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Synthesis, characterization and in vitro evaluation of new oxorhenium- and oxotechnetium-CCK4 derivatives as molecular imaging agents for CCK2-receptor targeting

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ABSTRACT

The goal of this study is to design new ^{99m}Tc-radiolabelled shortened CCK derivatives that might be suitable for the molecular imaging of cholecystokinin-2 receptors (CCK2-R), these receptors being over-expressed in a number of neuroendocrine tumors such as medullary thyroid cancer and small-cell lung cancer. For this purpose, we designed several modified CCK4 analogs bearing an ON_2S tetradentate chelating agent at the *N*-terminus, the CCK4 sequence representing the minimal peptide sequence that presents nanomolar affinity and activity towards the CCK2-R. Four peptide conjugates of general formula (Trt)SN_2OPh–$(X)_n$–CCK4 ($X = \beta$-alanine or 6-aminohexanoic acid spacers; $n = 0$, 2, 4) and their oxorhenium peptide conjugates have been synthesized and characterized. *In vitro* evaluation of these compounds showed a close relationship between the nature and the length of the spacer and the corresponding binding affinity values. The most promising oxorhenium complex **5-Re** exhibited potent CCK2-receptor agonist properties in promoting the production of inositol phosphate in COS-7 cells ($EC_{50} = 5.17$ nM). Preliminary ^{99m}Tc-radiolabelling studies with peptide conjugates **3** or **5** led exclusively to the corresponding ^{99m}TcO-complexes **3-Tc** and **5-Tc**, which exhibited high resistance towards an excess of cysteine and satisfactory stabilities in human serum. To conclude, the promising *in vitro* characteristics of compounds **5-Re**, **5-Tc** illustrate the feasibility to develop stable radiolabelled shortened CCK4 derivatives with a nanomolar CCK2-R affinity.

1. Introduction

Interest in the development of radiolabelled compounds such as antibodies or peptides continues to be stimulated by the suitability of such attractive tools to be used as receptor targeting diagnostic/therapeutic agents in nuclear medicine [1]. Currently, their relative facility of synthesis and their ability to tolerate the harsh conditions (pH, temperature…) of chemical modification and/or radiolabelling, their rapid blood clearance and high tumor-to-background ratios make peptides the most used targeting molecules [2]. Many regulatory peptide-based radiopharmaceuticals, ranging from neuropeptide-Y and RGD peptides to somatostatin and gastrin analogues have been developed to target receptor systems which are over-expressed in particular types of cancer [3]. The best examples are represented by the ^{111}In-DTPA-octreotide (Octreoscan™) [3], the first FDA-approved diagnostic radiopeptide widely used in cancer imaging and the ^{99m}Tc-depreotide (NeoTect®)[4] for imaging lung cancer.

Among these clinically relevant biological targets, we have focused on the cholecystokinin (CCK) receptor family and more particularly on the CCK receptors of the subtype 2 (CCK2-R) as model for development of such tumor-specific peptides. The cholecystokinin-2 receptors which belong to the G protein-coupled receptor superfamily are mainly present in gut mucosa and brain [5] and bind two natural ligands – cholecystokinin and gastrin-with nanomolar affinities. More interestingly, CCK2-receptors are over-expressed in different human tumors of neuroendocrine origin whereas they are not present in the corresponding normal tissues,

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as demonstrated by Reubi *and coll.* [6]. In particular, they are abundantly expressed in 92% of medullary thyroid carcinomas (MTC) but also in high percentage of small-cell lung cancers, some astrocytomas and stromal ovarian cancers and to a lesser extent in gastrointestinal tumors. On the basis of these findings, the specific targeting of CCK2-receptors with a variety of radiolabelled CCK/gastrin-related peptides has been intensively studied over the past years [7]. All of these peptide-based radiopharmaceuticals were characterized by an identical five amino acids sequence located at their biologically active C-terminus region. It has been shown that the presence of the carboxyl-terminal tetrapeptide amide CCK4 – Trp–Met–Asp–Phe–NH_2 - is crucial for receptor binding whereas the methionine residue can be replaced by leucine or norleucine (Nle) [7d].

A rapid overview showed that most of these studies have been performed with minigastrin (MG) or CCK8 derivatives radiolabelled with indium-111 or technetium-99 m [8,9]. Briefly, the radiolabelled MG analogs exhibited the greatest uptake in CCK2-receptor-positive tumors but they were also associated with high uptake in the kidney. Conversely, CCK8 analogs presented moderate tumor uptake but low kidney uptake. More surprisingly, a few studies were carried out on shorter peptides such as CCK4 peptide (= CCK fragment 30–33). It is well-known that the major drawback associated with small peptides is the loss of binding affinity upon coupling with a radiocomplex (chelator plus a given radioisotope). Thus, if the highest CCK2-R affinities were observed for radioiodinated CCK8 and minigastrin derivatives, the radioiodinated CCK4 and des-BOC-pentagastrine showed a loss of affinity by several orders of magnitude (10^{-7} M *vs.* nM range for CCK8 or MG), as shown by Behr *and coll.* [10]. Nevertheless, this loss of binding affinity can be minimized by incorporating an appropriate spacer between the chelating moiety and the peptide [11].

With these points in mind, we have taken an original strategy to target CCK2-receptor based on shortened CCK/gastrin-related peptides radiolabelled with technetium-99 m, this radionuclide presenting the ideal imaging characteristics (140 KeV γ emitter, relatively short half-life of 6 h) combined with a convenient availability from the commercial $^{99}Mo/^{99m}Tc$ generator. Our derivatives incorporate the design features illustrated in Fig. 1; these include: (i) the tetrapeptide amide Trp–Nle–Asp–Phe–NH_2 whereas the methionine has been replaced by the norleucine (ii) a ^{99m}TcO-specific tetradentate $PhON_2S$ chelator (iii) a tethering moiety between the chelator and the peptide. To avoid the introduction of new enzymatic cleavage sites, homoaminoacids like β-alanine (β-Ala) or 6-aminohexanoic acid (Ahx) were chosen as spacer. Moreover, a recent study on bombesin analogs demonstrated that the additional introduction of a (β-ala)$_2$ linker between the peptide sequence and the chelator led to an improved *in vivo* tumor uptake [12].

In the present study we investigated the rapid synthesis and characterization of a range of new peptide conjugates of general formula (Trt)SN_2OPh–$(X)_n$–CCK4 ($X = \beta$-ala or Ahx; $n = 0, 2, 4$; Trt = trityl group). The biological evaluation of the corresponding oxometallic peptide conjugates is performed by using either the technetium-99 m or its congener, the rhenium-185/187. The latter metal is used because it possesses chemical properties similar to those to technetium, particularly in the +V oxidation state, and it provides a non-radioactive alternative to working with technetium radioisotopes [13]. Thus, the first *in vitro* results of these bioconjugates and two oxorhenium complexes showed the impact of the nature and the length of the spacer on the CCK2-R affinity. In addition, one of these CCK4 analogs (compound **5**) led to both a ReO-complex which presents, for the first time, a nanomolar binding affinity towards CCK2-receptors and a ^{99m}TcO-radiolabelled peptide conjugate with a satisfactory stability in human serum, making it a promising candidate as molecular imaging agent for CCK2-positive tumors targeting.

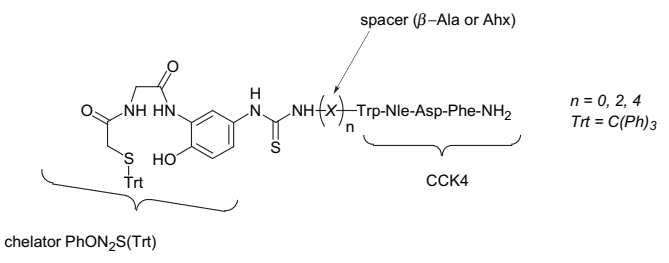


Fig. 1. Design of (Trt)SN_2OPh–$(X)_n$–CCK4 peptide conjugates.

2. Material and methods

All chemicals were of the highest commercially available purity and all solvents were freshly distilled by standard methods before use. Rhenium(VII) oxide, purchased from Aldrich Chem. Co, was converted to trichlorooxobis(triphenylphosphine)rhenium(V), $ReOCl_3(PPh_3)_2$, according to published protocols [14]. Preparation of the intermediate ligand H_2N–$PhON_2S$(Trt) has been described previously [15]. CCK4 analogs were purchased from NeoMPS (Strasbourg, France). NMR and Infra-Red spectra of compound **1** were recorded on a Bruker AC 300 (300 MHz) spectrometer and a Bruker Vector 22 spectrophotometer, respectively. Mass spectra were obtained on a Perkin Elmer Sciex API 365 (electrospray), a DSQ2 Thermofisher (chemical ionisation) or a Nermag 10-10 (FAB) mass spectrometers. $[^{99m}TcO_4]^-$ was eluted as a physiological saline solution from a commercial $^{99}Mo/^{99m}Tc$ generator.

Analytical and semi-preparative RP-HPLC were performed using a Waters system equipped with a diode-array detector or a Waters 600E gradient chromatography coupled to a UV-visible detector (ICS) and a γ-detector (Raytest) for the ^{99m}Tc-compounds. Analytical and semi-preparative separations for non-radioactive compounds were achieved on 250×4.6 mm (5 μm) and 250×10 mm (5 μm) Phenomenex Luna C18 RP columns, respectively. The flow rate was 1 mL/min for analytical runs and 5 mL/min for semi-preparative purifications. In all runs, the eluent was 0.1% TFA in H_2O (Solvent A) and acetonitrile (solvent B). For the analytical control and semi-preparative separation, the linear gradient used was 85% A and 15% B to 100% B over 35 min. RP-HPLC analysis and purification of ^{99m}Tc complexes were carried out on a Nucleodur C18 endcapped RP column (Macherey-Nagel analytical column 125×4 mm, 5 μm). The RP-HPLC conditions were as follows: flow rate was 1 mL/min, eluent was 0.1% TFA in H_2O/MeOH 40/60 (Solvent A) and 0.1% TFA in H_2O/MeOH 10/90 (solvent B) and gradient system was 0–17 min, 100% A to 100% B; 17–22 min, 100% B; 22–30 min, 100% B to 100% A. In all analytical and semi-preparative separations, the wavelength used for UV detection was 275 nm. Then, radiochemical purity was assessed by thin layer chromatography on Alugram C18 W plates (eluent MeOH/H_2O/TFA: 70/30/0.1) using a Berthold LB 2832 detector coupled to a radiochromatogram scanner.

2.1. SCN-$PhON_2S$(Trt) **1**

According to a modification of a literature procedure [15], to a solution of H_2N-$PhON_2S$(Trt) (350 mg, 0.7 mmol) in THF (10 mL) thiophosgene (540 μL, 7.0 mmol) was added. The solution was stirred for one hour at room temperature under nitrogen, and the solvent was then removed under reduced pressure. The residue was taken in acetone/H_2O solution and the resulting precipitate was filtered off, washed with cold acetone and dried under vacuum

to give the title compound as a white powder (341 mg, 90% yield). ^{1}H NMR (DMSO-d_6) δ_H(ppm): 2.86 (s, 2H, CH_2S), 3.85 (d, $J = 5.6$ Hz, 2H, CH_2N), 6.88 (d, $J = 8.6$ Hz, 1H, H_{Ar}), 7.01 (dd, $J = 8.6$ Hz and 2.6 Hz, 1H, H_{Ar}), 7.31 (m, 15H, $H_{Ar\ Trt}$), 7.99 (d, $J = 2.6$ Hz, 1H, H_{Ar}), 8.34 (s, 1H, NH), 9.27 (s, 1H, NH), 10.59 (s, 1H, OH); ^{13}C {^{1}H} NMR (DMSO-d_6) δ_C(ppm): 36.3 (CH_2S), 43.7 (CH_2N), 66.6 (C_{Trt}), 116.0, 118.8 ($2CH_{Ar}$), 120.9 (CS), 122.1 (CH_{Ar}), 127.4 (C_{Ar}), 127.3, 128.6, 129.6 ($15CH_{Ar\ Trt}$), 132.6 (C_{Ar}), 144.5 ($3C_{Ar\ Trt}$), 147.5 (C_{Ar}), 168.4 (2CO); MS (DCI/NH_3): 540 [$M + H^+$], 557 [$M + NH_4^+$]; IR (KBr): $\nu_{C=O} = 1657$, 1698 cm^{-1}; $\nu_{NCS} = 2124$ cm^{-1}.

2.2. Synthesis of bioconjugates **2**–**5**, general procedure

A solution of **1** (3 eq.) in anhydrous DMF was added to a vial containing a defined CCK4 derivative (1 eq.) solution in anhydrous DMF. Subsequently, a 10-fold molar excess of freshly distilled Et_3N was added to the mixture. The conjugation mixture was vortexed and incubated for one night at room temperature. Then, the solution was dried under vacuum and the crude bioconjugate was purified by semi-preparative RP-HPLC (see above for elution conditions) or by precipitation in CH_2Cl_2.

2.2.1. Bioconjugate **2**

8.4 mg (15.6 μmol) of **1** in DMF (50 μL), 3.0 mg (5.2 μmol) of CCK_4 in DMF (50 μL) and 7.3 μL (52 μmol) of Et_3N give 2.2 mg of **2** after RP-HPLC purification (38% yield); FAB$^+$-MS: *m*/*z* 1140 [$M + Na^+$]; RP-HPLC: $t_r = 23.6$ min.

2.2.2. Bioconjugate **3**

67.3 mg (124.8 μmol) of **1** in DMF (1 mL), 30 mg (41.6 μmol) of (β-ala)$_2$-CCK_4 in DMF (2 mL) and 58 μL (416 μmol) of Et_3N give 36.7 mg of **3** after precipitation in CH_2Cl_2 (70% yield); ESI$^+$-MS: *m*/*z* 1260 [$M + H^+$], 1282 [$M + Na^+$], 1298 [$M + K^+$]; RP-HPLC: $t_r = 22.6$ min.

2.2.3. Bioconjugate **4**

3.8 mg (6.96 μmol) of **1** in DMF (50 μL), 2.0 mg (2.32 μmol) of (β-ala)$_4$-CCK_4 in DMF (50 μL) and 3.2 μL (23.2 μmol) of Et_3N give 0.9 mg of **4** after RP-HPLC purification (28% yield); FAB$^+$-MS: *m*/*z* 1402 [$M + H^+$], 1424 [$M + Na^+$], 1440 [$M + K^+$]; RP-HPLC: $t_r = 20.0$ min.

2.2.4. Bioconjugate **5**

4.0 mg (7.47 μmol) of **1** in DMF (50 μL), 2 mg (2.49 μmol) of (Ahx)$_2$–CCK_4 in DMF (50 μL) and 3.5 μL (25.0 μmol) of Et_3N give 1.4 mg of **5** after RP-HPLC purification (42% yield); FAB$^+$-MS: m/z 1366 [$M + Na^+$], 1382 [$M + K^+$]; RP-HPLC: $t_r = 21.8$ min.

2.3. Synthesis of rhenium bioconjugates **2**-**Re**–**5**-**Re**, general procedure

To a given bioconjugate (1 eq.) and sodium acetate trihydrated (4 eq.) dissolved in methanol, $ReOCl_3(PPh_3)_2$ (1.1 eq) was added. The mixture was vortexed and incubated for one night at 65 °C. After cooling, the brown solution was evaporated to dryness. The crude bioconjugate was purified by semi-preparative RP-HPLC (see above for elution conditions) or by precipitation in CH_2Cl_2. Each oxorhenium complex was obtained as the sodium salt.

2.3.1. Rhenium bioconjugate **2**-**Re**

2.0 mg (1.79 μmol) of **2** in MeOH (80 μL), 1.0 mg (7.35 μmol) of $CH_3COONa.3H_2O$ and 1.6 mg (1.97 μmol) of $ReOCl_3(PPh_3)_2$ give 1.35 mg of **2**-**Re** as a brown powder after RP-HPLC purification (69% yield); ESI$^-$-MS: *m*/*z* (abundance) 1075 (100%) [M^-]; RP-HPLC: $t_r = 16.4$ min.

2.3.2. Rhenium bioconjugate **3**-**Re**

10 mg (7.9 μmol) of **3** in MeOH (2.0 mL), 4.3 mg (31.6 μmol) of $CH_3COONa.3H_2O$ and 7.3 mg (8.69 μmol) of $ReOCl_3(PPh_3)_2$ give 7.4 mg of **3**-**Re** as a brown powder after precipitation in CH_2Cl_2 (75% yield); ESI$^-$-MS: *m*/*z* (abundance) 1238 (100%) [$M^- + Na^+ - H^+$], 1261 (30%) [$M^- + 2Na^+ - 2H^+$]; RP-HPLC: $t_r = 9.8$ min.

2.3.3. Rhenium bioconjugate **4**-**Re**

0.8 mg (0.57 μmol) of **4** in MeOH (20 μL), 0.3 mg of $CH_3COONa.3H_2O$ and 0.5 mg of $ReOCl_3(PPh_3)_2$ give 0.5 mg of **4**-**Re** as a brown powder after RP-HPLC purification (62% yield); ESI-MS: *m*/*z* (abundance) 1359 (100%) [M^-]; RP-HPLC: $t_r = 12.6$ min.

2.3.4. Rhenium bioconjugate **5**-**Re**

2.0 mg (1.49 μmol) of **5** in MeOH (100 μL), 0.8 mg (5.96 μmol) of $CH_3COONa.3H_2O$ and 1.36 mg (1.64 μmol) of $ReOCl_3(PPh_3)_2$ give 1.34 mg of **5**-**Re** as a brown powder after RP-HPLC purification (68% yield); ESI$^-$-MS: *m*/*z* (abundance) 1301 (100%) [M^-]; RP-HPLC: $t_r = 18.5$ min.

2.4. Inositol phosphate production measurement

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (SCF) and 0.5% penicillin/streptomycin and were transfected with 2 μg of human CCK2-R using the DEAE/dextran method [16]. 24 h after transfection, transfected cells were split into 24-well plates. The cells were labelled overnight with 2μCi/well of *myo*- [^{3}H]inositol in DMEM with serum, washed and then stimulated for 1 h at 37 °C with either gastrin or CCK4 derivatives **2**, **3**, **5**, **3**-**Re**, **5**-**Re** (10^{-11} M to 10^{-6} M) in buffer pH 7.45 (20 mM Hepes, 135 mM NaCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose and 0.5% BSA). After stimulation, the cells were lysed with MeOH/HCl, the content was analyzed by strong anion exchange chromatography and the IP production was determined as previously described [17]. EC_{50} and agonist properties were determined using Graph-Pad Prism program software. The EC_{50} values obtained for the series of bioconjugates and oxorhenium complexes are summarized in Table 1.

2.5. Radiolabelling of **3** and **5**

In a borosilicated vial containing buffer (pH = 8.6; 100 μL), a solution of bioconjugate **3** or **5** in methanol (1 mg/mL, 100 μL) and $Na^{99m}TcO_4$ generator eluate (40 μL, 6 mCi) were added. After addition of a fresh $SnCl_2$ solution in MeOH (25 μL, 10^{-2} M solution), the vial was sealed with a teflon-lined cap and the mixture was heated at 75 °C for 30 min. After cooling, the resulting complexes were purified by RP-HPLC (see above for elution conditions). The radiolabelling yield was >95%. The radiochemical purity assessed by ITLC was >98% after RP-HPLC purification for each complex. t_r of **3**-**Tc** = 11.02 min. and t_r of **5**-**Tc** = 13.34 min.. In these same analytical RP-HPLC conditions, the rhenium complexes **3**-**Re** and **5**-**Re** were eluted at 10.37 min. and 12.13 min., respectively.

Table 1
EC_{50} values (nM) for bioconjugates **2**, **3**, **5** and complexes **3**-**Re** and **5**-**Re.**

Bioconjugate	EC_{50} (nM)	Complex	EC_{50} (nM)
Gastrin	0.41 ± 0.12		
2	84.54 ± 13.39	**2**-**Re**	n.d.[a]
3	3.10 ± 0.59	**3**-**Re**	22.86 ± 3.01
4	n.d.[a]	**4**-**Re**	n.d.[a]
5	5.01 ± 0.93	**5**-**Re**	5.17 ± 1.14

[a] n.d. non determined.

2.6. In vitro stability

Stability versus cysteine: Cysteine challenge experiment was carried out on the purified complexes **3**-**Tc** and **5**-**Tc** using an excess of cysteine (250:1 cysteine/complex molar ratio). In a borosilicated vial containing phosphate buffer (200 μL, 0.2 M, pH 7.2), water (100 μL) and purified technetium complex **3**-**Tc** or **5**-**Tc** (50 μL), an aliquot (50 μL) of a freshly prepared aqueous solution of L-cysteine (10 mM) was added. The vial was sealed with a teflon-lined cap and the solution was stirred and incubated at 37 °C for various time intervals (1, 6 and 12 h). Periodically incubate aliquots were removed and analysed by RP-HPLC.

In vitro stability: The *in vitro* stability of the purified complex **5**-**Tc** was evaluated by monitoring the radiochemical purity (RCP) at different time points using the following procedure: in a borosilicated vial, **5**-**Tc** (50 μL, approximately 1 mCi) was added to (i) 1 mL of fresh human plasma, (ii) 1 mL of homogenate of mouse liver. The resulting mixtures were incubated at 37 °C and analyzed at appropriate time points (30 min., 1, 2 and 4 h). For human plasma, 50 μL-aliquots were withdrawn and treated with 200 μL of ethanol to precipitate proteins. After centrifugation, the supernatant was analyzed by RP-HPLC. For homogenate of mouse liver, samples were precipitated with acetonitrile, centrifuged and analyzed by RP-HPLC using the radiolabelling elution conditions.

3. Results and discussion

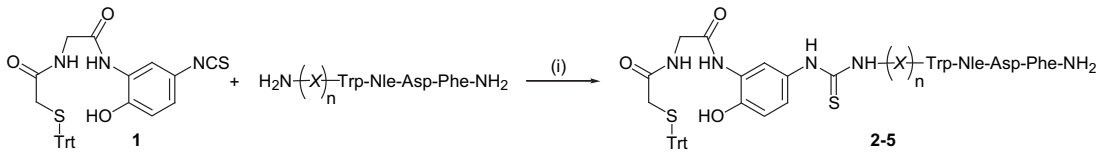
A high binding affinity of peptide-based radiopharmaceuticals is crucial for effective tumour imaging and therapy. To achieve this goal, design and synthesis of radiolabelled peptide having high receptor affinity and being stable under physiological conditions is essential. The latter criteria involved to tag the biomolecule with a very stable metal chelator complex. In recent works, we developed an amine-functionalized tetradentate bifunctional chelator containing amido, alcohol and thiol functions named H_2N–$PhON_2S$(Trt) which exhibited excellent chelating properties and high *in vitro* stabilities of the corresponding technetium and rhenium complexes [15,18]. This chelator was obtained in five steps with an overall yield of 46%. Biodistribution studies of the corresponding ^{99m}TcO-complex performed in healthy male Wistar rats at 5 and 30 min post-injection (p.i.) showed no particular organ uptake [19]. Briefly, the complex was preferentially eliminated *via* the renal-urinary excretion route, as revealed by the 17% ID in urine at 30 min p.i. The rapid clearance of this compound from the bloodstream (18.37% at 5 min., 8.59% ID/organ at 30 min.), indicated its high stability against exchange reactions with blood proteins and no specific uptake or long-term retention in organs or tissues. Then, the minimal activity accumulation in the stomach (1.02% ID/organ at 30 min.) indicated that $^{99m}TcO_4^-$ was not produced in relevant amount during biodistribution [20], providing important evidence that this ligand system is capable of stabilising ^{99m}TcO core even under *in vivo* conditions.

For bioconjugation purposes, H_2N–$PhON_2S$(Trt) has been converted to the isothiocyanate derivative **1** with 90% yield, by reaction with thiophosgene in dry THF followed by precipitation in acetone/water mixture (Fig. 2). The presence of the -NCS function was confirmed by its 2124 cm^{-1} IR band. The bioconjugation reactions have been performed by solution-phase synthesis technique, this technique being particularly adapted for large-scale peptide production [21]. In a typical protocol, an excess of **1** (3 eq.) was added to one equivalent of the CCK4 derivative in anhydrous DMF (Fig. 2). After overnight coupling at 37 °C and evaporation of the DMF, the different CCK4 bioconjugates **2**–**5** were purified by RP-HPLC or precipitation, in milligram-scale, from low to good yields (28–70%) and were characterized by positive FAB- or ESI-MS. The HPLC chromatographic analyses confirmed the purity of each compound **2**–**5** as they showed only one peak, with retention times ranging from 20.0 to 23.6 min. It is noteworthy that serious problems of solubility occurred during the HPLC purification of the bioconjugate **4**, explaining the low yield associated to this compound.

The complexation of ReO^{3+} with this type of ON_2S chelating moiety results in deprotonation of the two amide and alcohol groups and detritylation of the sulphur atom to produce metal chelate structures that have an overall negative charge. So, these oxorhenium complexes were prepared from ligand exchange reactions of bioconjugates with $ReOCl_3(PPh_3)_2$ as Re^VO starting material, in the presence of a deprotonating agent (Fig. 2). The bioconjugate and the Re^VO precursor were always used in a 1/1.1 molar ratio. Thus, the reaction of bioconjugates **2**–**5** with $ReOCl_3(PPh_3)_2$ in the presence of sodium acetate as deprotonating agent in methanol followed by adequate purifications resulted in oxorhenium complexes as the sodium salts in 62–75% yields. If pure complex **3**-**Re** has been obtained by precipitation in dichloromethane, the others complexes required HPLC purifications, even after precipitation in CH_2Cl_2. The cleavage of trityl group was accomplished during the coordination of the ligand to the ReO^{3+} core and this is in agreement with the acidic contribution of the metal in the mechanism of sulfur detritylation, as demonstrated previously [22]. Mass spectra data for rhenium bioconjugates **2**-**Re**–**5**-**Re** were obtained by electrospray mass spectrometry in the negative-ion detection mode. The anion M^- or the sodium adduct $[M + Na^+ - H^+]$ were detected; both of these ions exhibited the characteristic Re isotopic pattern, as illustrated in Fig. 3 for compound **5**-**Re** (see also supplementary data for compound **3**-**Re**). The LC-MS chromatogram of **2**-**Re** revealed a large peak with a shoulder. This result suggests the presence of at least two different structural isomers. The peptide moiety being directly coupled to the chelator, its interaction with the Re = O bond is possible. Therefore, these two products should be the *anti* and *syn* isomers with respect to the rhenium oxo and the peptide moiety. Unfortunately, it was not possible by HPLC to achieve isomeric resolution under a wide range of chromatographic conditions. Therefore, the mixture of the two isomeric forms of **2**-**Re** was not engaged for *in vitro* studies. Actually, the use of a mixture of isomers may have significant impact on the biological properties of a radiopharmaceutical [23]. the bioconjugate **4** and its oxorhenium complex **4**-**Re** were not biologically tested because of insufficient solubility in water.

The pharmacological profiles of bioconjugates **2**, **3**, **5** and oxorhenium complexes **3**-**Re**, **5**-**Re** were investigated by measuring their abilities to stimulate inositol phosphate (IP) formation in COS-7 cells transiently transfected with human CCK2-receptor. Firstly, all the compounds tested behaved as agonists and were able to induce IP production (Fig. 4). The agonist character of these compounds on the CCK2-R might be interesting for a therapeutic use with β^--emitter isotopes of rhenium (rhenium-186/188) [24]. Indeed, it is well-known that the success of the therapeutic strategy not only relies on the amount of radiolabelled bioconjugate that can be concentrated within the tumor cells but also on the rate of internalization of both radiolabelled bioconjugate and receptor [25].

Secondly, as listed in Table 1, some of the tested compounds show from slight to huge differences for potency values (EC_{50} data). A nanomolar potency of the bioconjugate suggests a high binding affinity of the bioconjugate to the CCK2-receptor, as previously reported [17,26]. In agreement with the EC_{50} values of bioconjugates **2**, **3** and **5** (Table 1), we observed a loss of binding affinity by two orders of magnitude for compound **2** ($\approx 10^{-7}$ M *vs.* nM range for the reference (gastrin)), *i.e.* compound without spacer.



Compound	n	X	Complex	n	X	M
2	0		**2-Re**	0		Re
3	2	β–Ala	**3-Re**	2	β–Ala	Re
4	4	β–Ala	**4-Re**	4	β–Ala	Re
5	2	Ahx	**5-Re**	2	Ahx	Re
			3-Tc	2	β–Ala	Tc
			5-Tc	2	Ahx	Tc

Fig. 2. Synthesis of bioconjugates and rhenium complexes. *Reagents and conditions* (i) Et_3N (10 eq.), dry DMF, 37 °C., one night, (ii) $ReOCl_3(PPh_3)_2$ (1.1 eq.), $AcONa.3H_2O$ (4 eq.), MeOH, 65 °C, one night or $^{99m}TcO_4^-$, excess of $SnCl_2$, MeOH, buffer pH 8.6, 75 °C, 30 min.

This result is consistent with previous studies using a CCK4 tagged by a radioiodinated Bolton-Hunter moiety [10a]. In contrast, the receptor affinity of the two bioconjugates containing a spacer was similar and in the nanomolar range. As expected, the incorporation of a flexible spacer between the chelator and the CCK4 sequence show a significant improvement of the CCK2-R affinity. Furthermore, the CCK2-R affinity seems more influenced by the length of the spacer than its own nature (hydrophilic/lipophilic character). Both bioconjugates **3** and **5**, which bear a hydrophilic β-ala and a lipophilic Ahx spacer respectively, exhibited analogous EC_{50} values ($3.10\ 10^{-9}$ M for **3** and $5.01\ 10^{-9}$ M for **5**) and similar HPLC retention times (22.6 min. for **3** and 21.8 min. for **5**). This first result might suggest that the lipophilic character of the four bioconjugates is mostly imposed by the presence of the bulky trityl group (Trt) in such molecules.

Another outcome of this present study was the different behaviour of our two oxorhenium complexes. Compound **3**-**Re** showed an almost complete loss of CCK2-R affinity while compound **5**-**Re** was able to recognize the CCK2-receptors with high binding affinity (nanomolar range). The own nature of the spacer could explain this difference. It has been reported previously, in the case of bombesin derivatives that changes at the C-terminus part of the peptide have little influence on the binding affinity if the lipophilic character is retained [27]. In our case, the lipophilic Ahx spacer could balance the hydrophilic character of our anionic complex Ph-ON_2S(ReO). The retention time of the bioconjugate **5** and its rhenium complex **5**-**Re** are similar like their EC_{50} values ($t_r \approx 19$ min. and $EC_{50} \approx 5 \times 10^{-9}$ M). In return, the β-ala spacer enhanced the hydrophilicity of the oxorhenium complex **3**-**Re** illustrated by the huge difference of the retention time between the bioconjugate **3** and its rhenium complex **3**-**Re** (22.6 min. *vs.* 9.8 min.). This enhancement of the hydrophilic character may be responsible of the loss of the CCK2-R affinity. These findings conjugated with previous studies [27,28] show that the length and the composition of the spacer could have a tremendous impact on the binding affinity of the tested bioconjugates. Therefore, the most potent CCK2-receptor binding peptide-based on CCK4 moiety is that bearing a double Ahx spacer, *i.e.* the compound **5**. To the best of our knowledge, complex **5**-**Re** exhibited the highest CCK2-R binding affinity value never obtained for a CCK4-based bioconjugate, compared to radioiodinated CCK4 and pentagastrine [10b].

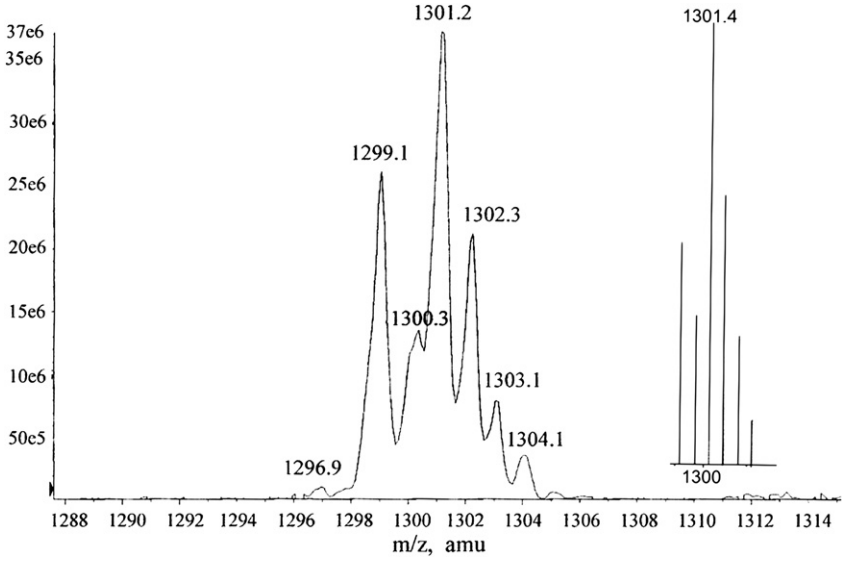


Fig. 3. Theoretical and experimental isotopic profiles for complex **5**-**Re**.

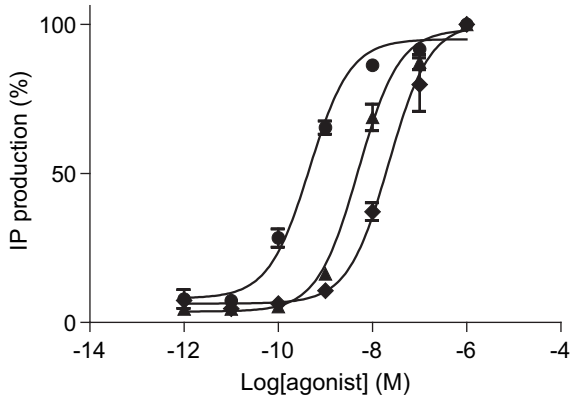


Fig. 4. Stimulation of inositol phosphate (IP) production in COS-7 cells transfected with the CCK2-R. Cells were treated with either gastrin as reference (●), compound **3-Re** (◆) and compound **5-Re** (▲). Results are the mean ± SEM of double separate measurements of IP production, each in duplicate. EC_{50} values are given in Table 1.

^{99m}Tc-labelling of the bioconjugates **3** and **5** was performed *in situ* in a methanol/buffer solution pH 8.6 (1/4: v/v) by direct reduction of sodium pertechnetate in the presence of an excess of tin chloride at 75 °C during 30 min (Fig. 2). Both complexes **3-Tc** and **5-Tc** were obtained in high yield (more than 95%, see supplementary data) and with excellent radiochemical purity after HPLC purification. They were characterized by comparing their HPLC profiles with the profiles of the corresponding rhenium complexes, as illustrated in supplementary data. The ^{99m}TcO-complexes were stable in our buffer and inert towards transchelation by an excess of cysteine (less than 5% of ^{99m}Tc dissociated after 12 h).

The *in vivo* stability, as well as the binding affinity, is important parameters to judge a new compound. So, to predict this *in vivo* stability, we investigated stability in fresh human serum and mouse liver homogenate at 37 °C for ^{99m}TcO-complex **5-Tc**. As shown in Fig. 5, this complex displayed a satisfactory stability in human serum (84% of intact radiolabelled peptide at 4 h). As expected, incubation in liver resulted in rapid decrease of activity. At 1 h, about 50% of the activity was associated with the peptide. This rapid degradation should be a result of an enzymatic cleavage by endogenous peptidases and proteases [11]. The metabolic products are more hydrophilic than the original radiopeptide. We demonstrated than none of them corresponded to the radiocomplex, excluding a cleavage of the thiourea linkage between the radiochelator and the spacer. Thus, observed metabolites seem to form by cleavage of peptide bond(s) in the peptide backbone or between the peptide and the spacer [29]. However, these results are similar to those previously reported for ^{99m}Tc-radiolabelled minigastrin [8d]. Moreover, the high resistance towards an excess of cysteine coupled to convenient serum stability must be taken into account to assess the ability of our CCK4 derivatives as molecular imaging agents for CCK2-receptor targeting.

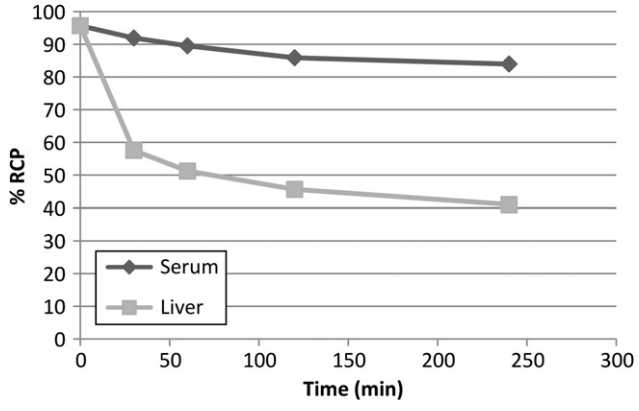


Fig. 5. Stability of radiolabelled bioconjugate **5-Tc** in (a) human serum, (b) mouse liver homogenate.

4. Conclusion

In short, the synthetic protocol described herein provides an easy access to a range of original shortened CCK derivatives, in good yields. Two of these bioconjugates provided well-defined ReO-complexes and formed stable ^{99m}TcO-complexes in high yield, under mild conditions. *In vitro* studies showed notably (i) the necessity to introduce a spacer between the chelator and the tetrapeptide, (ii) the influence of the nature of the spacer, the use of a lipophilic Ahx linker leading to an oxorhenium complex with nanomolar binding affinity, (iii) the agonist character of our compounds, the cell internalization being an interesting property for radiotherapy with rhenium-186/188. We demonstrated for the first time the feasibility to develop a potent radiolabelled CCK/gastrin-related peptides based on CCK4 moiety which exhibited a relevant binding affinity for CCK2-receptor and a satisfactory stability in serum. Therefore, bioconjugate **5** provides a new template to design new CCK4-based peptide conjugates. Further *in vivo* studies to investigate the suitability of these compounds as CCK2-R target-specific radiopharmaceuticals are under progress.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2009.09.043.

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