A convenient solid-phase synthesis methodology for preparing peptide-derived molecular imaging agents — Synthesis, characterization, and in vitro screening of Tc(I) – chemotactic peptide conjugates

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Abstract: A versatile solid-phase synthesis strategy for preparing peptide–chelate conjugates was developed. The methodology was optimized using a series of ligands, designed to bind Tc(I)/Re(I), and a chemotactic peptide fMFL, which was exploited as a model targeting vector. The peptide derivatives were prepared in parallel using a conventional automated peptide synthesizer in multi-milligram quantities, which provided sufficient material to perform complete characterization, radiolabelling, and in vitro screening studies. Because of the robust nature of the metal–chelate complexes, the Re complex of a chelate–peptide conjugate was prepared on the resin using the same methodology employed to prepare the free ligand conjugates. As such, the reported methodology is amenable to the preparation of libraries of novel Tc radiopharmaceutical ligands and their corresponding Re reference standards in which several factors, including peptide sequence, site of derivatization, and both the type and length of the spacer, can be easily varied.

Key words: radiopharmaceuticals, technetium, rhenium, peptides, solid-phase synthesis.

Résumé : On a développé une stratégie versatile de synthèse en phase solide de produits conjugués peptide–chélate. On a optimisé la méthodologie en utilisant une série de ligands qui ont été développés pour se lier au Tc(I)/Re(I) et au peptide chimiotactique fMFL qui a été exploité comme vecteur modèle de ciblage. On a préparé les dérivés peptidiques en parallèle en faisant appel à un synthétiseur automatique conventionnel de peptides à des niveaux de plusieurs milligrammes, ce qui fournissait des quantités suffisantes de matériel pour en faire une caractérisation complète, un marquage radiochimique et des études in vitro. En raison du fait que les complexes métal–chélate sont très robustes, un complexe du Re d'un produit conjugué chélate peptide a été préparé sur la résine en faisant appel à la même méthodologie qui avait été utilisée pour préparer les conjugués libres du ligand. De cette façon, la méthodologie qui a été mise au point peut être amener à la préparation de librairies de nouveaux ligands radiopharmaceutiques du Tc et des produits de référence du Re dans lesquels il est possible de faire varier plusieurs facteurs, y compris la séquence peptidique, le site de formation du dérivé ainsi que le type et la longueur du groupe d'écartement.

Mots clés : radiopharmaceutiques, technitium, rhénium, peptides, synthèse en phase solide.

[Traduit par la Rédaction]

Introduction

Small peptides have been shown to be highly effective targeting agents for delivering radionuclides to specific receptors, including those associated with tumours, infection, and thrombosis (1–8). Peptides are able to reach their target rapidly, thus avoiding the need to use radionuclides with excessively long half-lives, and they typically clear from nontarget tissues quickly, which improves image contrast and quality (1, 9). Peptides can be prepared in a cost-effective manner via automated solid-phase synthesis, and they can be designed to target practically any receptor (10–12).

The traditional strategy for linking radiometals to peptides involves appending a bifunctional chelate (BFC) to either the N or C termini of the peptide. This was typically performed in solution (1, 6), which can lead to numerous side products, particularly if lysine residues are present in the peptide. To avoid the poor regioselectivity of this approach, which often results in the need to employ extensive HPLC purification to isolate the desired product (13), several groups began to exploit the advantages of solid-phase synthesis (SPS) to prepare peptide–ligand conjugates directly on polymer supports

Received 3 August 2005. Published on the NRC Research Press Web site at http://canjchem.nrc.ca on 3 February 2006.

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 doi: 10.1139/V05-224

(14, 15). Using this approach, orthogonal protecting groups can be employed, and large excesses of reagents can be used to drive reactions to completion while byproducts are removed by simple filtration (10, 15–17). SPS of this type is amenable to automation and can therefore be used to prepare libraries of compounds (18–21).

Current SPS methods used to prepare peptide-targeted radiotracers derived from ^{99m}Tc (the most widely used radionuclide in diagnostic medicine (22–24)) typically involve conjugating a ligand to the N-terminus of the resin-bound peptide. For instance, Hoffman and co-workers (25) prepared a series of bombesin derivatives using N_3S triamide chelates linked to the N-terminal amino acid through different spacer groups. Egli et al. (26) attached a protected histidine derivative to the N-terminus of neurotensin as a way of tagging the peptide with the $[^{99m}Tc(CO)_3]^+$ core. More recently, two strategies for incorporating ligands within the backbone of the peptide have been reported. Blower and co-workers (15) prepared a derivative of Fmoc-lysine that contained a hydrazine substituent as a way of incorporating a ^{99m}Tc binding ligand within peptides. We recently reported the synthesis of amino acid derived chelates for Tc(I), which can also be incorporated regioselectively into the backbone of a peptide (17, 27). A unique feature of this system is that the Re complex can be prepared in parallel with the free ligand, owing to the stability of the low spin d^6 Re(I) complex.

This paper describes a new approach for preparing peptide–Tc ligand conjugates. The reported method is attractive in that a chelate can be attached at any position within the backbone of the peptide and not simply to the terminal amino or acid groups. Specifically, the approach involves using an on-resin orthogonal protecting group strategy, where a Tc(I) chelate is linked to a specific lysine residue. This methodology will ultimately be useful for creating libraries of tracers as a means of rapidly identifying new radiopharmaceuticals.

Experimental section

Unless otherwise stated, all reagents and solvents were ACS grade or higher and used without further purification. Polystyrene-based *N*-α-9-fluorenylmethoxycarbonyl (Fmoc)-glycine loaded SASRIN resin (0.69 mmol/g, 1% divinylbenzene, 200–400 mesh) was obtained from Bachem Inc. Fmoc-protected amino acids were purchased from NovaBiochem Inc., Bachem Inc., and Advanced ChemTech Inc. Fluorescein-labelled fNLFNTK was purchased from Molecular Probes Inc. (product No. F-1314).

Peptides were analyzed by electrospray mass spectrometry using a Micromass Quattro Ultima instrument in positive ion mode. Samples were dissolved in 50:50 ACN–H_2O prior to analysis. FT IR spectra were acquired on a Bio-Rad FTS-40 FT IR spectrometer. Analytical HPLC on nonradioactive compounds was performed using a Varian Pro Star model 330 PDA detector, model 230 solvent delivery system, and a C-18 Microsorb column (4.6 × 250 mm, 300 Å – 5 μm). Semipreparative HPLC on nonradioactive compounds was carried out using a Varian Pro Star HPLC fitted with a model 320 UV detector, a model 215 solvent delivery system, and a C-18 Microsorb column (10 × 250 mm, 300 Å – 5 μm). The elution protocol for both analytical and semipreparative experiments was a linear gradient of from 25% acetonitrile containing 0.1% TFA to 50% acetonitrile containing 0.1% TFA over 15 min. The flow rates were 1.0 and 4.2 mL/min for the analytical and semipreparative experiments, respectively. All HPLC experiments were monitored at λ = 254 and 214 nm. Radio–HPLC experiments were performed on a Varian Prostar HPLC equipped with an autosampler (model 410), a UV–vis detector (model 345), an NaI radiometric detector, Prostar Pumps (model 210), and a Vydac C18 25 cm × 4.6 mm column (5 μm pore size) fitted with a 2 cm guard column. The mobile phase consisted of the following: solvent A = pH 2–2.5 triethylammonium phosphate buffer (7 mL NEt_3, 4 mL concd. H_3PO_4 in 1000 mL water); solvent B = CH_3OH. The method employed a gradient run over 30 min at a flow rate of 1 mL/min. The gradient ramped from 5% to 100% B, beginning at 3 min and ending at 20 min.

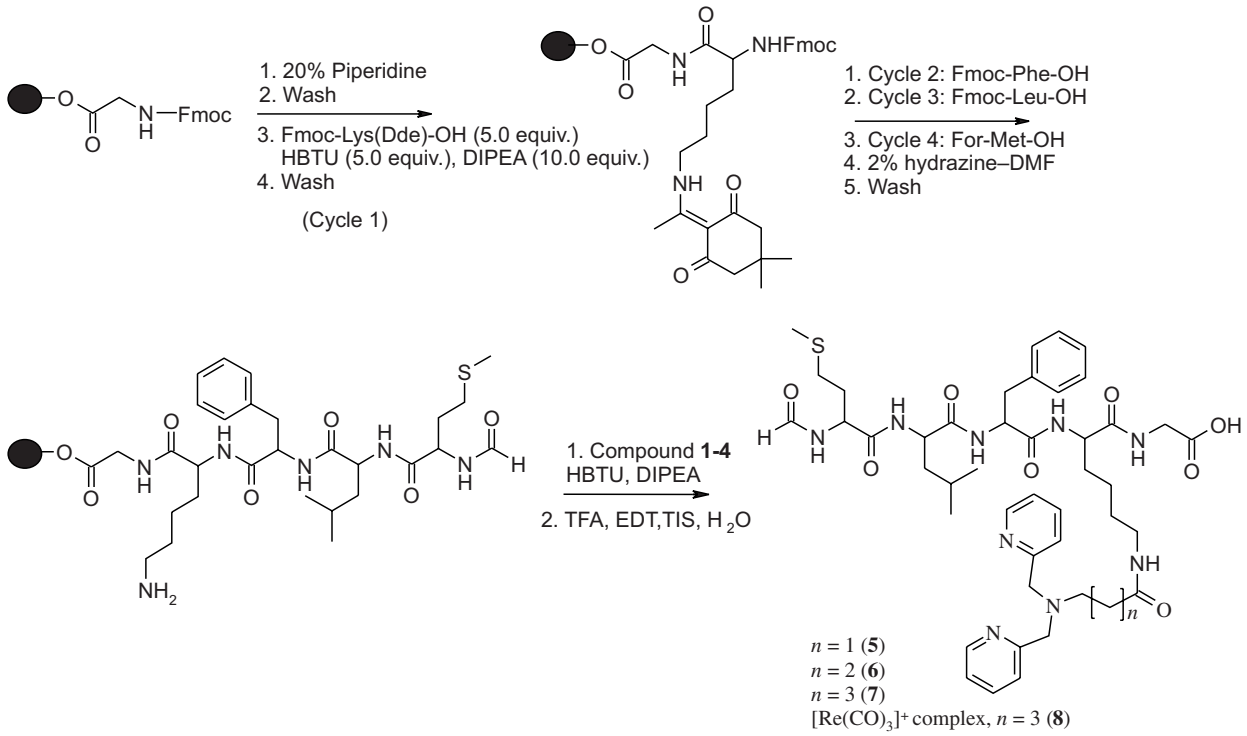
Solid-phase peptide synthesis

Fmoc-glycine loaded SASRIN resin (150 mg/well, 0.69 mmol/g) was added to four wells of an Advanced ChemTech 348 automated synthesizer, suspended in DMF (2 mL/well), and shaken at 600 rpm for 1 min. The wells were subsequently filtered, suspended in THF (2 mL/well), shaken at 600 rpm for 1 min, and drained for 90 s. The THF wash was repeated two more times. The DMF wash was then repeated a final two times to complete the general wash cycle. This procedure was used between every deprotection and coupling step. Fmoc deprotection was accomplished through the addition of a 20% *v*/*v* piperidine–DMF solution to the active vessels (2 mL/well) and shaking for 5 min at 600 rpm. Following filtration, the process was repeated, but with shaking for 10 min. The deprotected resin-bound amino acid was washed using the general wash procedure and subsequently coupled to the next Fmoc-protected amino acid using a standard HBTU coupling technique. A "cycle", according to Scheme 1, consists of Fmoc deprotection, a wash, and coupling the specified amino acid, followed by an additional wash.

Coupling reactions initially involved adding DMF (200 μL) to the active vessels followed by a fivefold excess of the protected amino acid as a 0.5 mol/L solution in DMF. Five equivalents of HBTU as a 0.5 mol/L solution in DMF were then added, followed by a 10-fold excess of DIPEA as 2.0 mol/L solution in DMF. The reaction block was subsequently shaken for 80 min at 600 rpm. Following filtration, the resin was washed using the general washing procedure prior to the start of the next cycle. Dde deprotection was accomplished through the addition of a 2% *v*/*v* hydrazine–DMF solution to the active vessels (2 mL/well) and shaking for 5 min at 600 rpm. Following filtration, the process was repeated, with shaking for 10 min. The free ε amine was then subjected to the general wash cycle and coupled to the desired chelate using the HBTU protocol described above, which was followed with a final wash.

Peptides were cleaved from the resin using a cocktail of TFA containing (*v*/*v*/*v*) EDT (2%), water (2%), and TIS (2%). The cleavage cocktail, cooled to 0 °C, was added to the resin, and the mixture was allowed to warm to room temperature over 60 min. The suspension was filtered into

Scheme 1. Synthesis of compounds **5**–**8**.



cold diethyl ether, and the resulting heterogeneous solution was centrifuged at 3000 rpm and –5 °C for 10 min. The resulting pellet was washed with cold ether (3 × 25 mL), dissolved in distilled–deionized water, and lyophilized, yielding a white solid.

Preparation of fMLFK(4m-Tc(CO)$_3^+$)G (9)

Technetium-99m was obtained in saline from a commercial ^{99}Mo/^{99m}Tc generator (Cardinal Health), and $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared using the commercially available Isolink kits (Mallinckrodt). Compound **7** (0.1 mg, 0.11 μmol) and 370 MBq (10 mCi) $[^{99m}Tc(CO)_3(H_2O)_3]^+$ were combined and heated at 75 °C for 60 min. After cooling to room temperature, the product was purified by HPLC (R_t = 12.1 min).

Ligand challenge experiments

Compound **9** in methanol (50 μL) and PBS buffer (1 mL, pH 7.2) was added to 0.1 mol/L solutions of cysteine in PBS buffer (pH 7.2) and histidine in PBS buffer (pH 7.2). The solutions were incubated at 37 °C for 24 h.

Leukocyte preparation

Whole human blood (10–15 mL) was collected in vacutainer tubes (Becton Dickinson) containing sodium heparin. The red blood cells were lysed using a modified ammonium chloride method (1 part whole blood to 23 parts 0.145 mol/L ammonium chloride solution containing 1.5 mmol/L potassium bicarbonate and 0.1 mmol/L EDTA). Tubes were incubated at room temperature for 15 min and then centrifuged in a Beckman Coulter Allegra 6R centrifuge at 900 rpm (400 g) for 7 min at 5 °C. The supernatant was removed, and the pellet of leukocytes were subsequently resuspended in Hank's Balanced Salt Solution (HBSS), containing 0.1% BSA, 10 mmol/L HEPES, and 1.5 mmol/L $CaCl_2$ (HBSS+). The cells were spun for 7 min at 400 g at 5 °C, the supernatant was removed, and the pellet was resuspended in HBSS+. Cell counts were performed using a hemacytometer and a Beckman Coulter Z2 Coulter particle count and size analyzer. Cell concentrations were adjusted to 2×10^6 mL^{-1}.

Flow cytometry

Samples were run on a Beckman Coulter EPICS XL, equipped with an argon laser (488 nm excitation wavelength). Fluorescence was measured using a 525 ± 15 nm band pass filter. The flow cytometer was calibrated with fluorescein isothiocyanate (FITC)-labeled beads (Quantum 26 beads, Bangs Laboratories, Inc, Fishers, Indiana) prior to sample analysis. The fluorescence intensity of the standard was provided by the manufacturer in units of molecules of equivalent soluble fluorochrome (MESF) and ranged from 5300 to 468 800. A linear fit of MESF vs. mean channel number of the beads was used to convert all subsequent mean channel values to MESF values.

Equilibrium-binding assay

The equilibrium-binding assay was used to determine the number of formyl peptide receptors (FPRs) on the surface of the neutrophils and the dissociation constant (K_d) of the fluorescein-labeled fNLFNTK (Molecular Probes) by preparing a saturated binding curve of peptide concentrations vs. fluorescence intensity (28, 29). Peptide stock solutions were made by dissolving the peptide derivative in DMSO and then diluting with HBSS+ to the desired concentrations, ensuring the concentration of DMSO was less than 0.1%. Cells at 1×10^6 mL^{-1} were equilibrated with 0.25, 0.50, 1.0, 3.0, 5.0, 10.0, and 20.0 nmol/L solutions of the peptide de-

rivative (L) in duplicate. The samples were incubated for 2 h at 4 °C in the dark and then run on the flow cytometer. Granulocytes were gated on the basis of forward and side scatter parameters using Expo 32 ADC software (Beckman Coulter). All samples were repeated in the presence of 30 μmol/L fMLF to account for nonspecific binding. The number of receptor–ligand complexes per cell (*B*) was determined using the following equation, where Q is a normalization factor used by Waller and *F* is the total fluorescence per cell:

$$B\,(\text{in ligand bound per cell}) = F\left(\frac{\text{MESF}}{\text{cell}}\right)\times \text{Q}$$

The level of nonspecific binding did not vary significantly with concentration of labeled peptide; thus, an average value of nonspecific binding was subtracted from the total fluorescence per cell, *F*, when determining the number of receptor–ligand complexes. The total number of *N*-formyl peptide receptors, R_{tot}, and the equilibrium dissociation constant, K_d, were evaluated by minimizing the squared residual of the equilibrium solution using a one-site binding model. The values of R_{tot} and K_d for each donor were solved simultaneously.

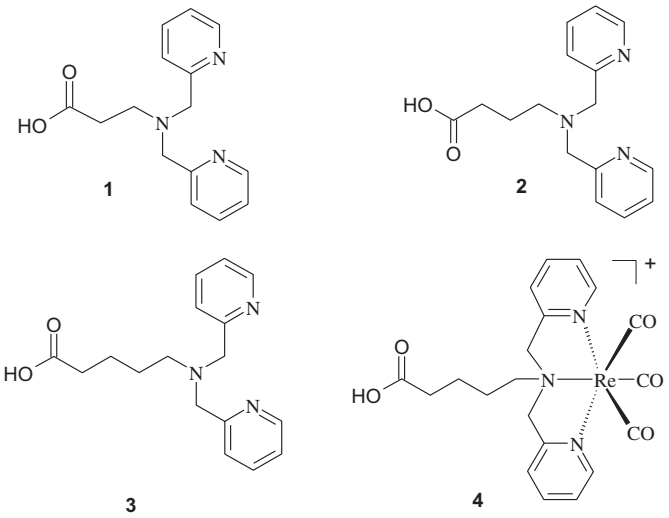
Results and discussion

The aim of this study was to develop a versatile bioconjugation strategy in which Tc ligands could be linked to peptides using a conventional automated peptide synthesizer. The method entailed incorporating lysine into the backbone of a peptide in such a manner that the ε-nitrogen could be selectively liberated and a Tc chelate added while the peptide was still linked to the resin. Using this approach, a number of important structural features, including the position and nature of the chelate, can be easily varied.

The blocking group selected for the ε-nitrogen of lysine was the 1-(4,4-dimethyl-2,6-dioxocyclohexyldiene)ethyl (Dde) protecting group, which is stable to the conditions used in typical Fmoc solid-phase synthesis, and which can be selectively liberated without affecting Boc protecting groups (30–32). The planned approach is amenable to any bifunctional chelating system containing a pendent acid group; however, for the work described here a series of dipyridyl amine ligands with linker arms of varying length were employed (Fig. 1) (17). These ligands form stable complexes with Tc(I), and their Re(I) complexes are sufficiently robust that the metal complex can be incorporated into peptides using the same methodology employed for the free ligand (33–37).

To develop the approach, the chemotactic peptide fMLFKG, which binds to the formyl peptide receptor (FPR) expressed on granulocytes, was used as a model system. Labelled versions of fMLF have shown potential utility for imaging sites of infection and inflammation (38–47). fMLF bioconjugates have traditionally been prepared in solution by conjugating hydrazine-type ligands (HYNIC) to the ε amine of fMLFK. This approach is not easily amenable to preparing libraries of fMLF derivatives, which would accelerate the process of identifying the appropriate derivative for clinical applications.

Fig. 1. Tc(I)/Re(I) ligands and the Re(I) complex of **3**.



The peptide fMLFK(Dde)G was prepared on an Advanced ChemTech 348 synthesizer using standard Fmoc–HBTU protecting groups – coupling protocols (Scheme 1). The Dde protecting group was removed by employing 2% hydrazine in DMF, and the reaction time was optimized using ninhydrin to ensure complete deprotection (48). Following removal of the Dde protecting group, the bifunctional chelates (**1**–**3**) were coupled to the resin-bound peptides using HBTU and DIPEA, and reaction times were again monitored to ensure complete coupling (48). The time required (80 min) for conversion to the desired product did not differ from standard amino acid coupling procedures.

Peptide conjugates **5**–**7** were cleaved from the resin using a cleavage cocktail containing 94% TFA, 2% triisopropylsilane, 2% ethanedithiol, and 2% water. Nitrogen was bubbled through the cocktail at 0 °C prior to and during use, to help prevent oxidation of methionine. Peptides were precipitated by trituration with cold diethyl ether and centrifuged, and the resulting pellet was washed with cold ether to remove any trace of the cleavage cocktail. Precipitates were then dissolved in distilled water and lyophilized to yield white solids.

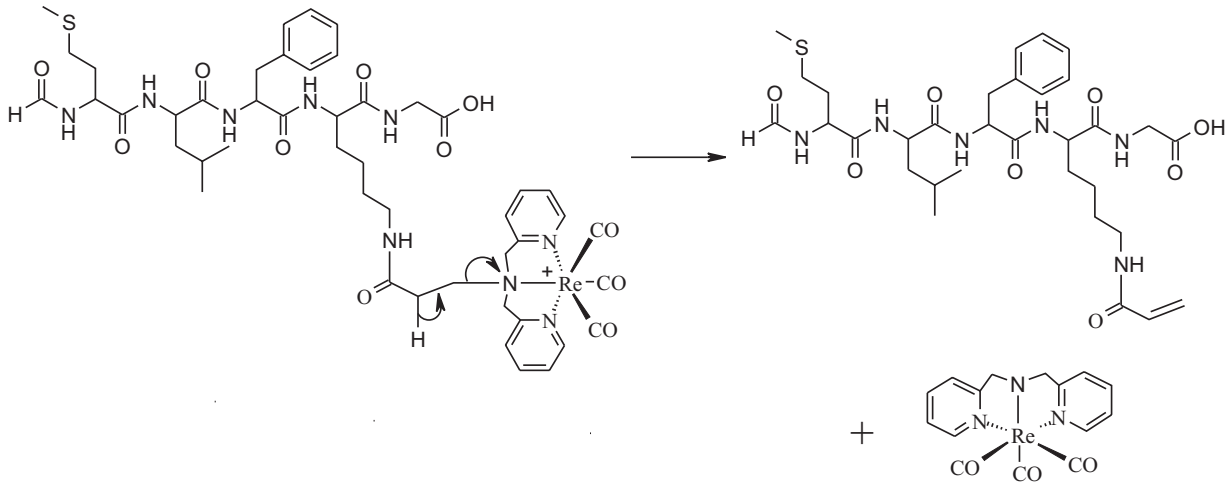
Peptides **5**–**7** were analyzed using electrospray mass spectrometry (ESMS), and in each case the observed molecular ion was consistent with the mass of the desired compound (Table 1). Analytical HPLC and LC–MS studies of each peptide indicated the presence of two peaks in a 3:1 ratio, corresponding to the same *m*/*z* values. Previous studies in our group and by others showed that the two peaks in the HPLC chromatogram are the result of epimerization of methionine, which is promoted by the electron-withdrawing nature of the formyl group (17, 49). Notwithstanding, the two isomers were readily separated using semipreparative HPLC.

One of the advantages of using M(I) (M = Tc, Re) ligands for methods involving solid-phase synthesis is the ability to prepare the Re complex in parallel with compounds containing the free ligand. Preparation of Re complexes is vitally important as they are needed for proper characterization of the ^{99m}Tc-analogues and for performing in vitro screening studies. For the method described here, the Re complex of **7**

Table 1. HPLC and ESMS data for compounds **5**–**8**.

Compound number	Elution time (min)	Found *m/z*	Calcd. *m/z*	Yield (%)	νCO (cm^{-1})
5	6.6	876.4	876.1	34	—
6	6.5	890.5	890.1	35	—
7	6.9	904.5	904.1	33	—
8	8.7	1174.4	1174.3	30	1933, 2041

Fig. 2. Proposed mechanism for the degradation of an Re(I)–peptide conjugate.



(**8**) was prepared successfully in parallel with the synthesis of compounds **5**–**7**, without modifying the basic strategy. After cleavage of the conjugate from the resin using the same cocktail described above, the product was isolated in comparable yields to the free ligands. Mass spectrometry clearly indicated the presence of the desired product (**8**), while IR showed two distinct metal CO stretches (1933 and 2041 cm^{-1}), which are consistent with values reported for analogous Re complexes (17, 33–37).

As an aside, attempts to prepare the peptide containing the Re(I) complex of **1** lead to a complex mixture of products according to HPLC. MS analysis of the precipitate formed after the addition of ether to the reaction mixture indicated the presence of the target compound, along with several degradation products. The likely cause of these impurities is elimination to form an αβ-unsaturated amide, which concomitantly results in liberation of a neutral metal complex (Fig. 2). By-products of this nature were not observed for the other ligands.

To ensure the products from the solid-phase synthesis can be radiolabeled, compound **7** (100 μg) was combined with 370 MBq (10 mCi) of $[^{99m}Tc(CO)_3(OH_2)]^+$, which was prepared using the commercially available Isolink kit (Scheme 2). After 60 min at 75 °C, the desired product **9** was isolated by HPLC in greater than 98% radiochemical purity in 63% radiochemical yield. Analytical HPLC of the purified product is shown in Fig. 3. The stability of the purified peptide **9** towards metal substitution was tested using a large excess of cysteine and histidine. The peptide and the two amino acids were heated at 37 °C for 24 h, whereupon HPLC analysis showed that the product was intact in both cases and that the radiochemical purities remained above 95% (Fig. 4).

Scheme 2. Synthesis of fMLFK(4m-$Tc(CO)_3^+$)G (**9**).

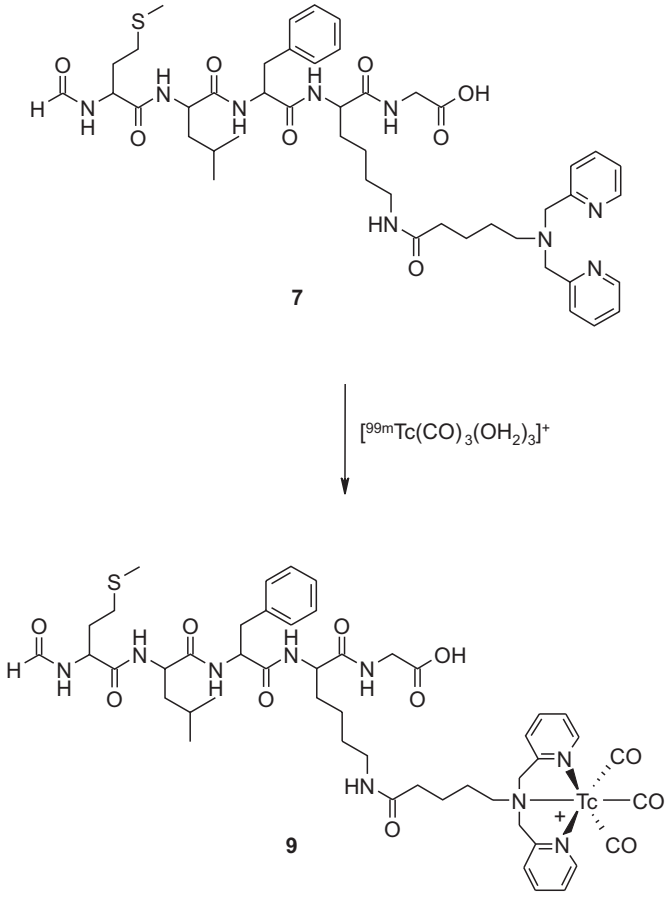


Fig. 3. γ-HPLC radio chromatogram (mV vs. time (min)) for purified **9**.

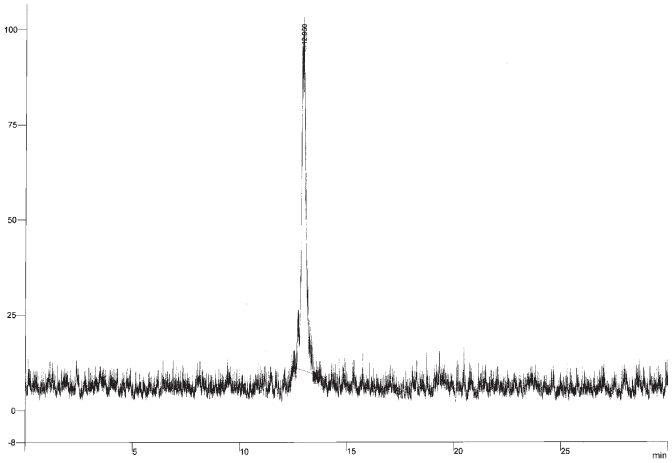
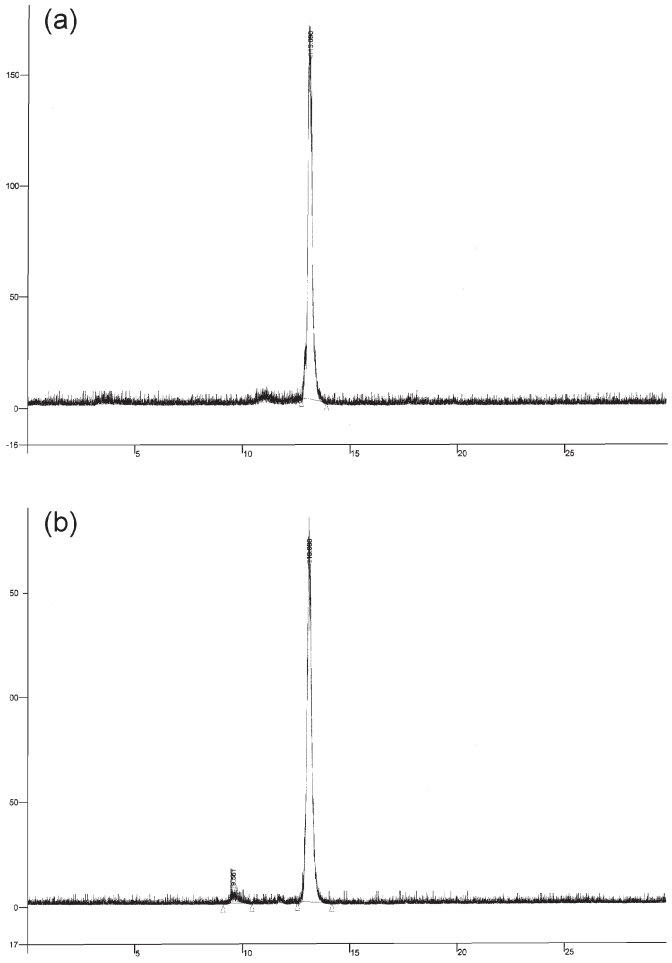


Fig. 4. γ-HPLC radio chromatograms (mV vs. time (min)) of the (*a*) cysteine and (*b*) histidine challenges of **9** after 24 h at 37 °C.



With the above methodology in hand it is possible to generate diverse libraries of compounds. The next step is to select the appropriate in vitro screening method that will indicate what compounds are the most promising for more advanced biological studies. In the example presented here, the ability of peptides **5–8** to bind to the FPR receptor was determined using flow cytometry. Flow cytometry is an attractive tool for screening novel molecular imaging agents because it is amenable to automation, which means that libraries can be evaluated rapidly, and it affords the opportunity to investigate specific cell populations. To evaluate the fMLF derivatives, human white blood cells were incubated with 1.0 nmol/L fluorescein-labelled fNLFNTK and varying amounts of the competitor in duplicate for 2 h at 0 °C in the dark. fMLF, which was used as a reference standard, exhibited a K_d that was in good agreement with the value reported in the literature (28, 50). Conjugates **5–8** had dissociation constants (Table 2) in the low nanomolar range, which is similar to the value for fMLF, thus demonstrating that incorporation of the ligand or Re complex does not have a negative impact on the ability of the peptides to bind the FPR.

Table 2. Dissociation constants (K_d) for compounds **5–8**.

Compound Number	Peptide sequence	K_d (nmol/L)
	fMLF	32±3
5	fMLFK(2M)G	21±6
6	fMLFK(3M)G	52±3
7	fMLFK(4M)G	55±8
8	fMLFK(4M-Re(CO)$_3^+$)G	67±6

Conclusions

The reported strategy offers a new alternative to existing solid-phase synthetic methodologies for preparing peptide-based radiotracers. The ligands and their rhenium complexes employed in this study can be incorporated into the backbone of peptides using traditional automated solid-phase synthesis methods. For instruments that have multiple reaction wells, the free ligand and Re–peptide conjugates can be prepared in parallel. The peptide derivatives described here were prepared in parallel in multi-milligram quantities, which provided sufficient material to allow for complete characterization, radiolabelling, and in vitro screening studies to be performed from the products of a single run of the synthesizer. The reported methodology can be used to prepare libraries of compounds in which several factors, including peptide sequence, site of derivatization, nature of the chelate, and both the type and length of the spacer can be varied, which will decrease the time and effort required to identify lead compounds.

Acknowledgements

We would like to acknowledge the Ontario Research and Development Challenge Fund (ORDCF) and the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding and for providing a scholarship for KAS.

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