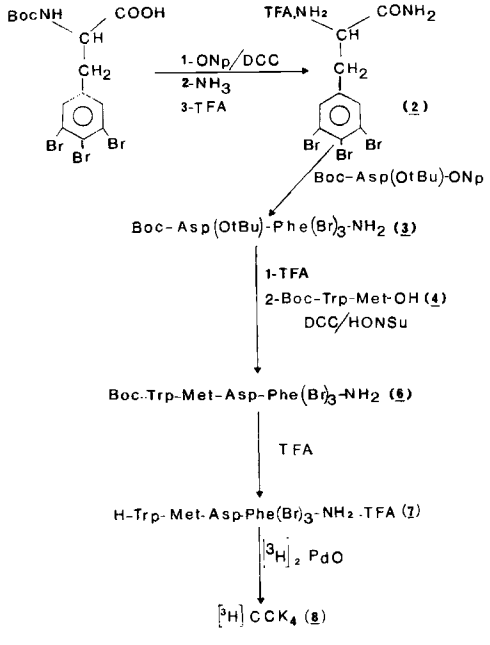

Figure 1. Synthetic route for the preparation of [^{3}H]CCK_4.

Accordingly, we report in this paper the synthesis of [^{3}H]CCK_4. The synthesis was performed in liquid phase using a fragment condensation method as illustrated in Figure 1. The L-3(3',4',5(-tribromophenyl)alanine (9) was used as the phenylalanine precursor. The catalytic tritiation was performed on a

methionine containing peptide (H-Trp-Met-Asp-Phe(Br)$_3$-NH_2.TFA (7), using PdO as catalyst. The reductive tritiation yielded [^{3}H]CCK_4 with a specific activity of 35 Ci/mmol (1295 GBq/mmol). This relatively low value as compared to the theoretical one (87 Ci/mmole) is probably due to sulphur poisoning of catalysts (10) decreasing the rate of tritiated and causing an exchange of tritium by hydrogen present in the reaction.

MATERIAL AND METHODS.

Chemistry.

All protected amino acids were from Bachem AG (Switzerland). Solvents were of analytical grade from Prolabo (France). The catalyst PdO was supplied by Engelhard (France). Melting points were determined on a Kofler apparatus and are reported uncorrected. Thin layer chromatography was carried out on silica gel plates with a fluorescent indicator using the following solvent systems : A : $CHCl_3$/MeOH (9:1), B : $CHCl_3$/MeOH (7:3), C : 2-propanol/conc. aq. ammonia (3:1), D : butanol/AcOH/H_2O (4:1:1), E : $CHCl_3$/AcOH/MeOH (95:5:3), F : $CHCl_3$/MeOH/AcOH (80:20:5), G : EtOAc/pyridine/AcOH/H_2O (120:20:6:11), H : EtOAc/pyridine/AcOH/H_2O (40:20:6:11).

The plates were developed with U.V., iodine vapor, ninhydrin or Ehrlich's reagent. The purity of the peptides was evaluated by HPLC (Waters apparatus) using a μ Bondapak C_{18} reverse phase column with U.V. (210 nm) and/or radioactivity detection. At each step of the synthesis the structure of the compounds and the lack of significant racemization was checked by ^{1}H NMR spectroscopy (Brucker WH 270 MHz). The FAB ionization was obtained with a FAB field source (Ion Tech Ltd, Teddington, UK) operated with Xenon at 8 KV and 1 mA. Glycerol or cesium iodide was used for calibration. Accelerating voltage was set at 6 kV. Tritium gas was made by the Commissariat à l'Energie Atomique (France). The automatic gas transfer unit for catalytic tritiation has been previously described (11). The specific activity was determined after tritium counting and either by measuring the weight of ^{3}H-phenylalanine after acid hydrolysis or by comparative quantification of an HPLC chromatogramm (U.V.

detection) with a known concentration of a non labelled CCK_4 sample. ^3H-Scannings of TLC plates were performed with a Berthols Scanner. Radioactivity [^3H] was determined using an Intertechnique liquid scintillation counter SL 3000. Peptide weight determination were carried out with a LKB Biochrom 4400 amino-acid auto-analyzer after hydrolysis with 6M HCl at 110°C for 24 h. ^3H-derivatives were purified by HPLC. The following abbreviations have been used : Boc, tert-butyloxycarbonyl ; H-Phe$(Br)_3$-OH, L-3(-3',4',5'-tribromophenyl) alanine ; MeOH, methanol ; EtOAc, ethylacetate ; AcOH, acetic acid ; DMF, dimethylformamide ; DMA, dimethylacetamide ; TFA, trifluoracetic acid ; DCC, N,N'-dicyclohexylcarbodiimide ; HONSu, N-hydroxysuccinimide ; DCU, N, N'-dicyclohexylurea ; ONp, paranitrophenol ; Boc_2O,di-t-butyldicarbonate ; Rt, retention time in HPLC ; FAB, fast atom bombardment. Other abbreviations used are those recommended by the IUPAC-IUB commission (12).

Boc-Phe(3',4',5'-Br_3)-NH_2 (1).

To a chilled solution of Boc-Phe(3',4',5'-Br_3)-OH (9) (480 mg, 0.96 mmol) in DMF (5 ml) was added paranitrophenol (176.2 mg, 1.15 mmol) and DCC (217.5 mg, 1.05 mmol). the reaction mixture was stirred for 1 h at 0°C and overnight at room temperature. After filtration of DCU the solvents were removed to give 0.59 g (91 %) of Boc-Phe(3',4',5',Br_3)-ONp, as a yellow solid : Rf (G) 0.73, Rf (A) 0.70 ; mp 205-207°C. A chilled solution of the above product (0.58 g, 0.86 mmol) in CH_2Cl_2 (20 ml) was saturated with NH_3 and the resulting mixture was stirred for 1 h at 0°C. After evaporation in vacuo, the residue was purified by chromatography on silica gel with the eluent CH_2Cl_2/MeOH (97:3) to give 0.42 g (87 %) ; Rf (A) 0.48 ; mp 183-184°C ; FAB-MS (MH^+) calc. 501, found 501.

Bos-Asp(OtBu)-Phe(Br_3)-NH_2 (3).

Compound 1 (200 mg, 0.40 mmol) was treated with TFA (3 ml) for 30 min at 0°C and 30 min at room temperature. After evaporation in vacuo, the oily residue was precipitated with dry ether to give 190 mg (92 %) of H-Phe (3',4',5'-Br_3)-NH_2. TFA (2) ; Rf (B) 0.36. To a chilled solution of 5 (150 mg, 0.29 mmol) in DMF (2 ml) containing DIEA (50 ml, 0.29 mmol) was added

Boc-Asp(OtBu)-ONp (143 mg, 0.35 mmol). The reaction mixture was stirred for 1 h at 0°C and for 5 h at room temperature. After evaporation in vacuo, the oily residue was purified by chromatography on silica gel with the eluent $CHCl_3$/MeOH/AcOH (9:0.5:0.3) to yield 170 mg (87 %) of 3 : Rf (B) 0.60 ; mp 140-143°C ; ^1H NMR (DMSO-d_6, TMS) δ : 7.75 (m, 1H, NH-Phe), 7.66 (s, 2H, CH-2',6'Phe), 7.42-7.20 (d, 2H, CO-NH_2), 7.05 (d, 1H, NH-Boc), 4.4 (m, 1H, CHα-Asp), 4.11 (m, 1H, CHα-Phe), 2.8 (m, 2H, CHβ-Phe), 2.35 (m, 2H, CHβ-Asp), 1.3 (s, 9H, Boc).

Boc-Trp-Met-OH (4).

To a chilled solution of Boc-Trp-Met-OCH_3 (13) (0.55 g, 1.2 mmol) in MeOH (5 ml) was added 1 M NaOH (1.5 ml). The solution mixture was stirred under N_2 for 1 h at 0°C and for 3 h at room temperature. After evaporation of the solvent, the residue was dissolved in cold water and extracted with ether. The aqueous phase was acidified with cold 1 M HCl and extracted with EtOAc. The organic layer was then washed with brine and dried on Na_2SO_4 to yield after evaporation 0.49 g (93 %) of 4 : Rf (B) 0.30 ; mp 140-142°C. ^1H NMR (DMSO-d_6, TMS) δ : 10.7 (s, 1H, NH-indole), 8.06 (d, 1H, NH Met), 7.5-6.9 (5H, Ar-H Trp), 6.64 (d, 1H, NH Boc), 4.31 (m, 1H, CHα Met), 4.16 (m, 1H, CHαTrp), 3-2.75 (m, 2H, CHβTrp), 2.4 (m, 2H, CHγMet), 1.95-1.8 (m, 2H, CHβMet), 1.25 (s, 9H, Boc).

Boc-Trp-Met-Asp-Phe(3',4',5'-Br_3)-NH_2 (6).

A solution of 3 (215 mg, 0.32 mmol) in CH_2Cl_2 (1.5 ml) and TFA (1.5 ml) was stirred for 1 h at 0°C and for 1 h at room temperature to give, after evaporation and precipitation from ether, 160 mg (80 %) of Asp-Phe(3',4',5'-Br_3)-NH_2. TFA (5) : Rf (B) 0.21. To a solution of 4 (104 mg, 0.24 mmol) in DMF (2 ml) at -10°C were added DCC (49 mg, 0.24 mmol) and HONSu (27.4 mg, 0.24 mmol). This reaction mixture was stirred for 30 min at -10°C, 2 h at 0°C and then 2 h at room temperature. To the above mixture at 0°C was added a solution of 5 (150 mg, 0.23 mmol) and Et_3N (33.3 µl, 0.24 mmol) in DMF (2 ml). The resulting mixture was stirred for 1 h at 0°C and overnight at room temperature. After evaporation in vacuo, the residue was precipitated from EtOAc

to give 161 mg (75%) of Boc-Trp-Met-Asp-Phe(3',4',5'-Br_3)-NH_2 (6) : Rf (F) 0.30 ; mp 210-212°C ; ^{1}H NMR (DMSO-D_6, TMS) δ : 10.71 (s, 1H, NH indol), 8.21-7.95 (m, 3H, NH-Met, -Asp, -Phe), 7.55 (s, 2H, -CH 2', 6' Phe), 7.50-6.88 (m, 7H, Ar-H Trp, CO-NH_2), 6.80 (d, 1H, NH-Boc), 4.43 (m, 1H, CHα-Phe), 4.31 (m, 2H, CHα-Met, -Asp), 4.15 (m, 1H, CHα-Trp), 2.93 (m, 2H, CHβ-Trp), 2.80 (m, 2H, CHβ-Phe), 2.50 (m, 2H, CHβ-Asp), 2.35-2.25 (m, 2H, CHγ-Met), 1.95 (m, 3H, S-CH_3), 1.70 (m, 2H, CHβ-Met), 1.23 (s, 9H, Boc).

H-Trp-Met-Asp-Phe(3',4',5'-Br_3)-NH_2.TFA (7).

A chilled solution of 6 (140 mg, 0.15 mmol) in CH_2Cl_2 (1 ml) and TFA/anisole (1 ml/ 70 µl) was stirred under N_2 for 45 min at 0°C and 45 min at room temperature to give after precipitation from ether-EtOAC 120 mg (85 %) ; Rf (H) 0.36 ; FAB-MS (MH^+ calc. 948, found 948). ^{1}H NMR (DSMO-d_6, TMS) δ : 10.7 (s, 1H, NH indole), 8.35-7.95 (3H, NH, Met, Asp, Phe), 7.55 (s, 2H, CH-2', 6' Phe), 7.50-6.88 (Ar-HTrp, CO-NH_2), 4.28-4.18 (m, 2H, CHα-Phe, Asp), 4 (m, 2H, CHα-Met), 3.58 (m, 1H, CHα-Trp), 3.08-2.90 (m, 2H, CHβ-Trp), 2.75-2.85 (m, 2H, CHβ-Phe), 2.35 (m, 2H, CHβ-Asp), 2.20-2.05 (m, 2H, CHγ-Met), 1.95 (s, 3H, SCH_3), 1.70 (m, 2H, CHβ-Met).

Catalytic hydrogenation of 7 in a solvent mixture DMA/NH_4OH (1:1) in the presence of PdO for 3 h at room temperature and atmospheric pressure afforded CCK_4.TFA as a major peak (over 80% determinated by integration of HPLC chromatogramm, U.V. : 210 nm) coeluted with CCK_4.TFA prepared separately starting from phenylalanine amide : Rf (H) 0.31 ; HPLC (Rt = 14.5 min), eluent 25 mM triethylammonium phosphate buffer (pH 3)/acetonitrile (80:20), column (250 x 4.6 mm), flow rate, 1.5 ml/min. FAB-MS (MN^+) calc. 710.7, found 710.7. ^{1}H NMR (DMSO-d_6, TMS) δ 10.8 (s, 1H, NH indole), 8.5 (d, 1H, NH-Met), 8.31 (d, 1H, NH-Asp), 8.0 (d, 1H, NH Phe) 7.53-6.86 (12 H, H aromatiques, $CONH_2$), 4.44 (m, 1H, CHα-Asp), 4.31 (m, 2H, CHα-Met, Phe), 3.90 (m, 1H, CHα-Trp), 3.14-2.93 (m, 2H, CHβ-Trp), 2.77-2.97 (m, 2H, CHβ-Phe), 2.59-2.38 (m, 2H, CHβ-Asp), 2.34 (m, 2H, CHγ-Met), 1.95 (s, 3H, SCH_3), 1.71-1.82 (m, 2H, CHβ-Met).

Tritium labelling.

A solution of H-Trp-Met-Asp-Phe (3',4',5'-Br_3)-NH_2. TFA (7) (2.3 mg, 2.4 mmol) in a solvent mixture NH_4OH-DMA (0.5 ml/0.5 ml) was taken in a tritiation reactor and was frozen (10). The catalyst (14 mg of PdO) was then dispersed on the surface and the reaction vial was connected to the automatic tritium gas transfer unit. After a vacuum of 10^{-4} Torr was reached, pure tritium gas (80 curies) was introduced and compressed to 1 bar and the catalyst was flushed for 15 min into the frozen solution. The reaction mixture was brought to 20° and was stirred for 3.5 h. To this mixture was then added β-mercaptoethanol (50 µl) and the catalyst was filtered through Millipore G_5. Labile tritium was eliminated by evaporation in a rotovapor with 1 % aqueous AcOH (80 ml). Total radioactivity recovered was : 71 mCi (2.627 GBq). The peptide was then purified with HPLC on a (300 x 7.8 mm), µ Bondapak C_{18} column with Et_3N-HCOOH buffer (0.025 M)/CH_3CN (80:20) as eluent (flow rate = 2.5 ml/min) with radioactivity and U.V. (210 nm) detection. The main peak coeluted with the reference was collected and rechromatographed giving a single peak by both U.V. and radioactivity detection (21 mCi, 788 MBq, yield : 30%). Quantitative and comparative determinations indicated that the specific radioactivity was found to be 35 Ci/mmol (1295 GBq/mmol). After several month of storage, in liquid nitrogen at a concentration (C = 5.10^{-5}M in water) in presence of β-mercaptoethanol (c=3.10^{-5}M), the tritiated ligand was recovered in a purity over 95 %.

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References.

1. Vanderhaeghen, J.J., Lostra, F., De Mey, J. and Gilles, C. - Proc. Natl. Acad. Sci. USA 77:1190 (1980).
2. Sakamoto, C., Williams, J.A. and Goldfine, I.C. - Biochem. Biophys. Res. Commun. 124:497 (1984).

3. Van Dijk, A., Richards, J.G., Trzeciak, A., Gilessen, D. and Möhler, H. - J. Neurosci. 4:1021 (1984).

4. Innis, R.B. and Snyder, S.H. - Proc. Natl. Acad. Sci. USA, 77:6917 (1980).

5. Agnati, L.F., Fuxe, K., Benfenati, F., Celani, M.F., Battistini, N., Mutt, V., Cavicchioli, L., Galli, G. and Hökfelt, T. - Neurosci. Lett. 35:179 (1983).

6. De Witte, P., Heidbreder, C., Roques, B.P. and Vanderhaeghen, J.J. - Neurochem. Int. 10:473 (1987).

7. Katsuura, G., Itoh, S. and Hsiao, S. - Peptides 6:91 (1985).

8. Hommer, D.W., Stoner, G., Crawley, J.N., Paul, S.H. and Skirboll, L.R. - J. Neurosci. 6:3039 (1986).

9. Leduc, R., Bernier, M. and Escher, E. - Helv. Chim. Acta, 66:960 (1983).

10. Bodanssky, M. and Martinez, J., Synthesis 333 (1981).

11. Morgat, J.L., Desmares, J. and Cornu, M. - J. Labelled Compd. Radiopharm., 11:257 (1975).

12. IUPAC-IUB commision. European J. Biochem. 138:9 (1984).

13. Toth, G.K., Penke, B., Zaraudi, M. and Kovacs, K. - Int. J. Peptide Protein Res. 26:630 (1985).