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Synthesis, characterization, and biological evaluation of new biotinylated ^{99m}Tc/Re-tricarbonyl complexes

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The synthesis and biological evaluation of three new biotinylated *fac*-[^{99m}Tc/Re(CO)$_3$]$^+$ complexes with the tridentate ligands L1, L2, and L3 are reported. L1–L3 contain the chelators 2-((5-aminopentyl)(pyridin-2-ylmethyl)amino)acetic acid, 2-(2-aminoethylthio)-3-(1*H*-imidazol-4-yl)propanoic acid, and 2-amino-3-(1-carboxy-2-(1*H*-imidazol-4-yl)ethylthio)propanoic acid, respectively, which are conjugated to biotin's carboxylate via their amine group. The *fac*-[Re(CO)$_3$(L1–L3)] complexes were synthesized and characterized by NMR and IR, where the (N,N,O) coordination for ReL1 and the (N,S,O) coordination for ReL2 and ReL3 were confirmed. The tracer complexes *fac*-[^{99m}Tc(CO)$_3$(L1–L3)] were synthesized in high yield and were found highly stable in 10^{-3} M L-histidine and L-cysteine over 24 h. Furthermore, they exhibited high binding affinity (>90%) for avidin. Rat plasma studies showed complete cleavage of biotin from ^{99m}TcL1 after 1 h and a low percentage of intact ^{99m}TcL2 and ^{99m}TcL3 with no biotin cleavage metabolites present, over 24 h. Similarly, rat urine analysis showed the presence of intact ^{99m}TcL2 and ^{99m}TcL3, while ^{99m}TcL1 was cleaved. Biodistribution studies of ^{99m}TcL2 and ^{99m}TcL3 revealed fast blood and tissue clearance.

Keywords: technetium; rhenium; tricarbonyl; biotin; avidin

Introduction

D-Biotin or vitamin H is a cofactor that participates in fatty acid biosynthesis as well as in other metabolic processes. The high-affinity binding of biotin to (strept)avidin (~10^{15} M^{-1}) makes this system very attractive for various biomedical applications. Radiolabeled biotin derivatives can be used as targeted diagnostic and therapeutic radiopharmaceuticals in nuclear medicine. They have been evaluated in the clinical practice for the scintigraphic diagnosis of skeletal infections with favorable results.[1,2] In addition, radiolabeled biotin derivatives have been evaluated in cancer pretargeted radioimmunoscintigraphy (RIS) and radioimmunotherapy (RIT), an approach with considerable advantages over the conventional RIS/RIT. The pretargeted RIS/RIT usually involves the systemic administration of a monoclonal antibody conjugated to (strept)avidin first, and after its localization at the tumor site, it is followed by the administration of radiolabeled biotin. The high affinity of biotin for avidin allows the accumulation of the radiolabeled biotin to the sites of the localized tumor-bound avidinylated antibodies with high specificity, while reduced radiation dose to healthy tissue is achieved owing to the faster pharmacokinetics of the radiolabeled biotin compared with the longer circulation time of radiolabeled antibodies in the conventional RIS and RIT.[3–5]

An early effort to synthesize In-111 radiolabeled biotin derivatives was reported, where conjugation of biocytin (D-biotin–lysine) to diethylene triamine pentaacetic acid or ethylenediaminetetraacetic acid chelators, resulted in *in vivo* binding to avidin.[6] However, biocytin has limited stability in serum, owing to biotinidase cleavage. Several later approaches to produce stable biotin derivatives were accomplished, where biotin was conjugated via different chelators and linkers, like ^{99m}Tc-labeled derivatives of oxotechnetium mercaptoacetyltriglycine,[7] [^{99m}TcN(PNP)(OS)],[8] and organometallic *fac*-[^{99m}Tc(CO)$_3$(dipicolylamine)]$^+$ among others.[9,10] The radiolabeling of reduced 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid-biotin or dipicolylamine-biotin conjugates with ^{90}Y[11] or ^{99m}Tc-tricarbonyl[9], respectively, resulted in improved stability. Other efforts to produce stable derivatives of biotin considered the shielding effect of a variety of moieties linked to biotin in a way that high affinity for avidin is maintained.[12–14]

Technetium-99m and rhenium-186/188 are both radionuclides with optimal properties for the development of radiopharmaceuticals, ^{99m}Tc for imaging and $^{186/188}$Re for radiotherapy, and owing to their similar chemical properties, they are regarded as a matched pair of radionuclides. In the low oxidation state, organometallic *fac*-[^{99m}Tc(CO)$_3$]$^+$ core is highly stable *in vivo* and flexible to accommodate various tridentate ligands on its coordination sphere.[15] In our previous works, we developed tridentate (N,S,O) chelators, with imidazole N, thioether S, and carboxylate O donor atoms for complexation with the *fac*-[^{99m}Tc/^{188}Re(CO)$_3$] core,[16–18] among which derivatives of 2-alkylthio-3-(1*H*-imidazol-4-yl)-propanoic acid, where the alkylthio- side chain

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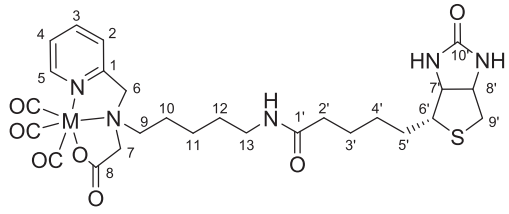
contains additional donor atoms that can be used for the conjugation of the biomolecule of interest.[18]

In this work, we sought to develop new biotinylated ^{99m}Tc-tricarbonyl complexes by using different tridentate chelators for complexation and linkers for biotin conjugation. Firstly, a known (N,N,O) chelator, 2-picolylmethylamine-*N*-acetic acid, was conjugated with biotin via a pentylamine chain, and furthermore, the (N,S,O) chelator we previously developed[17] was conjugated with biotin via cysteamine and cysteine linkers. Cysteine bears an additional carboxylate group that could confer different pharmacokinetic properties and stability to the biotinylated tracer complex. The rhenium and technetium complexes synthesized are depicted in Figure 1, and the objective of the study was the preliminary evaluation of their biological stability and avidin affinity.

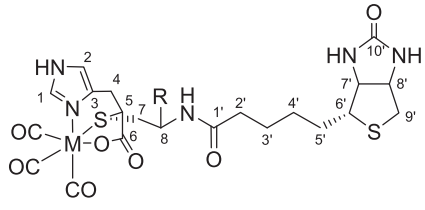
Experimental

General

All chemicals were of reagent grade and were used as such unless otherwise noted. Solvents for high-performance liquid chromatography (HPLC) were of HPLC grade, and solvents for mass spectroscopy (MS) were of MS grade. They were filtered through membrane filters (0.22 μm, Millipore, Milford, MA, USA) and degassed. The compounds $(NEt_4)_2[Re(CO)_3Br_3]$,[19] $[Re(CO)_3(H_2O)_3]Br$,[20] 2-chloro-3-(1*H*-imidazol-4-yl) propanoic acid,[21] and *tert*-butyl 5-(pyridin-2-ylmethylamino) pentylcarbamate[22] were prepared according to the procedures reported in the literature. The Sep-Pak C18 cartridges (Waters, Milford, MA, USA, 35 cc, 10 g) were preconditioned prior to use according to the manufacturer's protocol. Avidin was purchased from Aldrich and was used as a 1 mg/mL solution in 0.1 M phosphate-buffered saline (PBS) pH 7.4. For the ^{99m}Tc labeling, a kit containing 5.5 mg of $NaBH_4$, 4 mg of Na_2CO_3, and 10 mg of Na-K tartrate was purged with CO gas prior to addition of $Na^{99m}TcO_4$, as described in the literature.[23] Elemental analyses were performed on a PerkinElmer 2400 automated analyzer (Perkin Elmer, Waltham, MA, USA). ESI-MS data were obtained on a LCMS-2010 EV (Shimadzu, Duisburg, Germany). IR spectra were recorded as KBr pellets on a Spectrum BX spectrophotometer (Perkin Elmer, Boston, MA, USA) in the region 4000–500 cm^{-1}. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 300 MHz (Bruker, Milan, Italy) or an Agilent DD2 500 MHz spectrometer (Agilent, Santa Clara, CA, USA). HPLC analyses were performed on an Agilent HP 1100 series pump (Waldbronn, Germany), connected both to a Gabi (Raytest, Straubenhardt, Germany) gamma detector and an HP 1100 multiple wavelength detector. Reversed-phase HPLC analyses of the rhenium and technetium-99m complexes were achieved on an Agilent Eclipse XDB C18 column (25 cm, 4.6 mm, 5 μm, Agilent Technologies, Waldbronn, Germany) by applying a binary gradient system of 0% to 75% solvent B in solvent A (A = 0.1% TFA in water; B = methanol) at 0–15 min and 75% to 95% solvent B in solvent A at 15–20 min; 1 mL/min flow rate; UV detection at 254 nm. Size-exclusion HPLC analyses of the avidin-bound ^{99m}Tc-biotin complexes were performed on a BIOSEP-SEC-S3000 (Phenomenex, Torrance, CA, USA) column (300 × 7.8 mm, 5 μm), conditioned in 50 mM phosphate buffer pH 6.8 – 0.3 M NaCl and at flow rate 0.5 mL/min. Radio thin-layer chromatography (TLC) measurements were conducted on NaI scintillation counter (Caprac, Capintec, Ramsey, NJ, USA).



ReL1, ^{99m}TcL1



R= H, **ReL2, ^{99m}TcL2**

R= ^{9}COOH, **ReL3, ^{99m}TcL3**

Figure 1. Structure of the Re/^{99m}Tc complexes.

Ligand syntheses

*Synthesis of **L1***

*Ethyl 2-((5-(tert-butoxycarbonylamino)pentyl)(pyridin-2-ylmethyl) amino) acetate (**1**).* *tert*-Butyl 5-(pyridin-2-ylmethylamino)pentylcarbamate (370 mg, 1.26 mmol), ethyl bromoacetate (432 μL, 3.9 mmol), and triethylamine (544 μL, 3.9 mmol) were added in anhydrous tetrahydrofuran (20 mL), and the mixture was heated under reflux for 4 h. After cooling to room temperature, the reaction mixture was filtered, and the filtrate was evaporated to dryness. The crude product was purified by silica gel column chromatography (SiO_2, 230–400 mesh, 25 g) using dichloromethane–methanol, 97:3 v/v as eluent, and a brownish oil was afforded. Yield: 230 mg (48%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, *J* = 4.1 Hz, 1H, H-5), 7.67 (t, *J* = 7.6 Hz, 1H, H-3), 7.52 (d, *J* = 7.8 Hz, 1H, H-2), 7.16 (dd, *J* = 6.7, 5.5 Hz, 1H, H-4), 4.53 (bs, 1H, NH), 4.18 (q, *J* = 7.1 Hz, 2H, CH_2CH_3), 3.93 (s, 2H, H-6), 3.40 (s, 2H, H-7), 3.14–3.04 (m, 2H, H-13), 2.67 (t, 2H, H-9), 1.57–1.40 (s, 13H, H-10,12 and $C(CH_3)_3$), 1.40–1.23 (m, 5H, H-11 and CH_2CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4 (C-8), 159.6 (CONH), 156.0 (C-1), 149.0 (C-5), 136.5 (C-3), 123.0 (C-2), 122.0 (C-4), 79.0 ($C(CH_3)_3$), 60.3 (CH_2CH_3), 60.2 (C-6), 55.0 (C-7), 54.0 (C-9), 40.4 (C-13), 29.8 (C-12), 28.4 ($C(CH_3)_3$), 27.2 (C-10), 24.3 (C-11), 14.2 (CH_2CH_3). Anal. calc. for $C_{20}H_{33}N_3O_4{\cdot}0.25H_2O$: C, 62.56; H, 8.79; N, 10.94. Found: C, 62.59; H, 8.61; N, 10.74%.

*Ethyl 2-((5-aminopentyl)(pyridin-2-ylmethyl)amino)acetate (**1'**).* Compound **1** (800 mg, 2.08 mmol) was dissolved in aqueous HCl 1 M (20 mL) and was stirred at room temperature for 2.5 h. The solution was evaporated to dryness under vacuum, and the product was used without further purification. Yield: 776 mg (96%). ^{1}H NMR (300 MHz, MeOD) δ 8.96 (d, *J* = 5.4 Hz, 1H, H-5), 8.60 (td, *J* = 7.8, 1.2 Hz, 1H, H-3), 8.32 (d, *J* = 7.9 Hz, 1H, H-2), 8.09 (t, *J* = 6.6 Hz, 1H, H-4), 5.00 (s, 2H, H-6), 4.41 (s, 2H, H-7), 4.29 (q, *J* = 7.1 Hz, 2H, CH_2CH_3), 3.53–3.44 (m, 2H, H-13), 2.99 (t, *J* = 7.3 Hz, 2H, H-9), 1.99–1.86 (m, 2H, H-12), 1.78 (dt, *J* = 15.0, 7.7 Hz, 2H, H-10), 1.52 (dt, *J* = 15.0, 7.6 Hz, 2H, H-11), 1.31 (t, *J* = 7.1 Hz, 3H, CH_2CH_3). ^{13}C NMR (75 MHz, MeOD) δ 167.6 (C-8), 147.1 (C-1), 146.8 (C-5), 145.7 (C-3), 130.4 (C-2), 128.8 (C-4), 64.0 (CH_2CH_3), 57.3 (C-6), 56.3 (C-9), 54.8 (C-7), 40.3 (C-13), 27.8 (C-12), 24.7 (C-10), 24.3 (C-11), 14.3 (CH_2CH_3).

Synthesis of biotin tetrafluorophenyl ester. D-Biotin (170 mg, 0.70 mmol) was dissolved in anhydrous dimethylformamide (5 mL) at 50 °C. After cooling to room temperature, 2,3,5,6-tetrafluorophenol (128 mg, 0.77 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (148 mg, 0.77 mmol) were added, and the solution was stirred overnight. Dichloromethane (30 mL) was added, and the solution was extracted with water (3 × 30 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness under vacuum, to obtain a white solid. Yield: 180 mg (65%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.10–6.92 (m, 1H, H-Ph), 5.50 (s, 1H, NH),

5.03 (s, 1H, NH), 4.57–4.51 (m, 1H, H-8′), 4.39–4.32 (m, 1H, H-7′), 3.25–3.17 (m, 1H, H-6′), 2.96 (dd, J = 12.8, 5.0 Hz, 1H, H-9′), 2.76 (d, J = 13.2 Hz, 1H, H-9′), 2.73 (t, J = 7.4 Hz, 2H, H-2′), 1.93–1.72 (m, 4H, H-3′,5′), 1.65–1.52 (m, 2H, H-4′). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.4 (C-1′), 163.8 (C-10′), 146.0 (d, J = 244 Hz, C-TFP), 140.6 (d, J = 239 Hz, C-TFP), 103.1 (t, C-TFP), 62.4 (C-7′), 60.6 (C-8′), 55.4 (C-6′), 40.4 (C-9′), 33.1 (C-2′), 28.2 (C-4′/C-5′), 24.6 (C-3′). Anal. calc. for $C_{16}H_{16}F_4N_2O_3S$: C, 48.98; H, 4.11; N, 7.14. Found: C, 48.45; H, 3.94; N, 7.08%.

*Ethyl 2-((5-biotinamidopentyl)(pyridin-2-ylmethyl)amino)acetate (**L1$_{Et}$**).* Biotin-TFP ester (158 mg, 0.4 mmol), ethyl 2-((5-aminopentyl)(pyridin-2-ylmethyl)amino)acetate (**1′**) (327 mg, 0.84 mmol) and triethylamine (418 μL, 3 mmol) were added in methanol–acetonitrile (1:1 v/v, 10 mL), and the solution was stirred at room temperature for 4 h. The solvents were evaporated to dryness under vacuum, and the product was purified by silica gel column chromatography (SiO_2, 230–400 mesh, 15 g) using dichloromethane–methanol 9:1 v/v as eluent to afford a greenish solid. Yield: 170 mg (83%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (d, J = 4.2 Hz, 1H, H-5), 7.59 (td, J = 7.7, 1.6 Hz, 1H, H-3), 7.42 (d, J = 7.8 Hz, 1H, H-2), 7.08 (dd, J = 6.4, 5.3 Hz, 1H, H-4), 6.77 (s, 1H, NH), 6.55 (s, 1H, NH), 6.13 (s, 1H, NH), 4.46–4.36 (m, 1H, H-8′), 4.25–4.16 (m, 1H, H-7′), 4.06 (q, 2H, J = 7.1 Hz CH_2CH_3), 3.83 (s, 2H, H-6), 3.30 (s, 2H, H-7), 3.15–2.97 (m, 3H, H-6′,13), 2.80 (dd, J = 12.7, 4.5 Hz, 1H, H-9′), 2.64 (d, J = 12.7 Hz, 1H, H-9′), 2.57 (t, J = 7.1 Hz, 2H, H-9), 2.10 (t, J = 7.3 Hz, 2H, H-2′), 1.70–1.51 (m, 4H, H-5′,3′), 1.47–1.26 (m, 8H, H-10,11,12,4′), 1.17 (t, J = 7.1 Hz, 3H, CH_2CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3 (C-1′), 171.4 (C-8), 164.3 (C-10′), 159.4 (C-1), 148.9 (C-5), 136.6 (C-3), 123.1 (C-4), 122.1 (C-2), 61.7 (C-7′), 60.3 (CH_2CH_3), 60.2 (C-8′), 60.1 (C-6), 55.8 (C-6′), 55.0 (C-9), 54.1 (C-7), 40.5 (C-13), 39.3 (C-9′), 36.0 (C-2′), 29.3 (C-12), 28.3 (C-4′), 28.1 (C-5′), 27.1 (C-10), 25.8 (C-3′), 24.4 (C-11), 14.2 (CH_2CH_3). Anal. calc. for $C_{25}H_{39}N_5O_4S$: C, 59.38; H, 7.77; N, 13.85. Found: C, 58.80; H, 7.76; N, 13.37%.

*Synthesis of **L2***

*2-(2-Aminoethylthio)-3-(1H-imidazol-4-yl)propanoic acid (**2**).* 2-Chloro-3-(1*H*-imidazol-4-yl)propanoic acid (500 mg, 2.86 mmol) and cysteamine hydrochloride (340 mg, 3 mmol) were dissolved in a degassed mixture of water–ethanol (1:1 v/v, 10 mL), followed by dropwise addition of aqueous NaOH (4.5 mL, 2 M), under N_2. The mixture was stirred for 3 days. The pH was then adjusted to 6 by addition of aqueous HCl 5 M, and the solvents were evaporated to dryness. The residue was extracted in methanol (5 mL), and the filtrate was dried. The residue was recrystallized twice from water–ethanol, and a white crystalline solid was afforded. Yield: 285 mg (39%). MP: 182–185 °C. ^{1}H NMR (300 MHz, d_6-dmso) δ 7.78 (s, 1H, H-1), 6.92 (s, 1H, H-2), 3.63 (t, 1H, H-5), 2.91–3.11 (m, 3H, H-8,4β), 2.76–2.90 (m, 3H, H-7,4α). ^{13}C NMR (75 MHz, d_6-dmso) δ 173.4 (C-6), 134.4 (C-1), 133.4 (C-3), 116.7 (C-2), 48.9 (C-5), 39.8 (C-8), 29.0 (C-4), 28.1 (C-7). Anal. calc. for $C_8H_{13}N_3O_2S{\cdot}HCl{\cdot}0.5\ H_2O$: C, 36.85; H, 5.80; N, 16.12. Found: C, 37.21; H, 5.59; N, 16.14%.

*2-(2-Biotinamidoethylthio)-3-(1H-imidazol-4-yl)propanoic acid (**L2**).* Biotin-TFP ester (100 mg, 0.25 mmol), compound **2** (100 mg, 0.39 mmol), and triethylamine (143 μL, 1 mmol) were added in methanol–acetonitrile (1:1 v/v, 10 mL), and the solution was stirred at room temperature for 24 h. The solvents were evaporated to dryness, and the residue was dissolved in water (2 mL) and loaded on a conditioned Sep-Pak® C18 cartridge. The product was eluted with water–methanol (4:1 v/v), and after removal of the solvents, a white powder was afforded. Yield: 95 mg (82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.61 (s, 1H, H-1), 7.36 (s, 1H, H-2), 4.69 (dd, J = 7.6, 4.9 Hz, 1H, H-8′), 4.51 (dd, J = 7.8, 4.5 Hz, 1H, H-7′), 3.68 (t, J = 7.4 Hz, 1H, C-5), 3.56–3.37 (m, 3H, H-8,6′), 3.30 (dd, J = 15.0, 8.2 Hz, 1H, H-4), 3.16 (dd, J = 15.6, 6.9 Hz, 1H, H-4), 3.08 (dd, J = 13.0, 4.9 Hz, 1H, H-9′), 2.92–2.80 (m, 3H, H-7,9′), 2.35 (t, J = 7.0 Hz, 2H, H-2′), 1.86–1.56 (m, 4H, H-3′,5′), 1.56–1.44 (m, 2H, H-4′). ^{13}C NMR (75 MHz, D_2O) δ 178.1 (C-6), 176.7 (C-1′), 165.3 (C-10′), 133.2 (C-1), 131.0 (C-3), 116.7 (C-2), 62.2 (C-7′), 60.4 (C-8′), 55.5 (C-6′), 49.6 (C-5), 39.9 (C-9′), 38.7 (C-2′), 35.6 (C-8), 30.7 (C-4), 28.0 (C-7), 27.9 (C-4′), 27.8 (C-5′), 25.2 (C-3′). Anal. calc. for $C_{18}H_{27}N_5O_4S_2{\cdot}H_2O$: C, 47.04; H, 6.36; N, 15.24. Found: C, 46.72; H, 6.52; N, 14.92%.

*Synthesis of **L3***

*2-Amino-3-(1-carboxy-2-(1H-imidazol-4-yl)ethylthio)propanoic acid (**3**).* Compound **3** was prepared similarly as compound **2**: cysteine hydrochloride monohydrate (528 mg, 3 mmol) was used in the place of cysteamine hydrochloride followed by dropwise addition of aqueous NaOH (6 mL, 2 M). After stirring for 3 days, the reaction mixture was adjusted to pH 4 by addition of aqueous HCl 5 M and was concentrated to 2 mL under vacuum. The concentrate was loaded on a conditioned Sep-Pak® C18 cartridge, and the product was eluted with water to afford a white crystalline solid. Yield: 486 mg (50%). ^{1}H NMR (300 MHz, D_2O) δ 8.21 (s, 1H, H-1), 7.08 (s, 1H, H-2), 3.88–3.76 (m, 1H, H-8), 3.53 (t, 1H, H-5), 3.14–2.89 (m, 4H, H-4,7). ^{13}C NMR (75 MHz, D_2O) δ 177.7 (COOH), 172.6 (COOH), 133.6 (C-1), 131.3 (C-3), 116.6 (C-2), 53.8 (C-8), 49.7 (C-5), 31.3 (C-7), 27.8 (C-4). Anal. calc. for $C_9H_{13}N_3O_4S{\cdot}2HCl{\cdot}0.5H_2O$: C, 31.68; H, 4.73; N, 12.31. Found: C, 31.78; H, 4.80; N, 12.12%.

*2-Biotinamido-3-(1-carboxy-2-(1H-imidazol-4-yl)ethylthio)propanoic acid (**L3**).* Biotin-TFP ester (140 mg, 0.35 mmol), compound **3** (220 mg, 0.65 mmol), and $NaHCO_3$ (140 mg, 1.67 mmol) were dissolved in water–acetone (1:3 v/v, 9 mL), and the mixture was stirred at room temperature for 24 h. The product was purified by chromatography under the same conditions used for **L2**, and a white solid was afforded. Yield: 82 mg (47%). ^{1}H NMR (300 MHz, MeOD) δ 8.65 (s, 1H, H-1), 7.35 (s, 1H, H-3), 4.60–4.48 (m, 2H, H-8,8′), 4.42–4.33 (m, 1H, H-7′), 3.68 (t, J = 7.4 Hz, 1H, H-5), 3.30–3.17 (m, 3H, H-7,6′), 3.26 (dd, J = 15.0, 8.0 Hz, 1H, H-4), 3.11 (dd, J = 15.1, 6.9 Hz, 1H, H-4), 2.97 (dd, J = 12.8, 4.9 Hz, 1H, H-9′) 2.74 (d, J = 13.0 Hz, 1H, H-9′), 2.31 (t, J = 7.2 Hz, 2H, H-2′), 1.93–1.57 (m, 4H, H-3′,5′), 1.57–1.37 (m, 2H, H-4′). ^{13}C NMR (75 MHz, MeOD) δ 176.5, 176.4 (C-6/C-9[1]), 175.5 (C-1′), 166.1 (C-10′), 134.1 (C-3), 131.9 (C-1), 118.0 (C-2), 63.1 (C-7′), 61.4 (C-8′), 56.6 (C-6′), 54.2 (C-8), 48.5 (C-5), 40.9 (C-9′), 36.5 (C-2′), 34.3 (C-7), 29.2 (C-4′), 29.0 (C-5′), 28.0 (C-4), 26.4 (C-3′). Anal. calc. for $C_{19}H_{27}N_5O_6S_2{\cdot}H_2O$: C, 45.32; H, 5.80; N, 13.91. Found: C, 45.48; H, 5.72; N, 13.81%.

Syntheses of the rhenium complexes

fac-[Re(CO)$_3$(**L1**)] (**ReL1**)

To a solution of ligand **L1$_{Et}$** (80 mg, 0.16 mmol) in water (4 mL), aqueous NaOH (240 μL, 0.48 mmol) was added, and the reaction mixture was stirred for 20 min. The completion of hydrolysis was confirmed by HPLC, and then the pH was adjusted to 6 with aqueous HCl 1 M. $(NEt_4)_2[Re(CO)_3Br_3]$ (122 mg, 0.16 mmol) and methanol (5 mL) were added to the aforementioned solution, and the mixture was refluxed for 2 h. The volume of the reaction mixture was reduced under vacuum to 1.5 mL, and the solution was kept at 4 °C for 2 days. The precipitate formed was recrystallized by slow evaporation from water–methanol, and a greenish crystalline solid was afforded. Yield: 70 mg (59%). t_R (min) = 16.8. IR (KBr, cm^{-1}) 2023, 1896, 1637. NMR data are in Table 1. MS (ESI) (*m/z*): Calc. for $C_{26}H_{34}N_5O_7ReS$, M = 747.2. (+) Found: $[M+H]^+$, 748.1 (100%); $[M+Na]^+$, 770.1 (69%). (−) Found: $[M-H]^-$, 746.10 (67%); $[M+CH_2O_2-H]^-$, 792.1 (100%).

*fac-[Re(CO)$_3$(**L2**/**L3**)]* (***ReL2*** *and* ***ReL3***)

To a solution of $[Re(CO)_3(H_2O)_3]Br$ (0.06 mmol) in water (4 mL), **L2** (28 mg, 0.06 mmol) or **L3** (29 mg, 0.06 mmol) was added, and the mixture was refluxed for 1.5 h. The complex precipitated from water and was collected as a light yellow solid for **ReL2** or an off-white solid for **ReL3**.

ReL2: Yield: 31 mg (73%). t_R (min) = 14.8. IR (KBr, cm^{-1}) 2025, 1902, 1629. NMR data are in Table 1. MS (ESI) (*m/z*): Calc. for $C_{21}H_{26}N_5O_7ReS_2$, M = 711.1. (+) Found: $[M+H]^+$, 712.1 (100%); $[M+Na]^+$, 734.1 (31%). (−) Found: $[M-H]^-$, 710.1 (100%).

[1] C-9 corresponds to the carboxylate of the cysteine linker, marked as R in Figure 1.

Table 1. NMR analysis (500 MHz, 25 °C) of the rhenium complexes **ReL1** (CD_3OD), **ReL2** (CD_3OD), and **ReL3** (d_6-dmso)

	^{1}H NMR				^{13}C NMR		
	ReL1	**ReL2**	**ReL3**		**ReL1**	**ReL2**	**ReL3**
H-1	—	8.12	8.34	C-1	159.5	140.9	141.4
H-2	7.71 (d, J = 7.8 Hz)	7.08	7.17	C-2	123.6	116.1	116.4
H-3	8.08 (td, J = 7.8, 1.4 Hz)	—	—	C-3	140.2	134.1	133.5
H-4	7.53 (t, J = 6.6 Hz)	3.66 (dd, J = 17.8, 4.5 Hz)	3.49 (dd, J = 17.9, 4.3 Hz)	C-4	125.5	27.4	27.3
		3.30 (dd, J = 17.8, 3.7 Hz)	3.07 (dd, J = 17.9, 2.7 Hz)	C-5	152.1	46.0	45.5
H-5	8.81 (d, J = 5.1 Hz)	4.35 (t, J = 4.1 Hz)	4.20	C-6	67.9	181.0	177.0
H-6	4.72 (d, J = 15.6 Hz)	—	—	C-7	60.2	41.2	42.0
	4.53 (d, J = 15.5 Hz)			C-8	181.7	36.3	50.0
H-7	3.89 (d, J = 17.2 Hz)	3.30–3.26	3.39 (d, J = 13.9 Hz)	C-9	38.5	—	172.6[1]
	3.49 (d, J = 17.1 Hz)	3.19–3.10	3.19 (dd, J = 13.7, 3.6 Hz)	C-10	24.4	—	—
H-8	—	3.76–3.69	4.65–4.60	C-11	23.7	—	—
		3.56–3.47		C-12	28.1	—	—
H-9	3.27–3.16	—	—	C-13	69.7	—	—
H-10	1.98–1.79	—	—	C-1′	174.6	175.2	170.8
H-11	1.49–1.36	—	—	C-2′	35.4	35.2	34.9
H-12	1.79–1.54	—	—	C-3′	25.5	25.3	25.1
H-13	3.65–3.52	—	—	C-4′	28.8	28.4	28.0
NH	—	—	13.13, 8.11, 6.37, 6.32	C-5′	28.8	28.1	28.1
H-2′	2.21 (t, J = 7.3 Hz)	2.27 (t, J = 7.1 Hz)	2.15 (t, J = 7.3 Hz)	C-6′	55.6	55.7	55.5
H-3′	1.79–1.54	1.77–1.64	1.63–1.47	C-7′	62.0	62.0	61.0
H-4′	1.49–1.36	1.50–1.42	1.38–1.25	C-8′	60.6	60.2	59.1
H-5′	1.79–1.54	1.77–1.64	1.63–1.47	C-9′	39.6	39.6	40.1
H-6′	3.27–3.16	3.19–3.10	2.99–2.94	C-10′	164.6	164.6	162.7
H-7′	4.30 (dd, J = 7.8, 4.5 Hz)	4.27 (dd, J = 7.8, 4.5 Hz)	4.29–4.19	C≡O	196.8	196.7	195.8
H-8′	4.48 (dd, J = 7.8, 4.6 Hz)	4.45 (dd, J = 7.8, 4.9 Hz)	4.63		196.7	194.8	196.5
H-9′	2.92 (dd, J = 12.7, 5.0 Hz)	2.83 (dd, J = 12.8, 5.0 Hz)	2.71 (dd, J = 12.5, 5.1 Hz)		195.9	192.3	197.7
	2.70 (dd, J = 12.7, 3.2 Hz)	2.62 (d, J = 12.7 Hz)	2.52 (d, J = 12.6 Hz)				

ReL3: Yield: 35 mg (77%). t_R (min) = 14.6. IR (KBr, cm^{-1}) 2028, 1896, 1636. NMR data are in Table 1. MS (ESI) (*m/z*): Calc. for $C_{22}H_{26}N_5O_9ReS_2$, M = 755.1. (+) Found: $[M+H]^+$, 756.0 (100%); $[M+Na]^+$, 778.1 (20%). (−) Found: $[M-H]^-$, 754.0 (100%).

Synthesis of the technetium-99m complexes

A freshly prepared solution of the *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ (450 μL, 60–110 MBq) at pH 7 was added to a vial containing a solution of the ligands **L1$_{Et}$**, **L2**, and **L3** (50 μL of 10^{-2} or 10^{-3} or 10^{-4} M). The vial was sealed, flushed with N_2 for 5 min, and heated for 30 min at 80–85 °C. The reaction mixtures were analyzed by HPLC: **^{99m}TcL1**, t_R (min) = 17.0; **^{99m}TcL2**, t_R (min) = 14.9; and **^{99m}TcL3**, t_R (min) = 14.5.

In vitro studies

Histidine and cysteine challenge

The HPLC-purified ^{99m}Tc complexes (50 μL, approx. 10 MBq) were mixed with a solution of L-histidine or L-cysteine (10^{-3} M) in 0.1 M PBS, pH 7.4 (0.5 mL), and the mixtures were incubated at 37 °C for 24 h. The mixtures were analyzed by HPLC at 1-, 4-, and 24-h intervals.

Rat plasma stability studies

The HPLC-purified ^{99m}Tc complexes (50 μL, 10 MBq) were incubated in rat plasma (0.5 mL) at 37 °C for 24 h. Aliquots (100 μL) were taken at 1, 4, and 24 h. The proteins were precipitated using acetonitrile (300 μL) and separated by centrifugation. The supernatant was further analyzed by HPLC.

Avidin binding

The HPLC-purified ^{99m}Tc complexes (50 μL, 5 MBq) were mixed with an aqueous solution of avidin (50 μL, 1 mg/mL), and the mixtures were incubated at 37 °C for 30 min. Aliquots (2 μL) were analyzed by TLC as well as by size-exclusion HPLC. The SiO_2 TLC was performed in acetone–water (7:3 v/v), where avidin and avidin-bound ^{99m}Tc-complex stay at the origin, R_f = 0, and free ^{99m}Tc complex migrates to the solvent front, R_f =1.

In vivo studies

All animals were housed in proper animal facilities with food and water *ad libitum* in constant conditions of temperature and humidity and regular light cycles of 12/12 h light/dark and were handled humanely according to European Union guidelines. The experiment was approved by the Aristotle University Committee for Animal Experimentation.

Rat urine analysis

An injectable solution of 0.3–0.5 mL of the purified ^{99m}Tc complexes (6–7 MBq) was administered intraperitoneally to young male Fischer 344 rats. The rats were placed in metabolic cages, and their urine was collected for a period of 4 h. The urine samples were centrifuged for 30 min at 3500 rpm, and aliquots (150 μL) of the clear supernatant were analyzed by HPLC.

Biodistribution studies in mice

Ten-week-old male BALB/c mice of ~25 g weight were injected intravenously in the tail vein, each with ~370 kBq of the purified ^{99m}Tc-biotin complexes in 0.1 mL saline. Animals were sacrificed at 5 min and 1 h post injection (*p.i.*) by cervical dislocation followed by blood withdrawal and cardiectomy. Organs and tissues of interest were excised and weighed, and their radioactivity was determined using a NaI scintillation counter (Caprac®, Capintec). The activity of the tissue samples was decay corrected and calibrated by comparing the counts in the tissue with the counts of a standard solution corresponding to 1% of the injected dose. Counts of the sample and

calibration aliquots were measured in the gamma counter at the same time. The amount of activity in the selected tissues and organs is expressed as a percent of the injected dose per organ (%ID) or per gram tissue (%ID/g). Values are quoted as the mean ID ± standard deviation (SD) % of the four mice per group. Blood volume and muscle mass were estimated at 7% and 43% of body weight, respectively. Results are displayed in Table 2.

Results and discussion

Synthesis of ligands

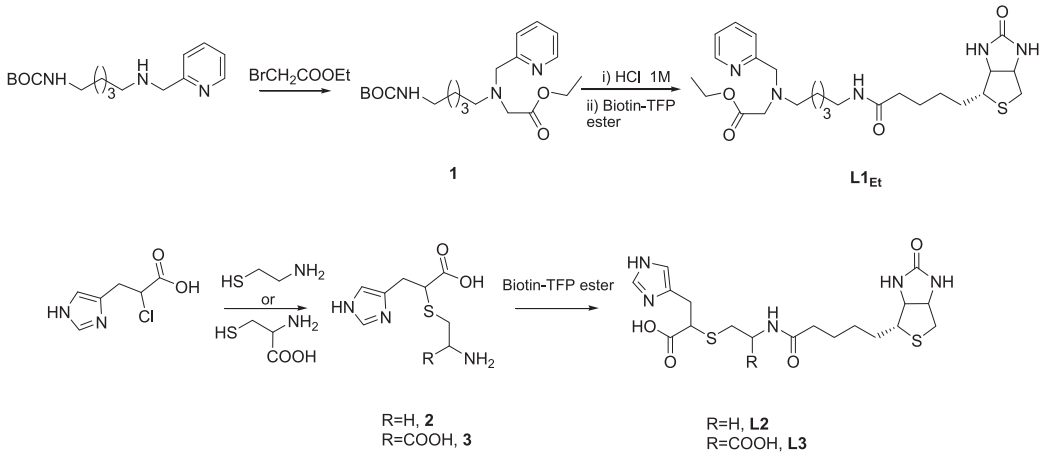
The synthetic route of the ligands **L1–L3** is presented in Scheme 1. Biotin-TFP ester was synthesized in moderate yield by treating D-biotin and tetrafluorophenol with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The bifunctional chelators 2-(2-aminoethylthio)-3-(1*H*-imidazol-4-yl)propanoic acid (**2**) and 2-amino-3-(1-carboxy-2-(1*H*-imidazol-4-yl)ethylthio)propanoic acid (**3**) were synthesized by reacting α-chloro-L-histidine with cysteamine and L-cysteine. Ligands $\mathbf{L1_{Et}}$, **L2**, and **L3** were prepared by reacting the activated biotin-TFP ester with ethyl 2-((5-aminopentyl)(pyridin-2-ylmethyl)amino)acetate **1′**, **2**, and **3**, respectively. $\mathbf{L1_{Et}}$ was purified by silica gel column chromatography in a high yield, and it was hydrolyzed in aqueous NaOH prior to rhenium complexation. Purification of **2** was accomplished by multiple crystallizations from water–ethanol. Purifications of **3**, **L2**, and **L3** were achieved by C18 chromatography in moderate to high yields.

Synthesis of rhenium complexes

Rhenium complex **ReL1** was prepared by reaction of equimolar amounts of hydrolyzed **L1** with $(NEt_4)_2[Re(CO)_3Br_3]$, and **ReL2** and **ReL3** were prepared by reaction of the **L2** and **L3** with $[Re(CO)_3(H_2O)_3]Br$, respectively. HPLC analysis of the reaction mixtures revealed one major peak in all cases. **ReL1** was purified by re-crystallization. **ReL2** and **ReL3** were obtained as pure solids from the reaction mixture. Rhenium complexes were characterized by spectroscopic methods (IR, NMR, and ESI-MS). Infrared spectroscopy of the complexes indicated the presence of the facially coordinated CO molecules with strong ν(CO) bands in the region 2028–1896 cm^{-1}. Nuclear magnetic resonance spectroscopy was used for structural characterization of the complexes, and the ^1H and ^{13}C NMR assignments of **ReL1**, **ReL2**, and **ReL3** are presented in Table 1. In the NMR spectra of the rhenium complexes, typical shifts of the protons and carbons associated with the donor atoms were observed, and the three facially coordinated CO ligands were detected at 192–198 ppm of the ^{13}C NMR spectra. **L1** coordinates with the *fac*-$[Re(CO)_3]^+$ core via the N pyridine, N tertiary amine, and O carboxylate as indicated by the distinctive shifts of H-6, H-7, and C-8 as shown in the literature for similar (N,N,O) complexes.[22] The protons of each H-6 and H-7 in $\mathbf{L1_{Et}}$ appear as singlets at 3.83 and 3.30 ppm, but upon coordination, each proton differentiates chemically and splits into two doublets with characteristic *J* couplings, for H-6 at 4.72 and 4.53 ppm (^2J = 15.6 Hz) and for H-7 at 3.89 and 3.49 ppm (^2J = 17.1 Hz).

Table 2. Biodistribution studies of ^{99m}**TcL2** and ^{99m}**TcL3** calculated as percent injected dose/organ and percent injected dose/gram tissue (n = 4)

	%ID/organ			
	5 min		1 h	
Organs	^{99m}TcL2	^{99m}TcL3	^{99m}TcL2	^{99m}TcL3
Blood	6.67 ± 0.13	9.28 ± 2.02	1.62 ± 0.13	1.47 ± 0.26
Heart	0.10 ± 0.01	0.16 ± 0.07	0.04 ± 0.01	0.04 ± 0.01
Liver	40.37 ± 0.96	21.44 ± 5.26	9.88 ± 0.85	3.27 ± 1.30
Lungs	0.30 ± 0.08	0.41 ± 0.10	0.07 ± 0.00	0.09 ± 0.03
Muscle	7.01 ± 0.84	13.42 ± 2.10	1.29 ± 0.10	1.43 ± 0.64
Kidneys	8.03 ± 1.06	8.26 ± 3.94	1.87 ± 0.34	0.72 ± 0.13
Spleen	0.08 ± 0.01	0.10 ± 0.02	0.12 ± 0.11	0.04 ± 0.01
Intestine	22.70 ± 0.32	22.47 ± 0.99	54.97 ± 8.91	61.55 ± 3.14
Skin	0.39 ± 0.06	0.34 ± 0.28	0.05 ± 0.02	0.02 ± 0.01
Stomach	0.23 ± 0.03	0.38 ± 0.07	0.45 ± 0.29	0.40 ± 0.15
Urine	6.64 ± 4.81	17.55 ± 5.46	20.25 ± 6.90	20.57 ± 4.72
	%ID/g			
	5 min		1 h	
Organs	^{99m}TcL2	^{99m}TcL3	^{99m}TcL2	^{99m}TcL3
Blood	5.03 ± 0.43	7.01 ± 1.57	1.15 ± 0.09	1.03 ± 0.18
Heart	1.15 ± 0.09	1.80 ± 0.74	0.47 ± 0.16	0.41 ± 0.10
Liver	32.43 ± 1.94	14.76 ± 1.41	7.76 ± 1.19	2.82 ± 1.14
Lungs	1.55 ± 0.45	2.56 ± 1.06	0.36 ± 0.07	0.47 ± 0.14
Muscle	0.81 ± 0.06	1.49 ± 0.40	0.15 ± 0.02	0.16 ± 0.07
Kidneys	30.06 ± 6.48	25.25 ± 11.98	5.65 ± 0.64	2.65 ± 0.31
Spleen	0.97 ± 0.12	1.04 ± 0.27	1.23 ± 1.15	0.37 ± 0.07
Intestine	14.10 ± 0.28	15.97 ± 0.51	31.94 ± 9.89	42.79 ± 5.97
Skin	2.82 ± 0.09	3.65 ± 2.32	0.36 ± 0.06	0.24 ± 0.08
Stomach	0.32 ± 0.18	1.53 ± 0.93	2.87 ± 1.80	2.44 ± 0.48



Scheme 1. Syntheses of the ligands.

The carboxylate C-8 shift to 181.7 ppm indicates its coordination to rhenium. The coordination of **L2** and **L3** with the *fac*-$[Re(CO)_3]^+$ core via the N(π) imidazole, the S thioether, and the O of the C-6 carboxylate was based on the NMR data of similar tripodal complexes as we have shown in our previous work.[17,18] In particular, the H-4 protons appear as two doublet of doublets at 3.30 ppm ($J = 15.0$, 8.2 Hz) and 3.16 ppm ($J = 15.6$, 6.9 Hz) in **L2**, and 3.26 ppm ($J = 15.0$, 8.0 Hz) and 3.11 ppm ($J = 15.1$, 6.9 Hz) in **L3**, while upon coordination, they are shifted to 3.66 ppm ($J = 17.8$, 4.5 Hz) and 3.30 ppm ($J = 17.8$, 3.7 Hz) in **ReL2**, and 3.49 ppm ($J = 17.9$, 4.3 Hz) and 3.07 ppm ($J = 17.9$, 2.7 Hz) in **ReL3**. Also, the H-5 proton is influenced and shifted from 3.68 ppm in **L2** and **L3** to 4.35 ppm in **ReL2** and 4.20 ppm in **ReL3**. Moreover, the shifts of C-6 at 181.0 and 177.0 ppm in **ReL2** and **ReL3**, respectively, are in the range observed for coordinated carboxyl groups.[17,18]

Radiochemistry

The complexes **^{99m}TcL1**, **^{99m}TcL2**, and **^{99m}TcL3** were synthesized in high radiochemical yield (>92%) by reaction of the precursor *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ with the ligands **$L1_{Et}$**, **L2**, or **L3** at 10^{-3} and 10^{-4} M ligand concentrations. For **^{99m}TcL1**, hydrolysis of **$L1_{Et}$** prior to the ^{99m}Tc-labeling was not necessary; it occurred simultaneously during the reaction. HPLC analysis of all reaction mixtures revealed the formation of single products, which were identified by comparison of their retention times with those of their co-injected rhenium analogs. The Re and ^{99m}Tc analogs exhibited similar retention times, which indicated their structural analogy: 16.8/17.0 min for **Re/^{99m}Tc-L1**, 14.8/14.9 min for **Re/^{99m}Tc-L2**, and 14.6/14.5 min for **Re/^{99m}Tc-L3**. Synthesis of **^{99m}TcL1** at 10^{-5} M concentration resulted in 43% yield, while **^{99m}TcL2** and **^{99m}TcL3** were formed in yields of 82% and 74%, respectively, at 10^{-5} M ligand concentration.

The tracer complexes were challenged against 10^{-3} M L-histidine and L-cysteine in 0.1 M PBS buffer pH 7.4 at 37 °C. Their stability was determined by means of HPLC at 1, 4, and 24 h, and it was verified that all complexes were stable after 24 h. In particular, **^{99m}TcL1** and **^{99m}TcL2** were 100% intact in histidine and cysteine at 24 h, while **^{99m}TcL3** was about 97% intact in histidine and 100% in cysteine at 24 h, respectively. These results show that all tracers are stable against these potent chelators, present in the biological media, which, in turn, indicates stability against transchelation *in vivo*.

Biotinidase is an enzyme with high activity in the serum (~4 U/L), responsible for the hydrolytic cleavage of biocytin to biotin,[24,25] and radiolabeled biotin derivatives, in the literature, are usually evaluated by incubating them in diluted serum (×10) and then measuring their binding to avidin, isolating thus the biotinidase action.[9,11,12,14] In our experiment, all the ^{99m}Tc complexes were incubated in undiluted fresh rat plasma in order to determine their overall stability through 24 h, assuming that the ^{99m}Tc chelates may interact not only with biotinidase but also with other biomolecules, leading to their biotransformation, addressing thus the chelate stability as well.

Plasma stability studies of **^{99m}TcL1** showed that the complex was quantitatively cleaved after 1 h to the biotin hydrolyzed product, which was observed by HPLC from the formation of a new species with different retention time, $t_R = 13.8$ min, from the original **^{99m}TcL1**, $t_R = 17.0$ min. The identity of this species was confirmed after labeling with $[^{99m}Tc(CO)_3]^+$ the unbiotinylated precursor of ligand **L1**, compound **1′**, which yielded the species **^{99m}Tc1′** with the same retention time at $t_R = 13.8$ min, as the metabolized **^{99m}TcL1** species (Figure 2).

^{99m}TcL2 and **^{99m}TcL3** behaved similarly in plasma studies and were found more stable than **^{99m}TcL1**. No biotin cleavage product was detected by HPLC, hypothesizing that the cleaved ^{99m}Tc-chelate fragment would have an earlier retention time than the biotinylated one, and as shown in Figure 3, no such distinct peak had formed. Furthermore, intact **^{99m}TcL2** and **^{99m}TcL3** at approximately 20% and metabolites at approximately 80% were observed, as early as 1 h. It is noteworthy to add that this picture stayed almost the same during the 24-h incubation period, where the amount of intact **^{99m}TcL2** and **^{99m}TcL3** remaining in the plasma at 24 h was approximately 10%. This result indicates that the enzymatic activity of the biological systems responsible for the biotransformation of the tracers was practically saturated within the first hour, and from then on, the biodegradation of the remaining tracers was much slower; therefore, intact complex was still present after 1 day.

The ^{99m}Tc-biotinylated complexes were evaluated for their binding affinity to avidin (0.5 mg/mL, approximately ≈ 7.41×10^{-6} M) in 0.1 M PBS buffer at pH 7.4 at 37 °C for 30 min. The percentages of bound tracer complexes were determined by TLC to be: 95 ± 2% for **^{99m}TcL1**, 92 ± 3% for **^{99m}TcL2**, and 91 ± 4% for **^{99m}TcL3**. The samples were further analyzed by size-exclusion HPLC where they all eluted at the same elution time as avidin at 20.1 min, while complexes **^{99m}TcL1**, **^{99m}TcL2**,

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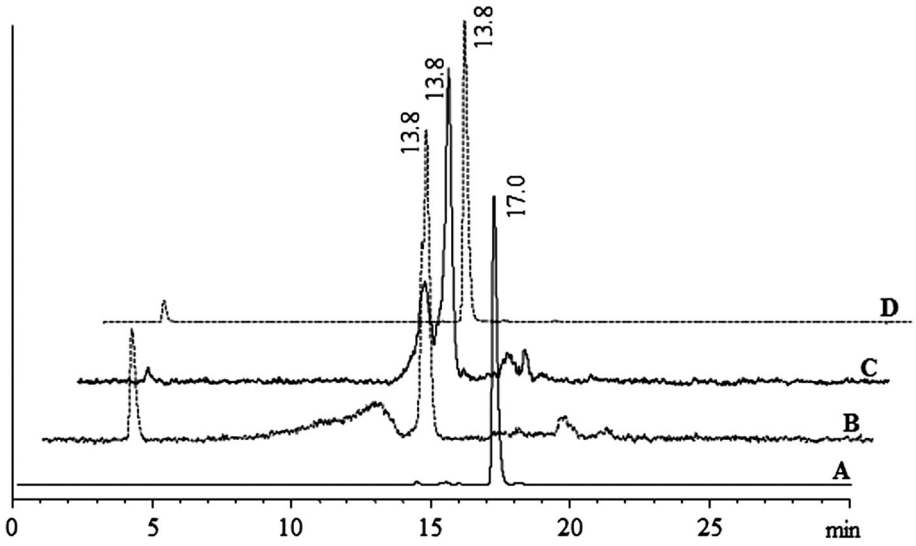

Figure 2. HPLC radiochromatograms of ^{99m}**TcL1**: (A) labeling reaction with 10^{-3} M $\mathbf{L1_{Et}}$, (B) rat plasma at 1 h, (C) rat urine, and (D) ^{99m}**Tc1′** labeling reaction with 10^{-3} M **1′**.

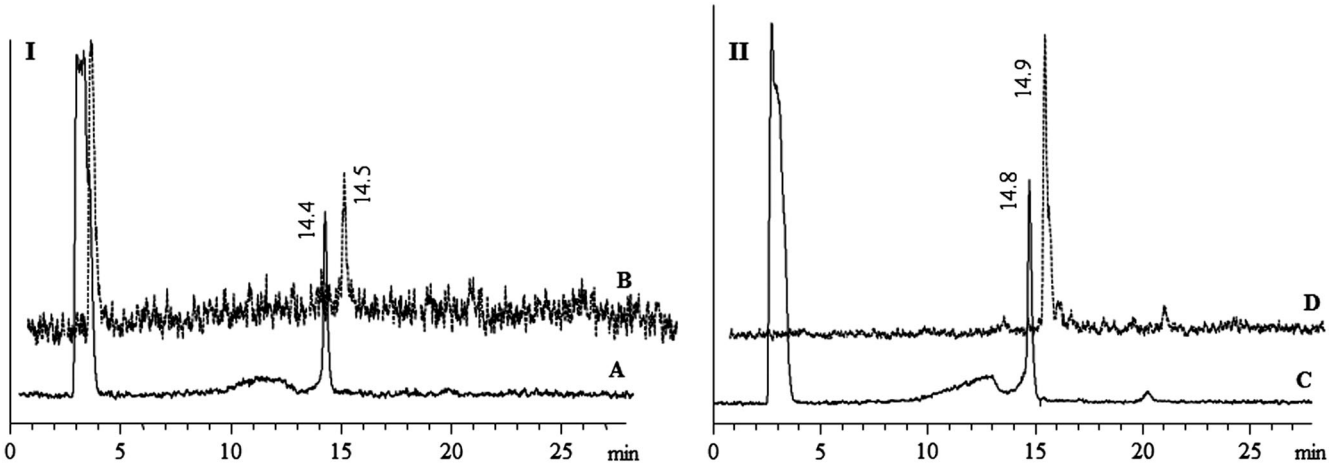

Figure 3. HPLC radiochromatograms of (I) ^{99m}**TcL2** and (II) ^{99m}**TcL3**, where A and C are rat plasma analyses at 4 h after incubation and B and D are rat urine analyses at 4 h after administration, respectively.

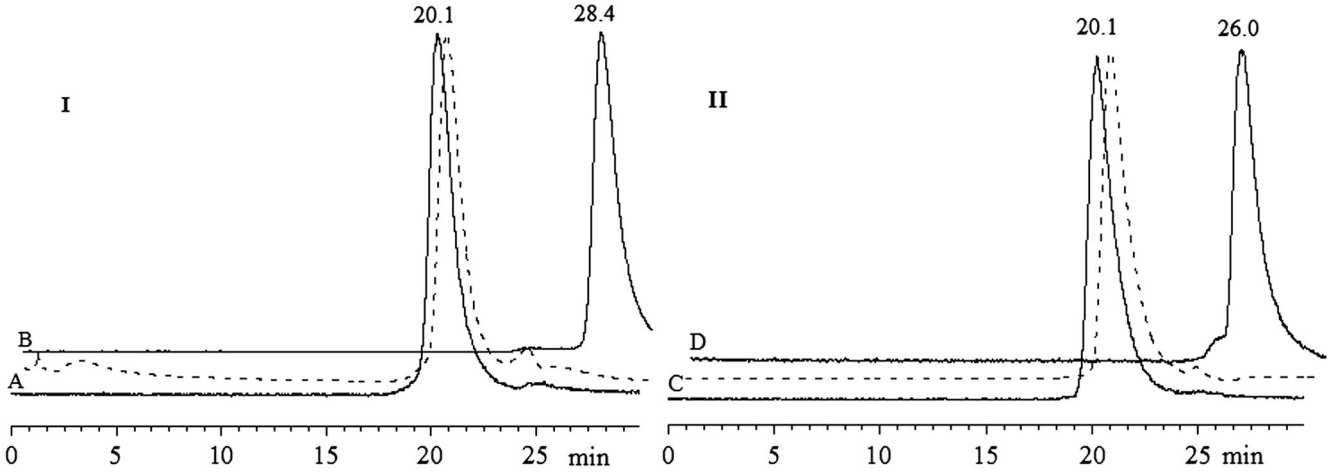

Figure 4. Size-exclusion HPLC chromatograms of (I) ^{99m}**TcL2** and (II) ^{99m}**TcL3**, where A and C correspond to the co-elution of the ^{99m}Tc complexes (solid lines, γ-detection) with avidin (dotted lines, UV detection) at 20.1 min, after incubation of the ^{99m}Tc complexes with avidin for 30 min at 37 °C. B and D (γ-detection) correspond to the elution of ^{99m}Tc complexes not treated with avidin, at 28.4 and 26 min, respectively.

and ^{99m}**TcL3** not treated with avidin eluted at later times, at 31.1, 28.4, and 26 min, respectively. From these chromatograms, it can be concluded that the binding of the tracers to avidin was quantitative. In Figure 4, the representative chromatograms of the stable tracers only, ^{99m}**TcL2**, and ^{99m}**TcL3** are shown.

In vivo studies

Rat urine analysis of ^{99m}**TcL1** had the same picture as the plasma studies, where no parent complex was found. The major metabolite was identified as the cleaved complex ^{99m}**Tc1′**, the hydrolysis product of ^{99m}**TcL1**, most probably due to biotinidase action, which was similarly observed in the plasma stability studies (Figure 2). On the other hand, intact complexes ^{99m}**TcL2** and ^{99m}**TcL3** were detected in the HPLC chromatograms of the collected rat urine, as shown in Figure 3.

Biodistribution studies (Table 2) in mice showed that both complexes ^{99m}**TcL2** and ^{99m}**TcL3** exhibited similar properties as expected, fast clearance from the hepatobiliary system, and low urinary excretion. In particular, 1 h after administration of ^{99m}**TcL2** and ^{99m}**TcL3**, it was observed that the majority of the injected dose had been cleared from circulation, being approximately 20% in the urine and 55–60% in the intestines (the tissue was counted with its contents). These results are similar with those of other radiolabeled biotin derivatives.[7]

It has been proposed in the literature[9] that the presence of the ^{99m}Tc-chelate moiety in the vicinity of the amide bond via which biotin is attached on the complexes protects it against biotinidase cleavage. In the aforementioned literature report,[9] stability of a biotinylated $[^{99m}Tc(CO)_3]^+$-type complex was observed, where a three-carbon chain separated the chelate from biotin amide bond. In another study, various substituents on the α-carbon of the amide bond were evaluated in terms of the protection they offer against biotinidase cleavage, among which the α-carboxyl group that was found to sufficiently shield the biotin amide.[13,14] From the blood plasma and urine analysis results of our tracers, it can be concluded that in the case of ^{99m}**TcL1**, complete cleavage takes place both *in vitro* and *in vivo*, and therefore, the five-carbon chain between the amide and the ^{99m}Tc-chelate moiety is too long to impart any stability. Tracers ^{99m}**TcL2** and ^{99m}**TcL3** were designed to exhibit higher stability against biotinidase, by using suitable structural elements, according to the literature:[9,13,14] the ^{99m}Tc-chelate moiety is located in a shorter distance of a two-carbon chain from the amide bond, and furthermore, in ^{99m}**TcL3**, there is the additional cysteine carboxylate in an α-position to the biotin amide bond. The results of ^{99m}**TcL2** and ^{99m}**TcL3** stability in plasma and urine are more complex to interpret, because, although no cleavage metabolites of biotin were detected in the HPLC analyses, hydrophilic metabolites were observed.

Conclusion

New biotinylated *fac*-$[^{99m}Tc/Re(CO)_3$(**L1-L3**)] complexes were synthesized and characterized, with high *in vitro* binding affinity for avidin. *In vitro* plasma stability and *in vivo* rat urine studies showed metabolic cleavage of biotin for ^{99m}**TcL1**. ^{99m}**TcL2** and ^{99m}**TcL3**, under the same conditions, exhibited higher stability although not entirely robust in the biological environment, compared to similar agents reported in the literature. However, their ability to accumulate in (strept)avidin-rich sites *in vivo* cannot be excluded.

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Conflict of interest

The authors did not report any conflict interest.

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