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Tritium labelling of two highly selective agonists for CCK-B receptors :

[^{3}H]propionyl-Tyr(SO_3Na)-gNle-mGly-Trp-(N-Me)Nle-Asp-Phe-NH_2 ([^{3}H]pBC 264)

[^{3}H]propionyl-γD.Glu-Tyr(SO_3H)-Nle-D.Lys-Trp-Nle-Asp-Phe-NH_2 ([^{3}H]pBC 254).

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SUMMARY

Among the CCK-B receptor agonists reported to date, the two modified peptides BC 264 and BC 254 display a high affinity and selectivity for this binding site and are highly protected from enzymatic degradation. Recently, we reported the biological properties of a tritiated analog of this agonist, [^{3}H]pBC 264, which fullfils all the criteria required for in vitro as well as in vivo studies of the CCK-B receptor. On the other hand, BC 254 displays a high affinity for the CCK-B binding sites in the guinea-pig (K_i = 0.56 nM) while its affinity in the rat is more than 60-fold lower, a difference which could be due to the occurrence of CCK-B receptor subtypes. In the present paper, we report the synthesis of [^{3}H]pBC 264 and of the new tritiated ligand [^{3}H]pBC 254 using [^{3}H] NPS (N-succinimidyl[2,3-^{3}H]propionate) as labelling agent. These two probes have high specific activity (70-100 Ci/mmol) and will enable extensive studies of the CCK-B receptors to be carried out.

Key words : Peptide synthesis, tritium labelling, cholecystokinin, CCK-B receptor.

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INTRODUCTION and RESULTS

Cholecystokinin, originally found in the gastrointestinal tract, is also present in high concentrations in the central nervous system where it is involved in the modulation of a variety of pharmacological processes (1,2). These actions are mediated by at least two classes of receptors designated CCK-A (or peripheral receptors) and CCK-B (or central receptors). To investigate the role of CCK in the brain, we have developed two series of selective CCK-B ligands.

The first series was designed from in vitro studies on the peptidase degradation (3) of the sulfated octapeptide CCK_{26-33} (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) and of its equiactive analog BDNL (4) (Boc-Tyr(SO_3Na)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2). The susceptible peptide bonds of BDNL were protected from degrading enzymes by a series of chemical modifications, i.e., retro-inversion of the amide bond between Met^{28} and Gly^{29} and N-methylation of the amide bond between Trp^{30} and Met^{31}. This led to BC 264 (5) (Boc-Tyr(SO_3Na)-gNle-mGly-Trp-(N-Me)Nle-Asp-Phe-NH_2) which showed the expected resistance to enzymatic degradation, associated with a high affinity and selectivity for CCK-B receptors and agonistic properties in electrophysiological and behavioral tests (6). These results prompted us to prepare a tritiated analogue of this agonist, i.e., [^{3}H]pBC 264 (7,8) in which the Boc group has been substituted by a tritiated propionyl group. This probe retains all the biological and physicochemical properties of BC 264, leading to the first radiolabelled CCK-B selective agonist reported. The other selective radiolabelled CCK ligands available endowed with high affinity and selectivity are the antagonists developed by Merk (9,10) and the recently reported [^{3}H] (N-methyl-Nle^{28-31})CCK_{26-33} (11), but the binding properties of these ligands are less favorable than those of [^{3}H]pBC 264 (12).

The second series of CCK-B ligand, was designed from conformational studies of CCK_8 in solution by ^{1}H NMR spectroscopy and fluorescence transfer measurements (13). These studies showed that CCK_{26-33} exists under folded conformations in solution, stabilized by a β turn between Asp^{26} and Gly^{29}. This spatial arrangement has been mimicked by modification and condensation of the side chains of Asp^{26} and Gly^{29}, leading to constrained CCK analogs, the most potent of which is BC 254 (14) (Boc-γD.Glu-Tyr(SO_3Na)-Nle-D.Lys-Trp-Nle-Asp-Phe-NH_2). This compound is a highly potent and selective CCK-B receptor ligand in the guinea-pig, but has a 60-fold lower affinity for CCK-B receptors in the rat (Table 1). It has been hypothesized that this difference could be explained by slight structural differences in rat and guinea-pig receptors or by

the occurrence of two CCK-B receptor subtypes present in different concentrations in these species (15). Furthermore, this compound behaves as an agonist in electrophysiological studies (16). Therefore, a radiolabelled analog of this agonist appeared suitable to further characterize the CCK-B binding sites as well as their possible receptor heterogeneity.

In this paper, we describe in detail the synthesis of [^{3}H]pBC 264 and of the new radioligand [^{3}H]pBC 254. Preliminary binding studies of [^{3}H]pBC 254 are also shown.

For both compounds, the N-terminal position was used to incorporate 2 tritium atoms by coupling N-succinimidyl [2,3-^{3}H]propionate, [^{3}H]-NPS (90-100 Ci/mmol), a reagent commonly used to label proteins (17), to the N-deprotected corresponding peptides 3 and 5 as illustrated in schemes 1 and 2. For the synthesis of compound 3, Fmoc protection was preferred to Boc protection to avoid acidic treatment of the tyrosine sulfate precursor. Later, we found that brief treatment with anhydrous TFA did not cleave the sulfate group, as shown for compound 5 in scheme 2. The unlabelled analogues pBC 264 and pBC 254 were synthesized in the same way and proved to have high affinities and selectivities for CCK-B receptors (see Table 1 for pBC 254 and reference 7 for pBC 264).

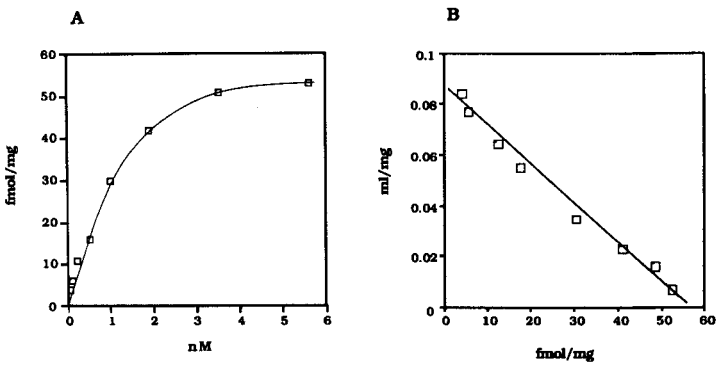
Table 1 : Apparent affinities (K_I, nM) and selectivities of BC 254 and pBC 254 for CCK-B (brain cortex) and CCK-A (pancreas) receptors of guinea pig and rat labelled by [^{3}H]pCCK$_8$ (0.2 nM).

	Guinea-pig			Rat		
	CCK-B	CCK-A	$\frac{\text{CCK-A}}{\text{CCK-B}}$	CCK-B	CCK-A	$\frac{\text{CCK-A}}{\text{CCKB}}$
BC 254	0.56 ± 0.05	2500 ± 640	4464	35.70 ± 1.80	1090 ± 309	30
pBC 254	0.69 ± 0.10	2660 ± 720	3855	66.5 ± 8.5	1150 ± 431	17

The K_I values are the means ± S.E. of at least four determinations performed in triplicate. These binding studies were performed as previously described in detail (15).

Using tritiated NPS, the radiolabelled probes were obtained in good yields and with high specific activities (98-100 Ci/mmol for [^{3}H]pBC 264 and 68-70 Ci/mmol for [^{3}H]pBC 254). Scatchard analysis of the binding isotherm of [^{3}H]pBC 254 to membranes of guinea-pig cortex (Fig. 1), showed that the ligand interacted with a high affinity, K_D = 0.82 ± 0.15 nM, apparently to a single class of binding sites, B_{max} = 50.8 ± 1 fmol/mg of protein. The specific binding was around 65% at the K_D concentration.

Figure 1 : Binding isotherm (A) and Scatchard plot (B) of the specific binding of [^{3}H]p BC 254 to guinea pig brain cortex determined at concentrations between 0.01 and 10 nM. The data are from a single representative experiment performed as previously described (15), with each point in triplicate. The nonspecific binding was determined using 1 µM CCK_{26-33}.



In conclusion, [^{3}H]pBC 264 and [^{3}H]pBC 254 have been synthesized by a very simple method using the commonly available [^{3}H]NPS, leading to the pure compounds endowed with high specific activities and good stability (purity greater than 80% after 4 months storage). Both agonists had a high affinity and selectivity for the CCK-B receptor. In particular, [^{3}H]pBC 264 shows very favorable physicochemical properties (water solubility, resistance to peptidase), very low non-specific binding and had been shown to cross the blood-brain barrier (7,8). On the other hand, [^{3}H]pBC 254 retains the binding properties of its parent compound BC 254, and seems to be a good candidate for the study of the CCK-B receptor heterogeneity.

EXPERIMENTAL PART

MATERIALS

All protected amino acids were from Bachem (Switzerland). Solvents were from Prolabo (France) and were of analytical grade. Anhydrous solvents were dried over 4-Å molecular sieves prior to use NPS (17), compound **1** (5) and compound **4** (14) were synthesized as previously described. [^{3}H]-NPS (90-110 Ci/mmol) was purchased from Amersham.

The structure of the compounds and all intermediates were established by ^{1}H NMR spectroscopy (Bruker WH 270 and 400 MHz). The purity was checked by thin layer

chromatography on silica gel plates (Merck) in the following solvent systems : (A) chloroform-MeOH (9:1) ; (B) EtOAc-pyridine-AcOH-H_2O (200:20:6:11) ; (C) EtOAc-pyridine-AcOH-H_2O (65:20:6:11) ; (D) EtOAc-pyridine-AcOH-H_2O (40:20:6:11). Plates were developed with UV, iodine vapor, ninhydrin or Ehrlich's reagent. Analytical and preparative HPLC was carried out on a LKB apparatus with a 250 x 4.6 mm nucleosil C18, 5 µm column using Et_3N-H_3PO_4 buffer (TEAP ; 0.025M ; pH = 6.5)-CH_3CN as eluent at a flow rate of 1.2 mL/min with UV (210 nm) detection. Melting points were determined with a Kofler apparatus and are given uncorrected. FAB mass spectra were recorded on a double-focussing VG 70-250 instrument. The radioactivity was determined using a LKB 1209 Rackbeta liquid scintillation counter. The concentration was estimated by comparing the area of the peak eluting on HPLC with those of different quantities of unlabeled standard. The following abbreviations have been used : NPS, N-succinimidyl propionate ; [^{3}H]-NPS, N-succinimidyl [2,3-^{3}H]propionate ; MeOH, methanol ; EtOAc, ethylacetate ; AcOH, Acetic acid ; TFA, trifluoro acetic acid ; DMF, dimethylformamide ; DMSO, dimethylsulfoxide ; ONP, p-Nitrophenyl ester ; Fmoc, 9-Fluorenylmethoxycarbonyl ; Boc, tert-butyloxycarbonyl. Other abbreviations used are those recommanded by the IUPAC-IUB Commission (Biochem. J. 1984, 219, 345). The standard three letter notation preceded by the prefix g represents the gem diamino alkyl residue derived from the specific amino acid. The prefix m represents the malonic acid derived residue.

METHODS

The synthesis of [^{3}H]p BC 264 and [^{3}H]p BC 254 are reported in schemes 1 and 2.

SCHEME 1

H - gNle - mGly - Trp - (N-Me)Nle - Asp - Phe - NH_2 **1**

↓ Fmoc-Tyr-ONp

Fmoc - Tyr - gNle - mGly - Trp - (N-Me)Nle - Asp - Phe - NH_2 **2**

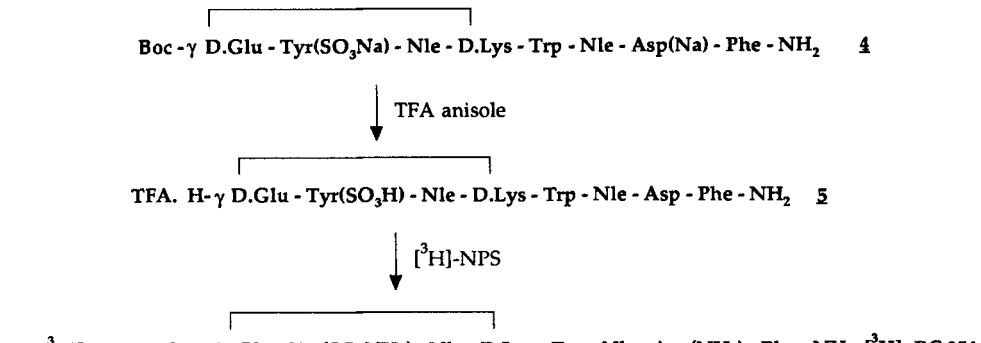
↓ 1) SO_3/Pyridine
↓ 2) $NaHCO_3$

H - Tyr(SO_3Na) - gNle - mGly - Trp - (N-Me)Nle - Asp(Na) - Phe - NH_2 **3**

↓ [^{3}H]-NPS

[2,3-(^{3}H)]propionyl - Tyr(SO_3Na) - gNle - mGly - Trp - (N-Me)Nle - Asp(Na) - Phe - NH_2 [^{3}H]pBC 264

SCHEME 2



I- Peptide synthesis

Fmoc-Tyr-gNle-mGly-Trp-(N-Me)Nle-Asp-Phe-NH_2 (2)

To a solution of Fmoc-Tyr-OH (1.279g, 3.17 mmol) in dry DMF (5 ml), cooled to 0°C, was added p-nitrophenol (441 mg, 3.17 mmol) and dicyclohexylcarbodiimide (660 mg, 3.17 mmol). The mixture was stirred for 1 h at 0°C and overnight at room temperature. After evaporation of the solvent in vacuo, the residue was dissolved in EtOAc (40 ml) and dicyclohexylurea was removed by filtration. The filtrate was washed with a 10% solution of $NaHCO_3$ (4 x 50 ml) and a saturated solution of NaCl (2 x 50 ml), dried (Na_2SO_4) and evaporated in vacuo to yield 1.58 g (95%) of crude Fmoc-Trp-ONp which was used without further purification (TLC Rf[A) 0.9). This compound (230 mg, 0.44 mmol) was added to a solution cooled to 0°C of H-gNle-mGly-Trp-(N-Me)Nle-Asp-PheNH_2 (1) (303 mg, 0.4 mmol) in dry DMF (3 ml). The mixture was stirred for 1 h at 0°C and overnight at room temperature under N_2. After evaporation of the solvent in vacuo, the residue was washed with ether and purified by chromatography on a silica gel column with the eluent EtOAc-pyridine-AcOH-H_2O (200:20:6:11) to yield 320 mg (72%) of a light yellow powder. mp = 188°C ; TLC Rf[B) 0.29, Rf(C) 0.79.

H-Tyr(SO_3Na)-gNle-mGly-Trp-(N-Me)Nle-Asp(Na)-Phe-NH_2 (3)

A solution of the preceding compound 2 (320 mg, 0.28 mmol) in dry DMF (5 ml) and dry pyridine (5 ml) was treated with an SO_3-pyridine complex (2 g) overnight at room temperature

under N_2 with vigorous stirring. After evaporation in vacuo at 35°C, the residue was taken up in cold saturated $NaHCO_3$ solution and stirred at room temperature for 3 h with the pH maintained at 9. After lyophilization, the inorganic salts were precipitated from MeOH and the filtrate was evaporated in vacuo. The residue was purified by chromatography on a silica gel column with EtOAc-pyridine-AcOH-H_2O (60:20:6:11) as eluent to give, after evaporation of the solvent in vacuo and lyophilization in 0.1 M NH_4OH, 121 mg (43%) of a product identified by NMR as the non protected sulfated peptide (3). mp = 218-224°C, dec ; TLC Rf(C) 0.13, Rf(D) 0.74 ; FAB-MS (MH+) Calc. 1051, found 1051 ; HPLC (Rt = 10.0 min) eluent TEAP-CH_3CN (66:34).

Propionyl-Tyr(SO_3Na)-gNle-mGly-Trp-(N-Me)Nle-Asp(Na)-Phe-NH_2

To a solution of the preceding compound 3 (50 mg, 49 µmol) in dry DMF (1 ml) was added NPS (10 mg, 60 µmol) and the mixture was stirred overnight at room temperature. After evaporation of the solvent in vacuo, the residue was washed with ether and purified by chromatography on a silica gel column using EtOAc-pyridine-AcOH-H_2O (65:20:6:11) as eluent to give 48 mg (90%) of a white powder which was lyophilized in 0.1 M NH_4OH (10 ml). mp = 195-200°C, dec ; TLC Rf(C) 0.18 ; FAB-MS (MH+) Calc. 1107, found 1107 ; HPLC (Rt = 12.2 min) eluent TEAP-CH_3CN (66:34).

TFA.H-γD.Glu-Tyr(SO_3H)-Nle-D.Lys-Trp-Nle-Asp-Phe-NH_2 (5)

Boc-γD.Glu-Tyr(SO_3Na)-Nle-D.Lys-Trp-Nle-Asp(Na)-Phe-NH_2 (4) (18 mg, 13.6 µmol) was treated with an ice cold mixture of TFA-anisole (9:1) (200 µl). The solution was stirred for 30 min at 0°C and 30 min at room temperature under N_2. Precipitation with dry ether (5 ml) afforded a white powder which was quickly washed with ether (4 x 5 ml) and dried in vacuo to yield 17 mg (93%) of compound. mp = 232-238°C, dec ; TLC Rf(C) 0.10, Rf(D) 0.80 ; FAB-MS (MH+) Calc. 1290, found 1290 ; HPLC (Rt = 15.9 min) eluent TEAP-CH_3CN (69:31).

Propionyl-γD.Glu-Tyr(SO_3NH_4)-Nle-D.Lys-Trp-Nle-Asp(NH_4)-Phe-NH_2

To a solution of the preceding compound 5 (9 mg, 6.7 µmol) in dry DMF (200 µl) was added Et_3N (1.2 ml, 8.6 µmol), NPS (1.4 mg, 8 µmol) and the mixture was stirred overnight at room temperature. After evaporation in vacuo, precipitation from MeOH/ether gave a white powder

which was dissolved in 0.1 M NH_4OH (10 ml) and lyophilized to yield 8 mg (90%) of 7. mp = 222-228°C, dec ; TLC R(C) = 0.15 ; FAB-MS (MH+) Calc. 1266, found 1266 ; HPLC (Rt = 18.7 min) eluent TEAP-CH_3CN (69-31).

II-Tritiation

(2,3-^{3}H)propionyl-Tyr(SO_3Na)-gNle-mGly-Trp-(N-Me)-Nle-Asp(Na)-Phe-NH_2

[^{3}H]-NPS (50 nmol, 5 mCi) in toluene (5 ml) was placed in a microvial. After evaporation of the organic solvent under a gentle stream of N_2, a solution of H-Tyr(SO_3Na)-gNle-mGly-Trp-(N-Me)Nle-Asp(Na)-Phe-NH_2 (105 µg, 100 nmol) in anhydrous DMSO (25 µl) was added. The mixture was stirred for 4 days at room temperature under N_2 and then diluted with 225 µl of acetonitrile-water (1:1). The resulting solution was purified by HPLC using a linear gradient rising from 28% to 38% acetonitrile in 30 min (under these conditions Rt(3) = 17.1 min, R_t(pBC 264) = 19.2 min). Fractions (30s) were collected and 1 µl of each was mixed with 5 ml scintillation liquid for quantitation of the eluted radioactivity (Fig. 2). Quantitative analysis of the labelled peptide was performed by UV (210 nm) during elution and subsequently confirmed by fluorescence (1_{EM} = 350 nm, 1_{EX} = 285 nm), using the unlabelled peptide as standard. Purity was checked by HPLC by coinjecting 1 µl of the labelled probe with the cold peptide under the preceding conditions and was greater than 95%. Thus, the reaction led to around 18.3 nmol (37%) of pure [^{3}H]pBC 264 with a specific activity of 98-100 Ci/mmol.

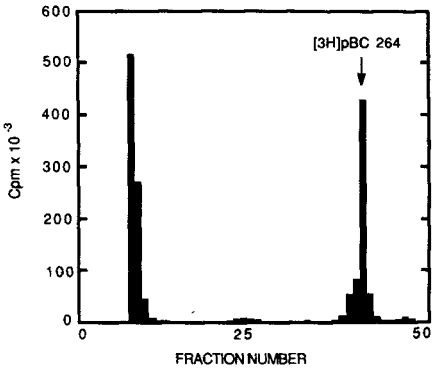
(2,3-^{3}H)propionyl-γD.Glu-Tyr(SO_3H)-Nle-D.Lys-Trp-(N-Me)Nle-Asp-Phe-NH_2

[^{3}H]-NPS (105 nmol, 10.5 mCi) in toluene was placed in a microvial. After evaporation of the organic solvent under a gentle stream of N_2, a solution of TFA-H-γD.Glu-Tyr(SO_3H)-D.Lys-Trp-Nle-Asp-Phe-NH_2 (271 µg, 210 nmol) and triethylamine (212 µl, 210 nmol) in dry DMSO (40 µl) was added. The mixture was stirred for 5 days at room temperature and then diluted with 360 µl of acetonitrile-water (1:1). The resulting solution was purified by HPLC. The elution was performed with 25 mM TEAP buffer, at pH 4.2 and acetonitrile, using a linear gradient rising from 34% to 38% acetonitrile in 15 min, (under these conditions, Rt(5) = 10.2 min and Rt(pBC 254) = 15.7 min). Fractions (30s) were collected, adjusted to pH 6.5 by 0.1 M NH_4OH and analysed

qualitatively and quantitatively as for [^{3}H]pBC 264, yielding 31.5 nmol (30%) of pure [^{3}H]pBC 254 with a specific activity of 68-70 Ci/mmol.

For both tritiated ligands, after storage at 4°C for 4 months the purity was checked by HPLC and was greater than 80%, illustrating the good stability of these probes.

Figure 2 : Purification of [^{3}H]p BC 264 by reverse-phase HPLC.



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