Rhenium and Technetium-oxo Complexes with Thioamide Derivatives of Pyridylhydrazine Bifunctional Chelators Conjugated to the Tumour Targeting Peptides Octreotate and Cyclic-RGDfK

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Supporting Information

ABSTRACT: This research aimed to develop new tumor targeted theranostic agents taking advantage of the similarities in coordination chemistry between technetium and rhenium. A γ-emitting radioactive isotope of technetium is commonly used in diagnostic imaging, and there are two β− emitting radioactive isotopes of rhenium that have the potential to be of use in radiotherapy. Variants of the 6-hydrazinonicotinamide (HYNIC) bifunctional ligands have been prepared by appending thioamide functional groups to 6-hydrazinonicotinamide to form pyridylhydrazide ligands (SHYNIC). The new bidentate ligands were conjugated to the tumor targeting peptides Tyr3-octreotate and cyclic-RGD. The new ligands and conjugates were used to prepare well-defined {M=O}5+ complexes (where M = 99mTc or natRe or 188Re) that feature two targeting peptides attached to the single metal ion. These new SHYNIC ligands are capable of forming well-defined rhenium and technetium complexes and offer the possibility of using the 99mTc imaging and 188/186Re therapeutic matched pairs.

INTRODUCTION

The technetium-99m isotope has excellent properties for detection with single photon emission computed tomography (SPECT) due to its low energy and nonparticulate gamma-ray emission (t1/2 = 6.01 h, Eγ = 141 keV γ-ray emission, λ < 10 pm). Despite recent concerns over production related shortages of technetium-99m and the advent of positron emission tomography technetium-99m retains its importance to nuclear medicine to the extent that the isotope is used in over 80% of nuclear imaging procedures worldwide.1 The heavier third row Group VII congener, rhenium, has an ionic radius similar to technetium due to the lanthanide contraction. Technetium and rhenium display similar coordination chemistry often resulting in essentially isostructural technetium and rhenium complexes. It is common for technetium and rhenium complexes to be essentially isostructural. There are two isotoopes of rhenium that are of potential use in targeted radiotherapeutics, rhenium-186 (t1/2 = 89.3 h, Eγ = 1.07 MeV β− particle emission, 137 keV γ-ray emission) and rhenium-188 (t1/2 = 16.9 h, Eγ = 2.12 MeV β− particle emission, 155 keV γ-ray emission). The similar coordination chemistry of technetium and rhenium offers the possibility of using their radioisotopes as an imaging (99mTc) and therapeutic (186/188Re) matched pair using a single targeted ligand to form essentially isostructural complexes.2−5

One approach to targeted imaging and therapy is to incorporate appropriate metal radionuclides into coordination complexes that are attached to biological targeting vectors such as tumor targeting peptides, antibodies or antibody fragments. Peptides that feature the −RGD− (arginine-glycine-aspartic acid) fibronectin fragment such as the cyclic-RGDfK pentapeptide (cRGDK) bind to ανβ3 integrin receptors that are overexpressed in certain invasive tumors including osteosarcomas, glioblastoma, melanomas, and breast cancer, and can be used to selectively target tumor cells.6−16 Metabolically stabilized somatostatin analogues such as octreotide and octreotate bind to somatostatin subtype 2 receptors (sstr2) that are overexpressed in many types of neuroendocrine tumors compared to relatively low levels of expression in other tissues and organs.17−21

Tumor targeting technetium based imaging agents can be prepared using 6-hydrazinonicotinamide (HYNIC) derivatives conjugated to targeting molecules as ligands to form
technetium(III) diazenido complexes.\textsuperscript{22–31} The HYNIC ligand forms remarkably stable technetium complexes, and manipulation of the carboxylate functional group to attach a variety of targeting molecules is generally straightforward. HYNIC binds to technetium through the terminal hydrazone nitrogen but probably forms bidentate complexes through coordination to the pyridyl nitrogen.\textsuperscript{26,32} In all the crystallographically characterized technetium and rhenium complexes with one or more HYNIC-like ligands, such as 2-hydrazinopyridine, the pyridyl nitrogen is also coordinated to the metal center.\textsuperscript{33} A variety of coligands such as tricine, nicotinic acid, EDDA, and phosphines (TPPTS, TPPDS, TPPMS = tri/bi/sodium triphenylphosphine tri/di/monosulfonate) are required to complete the coordination sphere and stabilize the metal oxidation state, and this leads to a high degree of uncertainty in the exact nature of the primary coordination sphere as well as challenges in ensuring structural homogeneity in the formulated product. Variation of the coligand can modify the in vivo metabolism and excretion.\textsuperscript{34} A well-established “ternary ligand system” involves combining the HYNIC ligand with tetradentate tricine and monodentate trisodium 3,3’,3”-phosphanetriyltris(benzenesulfonate) (TPPTS) coligands, but the possibility of forming multiple isomers adds complications.\textsuperscript{33,35–39}

Despite the superficial similarities in coordination chemistry between technetium and rhenium extrapolation of the HYNIC strategy to radioactive rhenium isotopes is challenging presumably due to their differences in kinetic lability and redox chemistry.\textsuperscript{33} Some of the difficulty in isolating pure Re-HYNIC-peptide conjugates can be understood by considering the reaction of [ReO$_2$]$^-$ with 2-hydrazinopyridine (used as model for HYNIC).\textsuperscript{34} This reaction results in relatively complex coordination chemistry due to the ability of the pyridylhydrazine derived ligands to coordinate as either monodentate or bidentate ligands and the existence of protic equilibria as well as the formation of complexes where two pyridylhydrazine derived units are coordinated to the rhenium (Figure 1).\textsuperscript{26,33,40–43}

Modification of the terminal hydrazinic nitrogen of hydrazinopyridine to incorporate an additional thiourea functional group results in a ligand system that is capable of forming well-defined, very stable complexes with [ReO$_3$]$^{3+}$ cores while retaining the bioconjugation possibilities well established for HYNIC.\textsuperscript{34,44} A preliminary communication reported the structural characterization of a Re$^V$-oxo complex featuring two pyridylphenylthiocarbazide (SHYNIC) ligands (Figure 1).\textsuperscript{44} In this manuscript we extend this concept by synthesizing a family of different substituted pyridylthiosemicarbazide ligands with carboxylate or ester functional groups that were used to tether octreotide and cyclic-RGD peptides to the ligands. The new ligands were used to prepare \{MO\}$_3^+$ complexes (where M = Tc or Re) that feature two targeting peptides attached to the single metal ion. These modified HYNIC ligands are capable of forming well-defined rhenium and technetium complexes and offer the possibility of using the two radionuclides as imaging and therapeutic matched pairs.

## RESULTS AND DISCUSSION

### Synthesis of H$_2$L$_1^{1–4}$, and Their Ester Derivatives, H$_2$L$_1^{4(OMe)}$ and \{ReO\}$_3^{3+}$ Complexes.

Synthesis of 6-hydrazinonicotinic acid (HYNIC), \textsuperscript{2} required treatment of 6-chloronicotinic acid (1) with aqueous hydrazine.\textsuperscript{17} Ligands H$_2$L$_1^{1}$ to H$_2$L$_4^{4}$ were prepared by reaction of either the ethyl, tert-butyl, phenyl, or nitrophenyl isothiocyanate with 6-hydrazinonicotinic acid (1) in anhydrous N,N-dimethylacetamide (DMA) (Scheme 1).

The rhenium complexes of the methyl ester derivatives of H$_2$L$_1^{1–3}$ complexes, [ReO(HL$_1^{1–3}$(OMe))$_2$]$^+$, can be prepared by reaction of either the trans-[ReOCl$_4$(PPh$_3$)$_2$] or [Bu$_4$N]-[ReOCl$_4$] with the two equivalents of ligand (Scheme 2). The IR spectra for the three complexes, [ReO(H$_2$L$_1^{1–3}$(OMe))$_2$]$^+$, display medium intensity bands at \textbar 960–963 cm$^{-1}$ characteristic of Re==O stretches.\textsuperscript{48} Bands, which occur between \textbar 1553 and 1557 cm$^{-1}$ due to carbonyl stretching of the ester functional group, shift approximately 150 cm$^{-1}$ lower in energy when compared to the metal-free ligands.

Analysis of the complexes by $^1$H NMR data reveals that the two coordinated ligands are magnetically equivalent, with three resonances at $\delta$ 8.63, 8.25, and 7.86 ppm corresponding to the six pyridinyl CH protons for [ReO(HL$_1^{1}$(OMe))$_2$]$^+$, and similar resonances for the phenyl and tert-butyl derivatives. The pyridine proton which is closest to the rhenium ion shifts from $\delta$ 6.55 ppm in free ligand to $\delta$ 7.86 ppm in the complex. The methyl ester functional group functions rise to singlets at $\delta$ 3.89 (DMF-d$_6$), 3.86 (CHCl$_3$-d), and 3.92 (DMF-d$_6$) for complexes [ReO((HL$_1^{1–3}$(OMe))$_2$]$^+$ respectively. The [ReO-(HL$_1^{1}(OMe))_2$]$^+$ complex was stable to cysteine and histidine challenge experiments with very little decomposition evident ($<5\%$), as detected by analysis by HPLC and UV/vis spectroscopy, when incubated at 37 °C for 24 h in the presence of a 100-fold excess of cysteine and histidine.

Red crystals of [ReO(HL$_1^{1}$(OMe))$_2$]CF$_3$CO$_2$ suitable for X-ray crystallographic analysis were obtained by evaporation of a solution of the compound that had been purified by semipreparative HPLC using an aqueous/CH$_3$CN mobile phase with 0.1% trifluoroacetic acid (Figure 2a). The compound crystallizes in the triclinic space group, P1, and the rhenium ion is in a distorted square pyramidal environment with the oxo group in the apical position relative to the pseudo basal plane of two five-membered chelate rings. Each thio-carbarylhydrazide functional group is doubly deprotonated and serves as a

![](image)

**Figure 1.** (a) 6-Hydrazinonicotinic acid. (b) Metal complex (M = Tc or Re) with 6-hydrazinonicotinamide (HYNIC) acting as a monodentate ligand. It is necessary to complete the coordination sphere with coligands (L). (c) Metal complex (M = Tc or Re) with 6-hydrazinonicotinamide (HYNIC) acting as a bidentate ligand. (d) Pyridylphenylthiocarbazide (SHYNIC) ligand. (e) Re$^V$-oxo complex featuring two SHYNIC ligands.\textsuperscript{44}
The potential of the substituted pyridylthiosemicarbazide (SHYNIC) ligands H2L1−4 to be modified with amino acids using standard solid phase peptide synthesis techniques was first accomplished by attaching L-lysine to H2L1 to give H2L1(Lys). The doubly N-protected lysine derivative, Nε-Boc-

Nγ-Fmoc-L-Lys, was immobilized on chlorotrityl resin, and the Nγ-Fmoc group was removed by treatment with piperidine. The ligand, H2L1, was added to the resin in a mixture of DMF followed by the coupling agent HATU (HATU = 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]-pyridinium-3-oxo hexafluorophosphate) in the presence of N,N-disopropylethylamine (DIPEA). The product was cleaved from the resin using trifluoroacetic acid that also resulted in the deprotection of the Nε-f-butoxycarbonyl group (Scheme 3). This lysine amino acid conjugate provides an amino acid with an appended chelator, which with appropriate protecting groups, could be incorporated into biological targeting molecules with total site specificity via solid-phase peptide synthesis.59–63

The rhenium complex, [ReO(HL1(Lys))]2+, was prepared by adding either trans-[ReOCl3(PPh3)2] or [BuN][ReOCl4] suspended in DMF to the reaction mixture, while the ligand remained immobilized on the resin. Performing the complexation while the ligand remained immobilized on the resin and with the amino group still protected ensured that the functional groups of the lysine did not complicate the coordination chemistry. When green trans-[ReOCl3(PPh3)2] is used as the starting material the green colored suspension gradually changes to colorless, and the resin beads turn dark red indicative of the formation of [ReO(HL1(Lys))]2+. After being stirred at room temperature, the resin was washed with dimethylformamide and dichloromethane and cleaved off the resin with a 10% trifluoroacetic acid/dichloromethane mixture (Scheme 3).

Analysis of [ReO(HL1(Lys))]2+ complex by electrospray ionization mass spectrometry (ESI-MS) reveals the expected peaks. Analysis by 1H NMR shows the singlet expected peaks. Analysis by 1H NMR shows the singlet attributed to the aromatic proton on the pyridine ring (pyH2) shifts upon coordination to the metal center from δ 8.48 in H2L1(Lys) to 8.58 ppm in [ReO(HL1(Lys))]2+. The four downfield 13C(1H) NMR signals in [ReO(HL1(Lys))]2, 173.0 (CO2H), 169.0 (Nε-C), 165.4 (CONH), and 163.6 (pyC), were assigned using HSQC and HMBC techniques.

### Synthesis of Peptide-Conjugated Ligands

H2L1−4(cRGDFK) and H2L1−3(Tyr-Octreotate) are cyclic pentapeptide, cRGDK, was prepared using standard solid phase peptide synthesis techniques with Fmoc (Fmoc = 9-fluorenlymethoxycarbonyl) protected amino acids, using HATU/DIPEA coupling methodology on chlorotrityl resin. The Fmoc protecting groups were removed with 20% piperidine in dimethylformamide and dichloromethane and cleaved off the resin with a 10% trifluoroacetic acid/dichloromethane mixture (Scheme 3).
DMF on the solid phase, followed by cleavage from the resin and cyclization using a small modification of published procedures. The ligands, H$_2$L$^1$−$^3$, were conjugated to cRGDfK using standard peptide coupling conditions (HATU, DIPEA) to give H$_2$L$^1$−$^3$(cRGDfK) (Scheme 4). The new conjugates were purified by semipreparative RP-HPLC and characterized by electrospray mass spectrometry and analytical HPLC. The reaction for the ethyl-SHYNIC derivative, H$_2$L$^1$(cRGDfK), resulted in a higher isolated yield (80%) than H$_2$L$^2$(cRGDfK) (24%) and H$_2$L$^3$(cRGDfK) (55%). Analysis of aqueous solutions of H$_2$L$^1$−$^3$(cRGDfK) by RP-HPLC revealed no degradation over a 24 h.

The {ReO}$^{3+}$ complexes of H$_2$L$^1$−$^3$(cRGDfK) were prepared by adding [ReOCl$_4$]$^{-}$ in DMF at ambient temperature followed by adding [ReOCl$_4$]$^{-}$ in DMF at ambient temperature. Figure 2. (a) ORTEP representation of [ReO(HL$^1$(OMe))$_2$]$^{2+}$ (50% probability ellipsoids). The trifluoroacetate counterion and hydrogen atoms (except those bound to nitrogen) are omitted. (b) Representation of hydrogen bonded centrosymmetric dimer with the two remaining H-bond donors capped by water molecules.

Table 1. Selected Bond Lengths (Å) and Angles (deg) for the Rhenium Complex for [ReO(HL$^1$(OMe))$_2$]CF$_3$CO$_2$.$^a$

<table>
<thead>
<tr>
<th>Bond Lengths</th>
<th>Bond Angles</th>
<th>Torsion Angles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re−O(1) 1.679(3)</td>
<td>Re−N(1) 2.049(4)</td>
<td>N(4)−C(1)−N(1)−Re 169.5(3)</td>
</tr>
<tr>
<td>N(1)−C(1) 1.350(6)</td>
<td>Re−S(1) 2.3917(11)</td>
<td>N(8)−C(9)−N(5)−Re 174.4(4)</td>
</tr>
<tr>
<td>C(6)−N(3) 1.434(6)</td>
<td>Re−N(2) 2.2904(11)</td>
<td>N(10)−C(9)−N(5)−N(6) 175.1(3)</td>
</tr>
<tr>
<td>C(14)−N(7) 1.359(6)</td>
<td>N(5)−C(6) 1.387(6)</td>
<td>C(2)−C(1)−N(1)−N(2) 167.1(4)</td>
</tr>
</tbody>
</table>

$^a$Trifluoroacetate counterion bond lengths and angles are not provided.

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by isolation and purification using semipreparative RP-HPLC (Scheme 4). Analysis of the complexes by ESI-MS revealed the 2+ molecular ion peaks at \( m/z \) 926.343, 954.372, and 974.371 for \([\text{ReO(HL1(cRGDfK))}_2]^+\), \([\text{ReO(HL2(cRGDfK))}_2]^+\), and \([\text{ReO(HL3(cRGDfK))}_2]^+\) respectively.

An intramolecular disulfide bridge between the second and seventh cysteine residues in Tyr3-octreotate improves the metabolic stability of the peptide, and this disulfide is often introduced by oxidation of the linear octapeptide with 2,2′-dithiodipyridine. Unfortunately the bioconjugation of ligands \( \text{H}_2\text{L}^1 - 3 \) to Tyr3-octreotate was complicated by degradation of the pyridylthiocarbazide (SHYNIC) ligands (\( \text{H}_2\text{L}^1 - 3 \)) in the presence of the two cysteine thiol containing residues in the linear peptide, leading to loss of \( \text{H}_2\text{S} \) identified in the ESI-MS by a loss of 34 atomic mass units. This loss of \( \text{H}_2\text{S} \) results in the formation of a carbodiimide form of the SHYNIC ligands. The formation of carbodiimides from thioureas is well-known.65 This degradation and loss of sulfur were not observed for RGD-based conjugates, suggesting that in the case of the octreotate conjugates, thiocarbazide-thiol-disulfide interchange/scrambling promotes the loss of \( \text{H}_2\text{S} \) from the ligands (Scheme 5).

As conventional off-resin oxidative cyclization methodologies were inadequate for this synthesis, \( \text{H}_2\text{L}^1 - 3 \)-(Tyr3-Oct) was prepared entirely on solid support, where intramolecular oxidation/cyclization preceded bioconjugation of \( \text{H}_2\text{L}^1 - 3 \). The eight-residue peptide was synthesized by sequential addition of the amino acid residues via solid-phase peptide synthesis, using acetamidomethyl (Acm) protected cysteine residues followed by in situ Acm removal and simultaneous disulfide bond formation using thallium(III) trifluoroacetate.66 Following cyclization, the preactivated SHYNIC derivative (\( \text{H}_2\text{L}^1 - 3 \)) is

### Table 2. Summary of Crystal Data and Structure Refinement for \([\text{ReO(HL}^1(\text{OMe}))_2]\text{CF}_3\text{CO}_2^a\)

<table>
<thead>
<tr>
<th>data collection</th>
<th>compound details</th>
<th>data collection</th>
<th>compound details</th>
</tr>
</thead>
<tbody>
<tr>
<td>empirical formula</td>
<td>( \text{C}<em>{20}\text{H}</em>{26}\text{N}_8\text{O}_5\text{ReS}_2\cdot\text{H}_2\text{O}\cdot\text{CF}_3\text{CO}_2 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V (( \text{Å}^3 ))</td>
<td>1551.05(13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Z} )</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( D_{\text{calc}} ) (( \text{Mg m}^{-3} ))</td>
<td>1.797</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \mu ) (( \text{mm}^{-1} ))</td>
<td>9.599</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( F(000) )</td>
<td>828</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( T ) (K)</td>
<td>130.00(10)</td>
<td>reflections measured</td>
<td>10363</td>
</tr>
<tr>
<td>( \lambda ) (( \text{Å} ))</td>
<td>1.54184</td>
<td>independent reflections</td>
<td>5568 [( R_{\text{int}} = 0.0449 )]</td>
</tr>
<tr>
<td>( a ) (( \text{Å} ))</td>
<td>10.9154(4)</td>
<td>final R indices [( I &gt; 2\sigma(I) )]</td>
<td>( R_1 = 0.0329 )</td>
</tr>
<tr>
<td>( b ) (( \text{Å} ))</td>
<td>11.1201(6)</td>
<td>( wR(F^2) = 0.0794 )</td>
<td>( \mu(R) = 0.0398 )</td>
</tr>
<tr>
<td>( c ) (( \text{Å} ))</td>
<td>13.2651(7)</td>
<td>final R indices (all data)</td>
<td>( wR(F^2) = 0.0831 )</td>
</tr>
<tr>
<td>( \alpha ) (deg)</td>
<td>86.355(4)</td>
<td>goodness-of-fit on ( F^2 )</td>
<td>1.027</td>
</tr>
<tr>
<td>( \beta ) (deg)</td>
<td>74.965(4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \gamma ) (deg)</td>
<td>87.273(4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \)Crystals were grown from a concentrated solution of the complex in methanol.

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**Scheme 3. "On-Resin" Formation of \( \text{H}_2\text{L}^1 \)-(Lys) and the Corresponding Rhenium-oxo Complex, \([\text{ReO(HL}^1(\text{Lys}))_2]^+\)**

- 1. DiPEA/CH\(_2\)Cl\(_2\)
- 2. Piperidine/DMF
- HATU
- DiPEA
- \( \text{H}_2\text{L}^1 \)
- DMF, RT

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**Scheme 4.** Analysis of the complexes by ESI-MS revealed the 2+ molecular ion peaks at \( m/z \) 926.343, 954.372, and 974.371 for \([\text{ReO(HL}^1(\text{cRGDfK}))_2]^+\), \([\text{ReO(HL}^2(\text{cRGDfK}))_2]^+\), and \([\text{ReO(HL}^3(\text{cRGDfK}))_2]^+\) respectively.

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**Scheme 5.** This degradation and loss of sulfur were not observed for RGD-based conjugates, suggesting that in the case of the octreotate conjugates, thiocarbazide-thiol-disulfide interchange/scrambling promotes the loss of \( \text{H}_2\text{S} \) from the ligands.
reacted at the deprotected D-phenylalanine N-terminus. Cleavage and deprotection of remaining protecting groups are achieved by treatment with trifluoracetic acid (Scheme 6).

The rhenium complexes of \( \text{H}_2 \text{L}^{1-3} \text{(Tyr}^3 \text{-Oct}) \) could be prepared on-resin or in-solution by treatment with \([\text{tBu}_4\text{N}]^{-} [\text{ReOCl}_4]^\text{−}\) in methanol (Scheme 6). The on-resin approach is potentially of interest in producing radioactive complexes in high specific activity as unreacted \([\text{ReO}_4]^\text{−}\) and other impurities such as colloidal rhenium could be readily removed by filtration of the resin. The pure complex can be cleaved from the resin with 50% trifluoracetic acid and is stable to this relatively high concentration of acid. Analysis by HPLC and ESI-MS confirmed the identity of the complexes, with the \([\text{ReO}-(\text{HL}^{1-3})(\text{Tyr}^3\text{-Oct})]_2]^+\) complexes showing signals in the ESI-MS that could be attributed to the 3+ molecular ion with expected rhenium isotope peak patterns (Figure 3).

**Scheme 4. Synthesis of \( \text{H}_2 \text{L}^{1-3}(\text{cRGDfK}) \) and \([\text{ReO}(\text{HL}^{1-3}(\text{cRGDfK}))_2]^+\)**

**Scheme 5. Suggested Mechanism for the Formation of a Carbodiimide from \( \text{H}_2 \text{L}^{1-3}(\text{Tyr}^3\text{-Oct}) \) Resulting in Loss of \( \text{H}_2\text{S} \)**

Preparation of \([^{188}\text{ReO}(\text{HL}^1(\text{Tyr}^3\text{-Oct}))_2]^+\). Preliminary radiolabeling of \( \text{H}_2 \text{L}^1(\text{Tyr}^3\text{-Oct}) \) with radioactive \(^{188}\text{Re} \) was performed using generator-produced \([^{188}\text{ReO}_4]^\text{−}\). A solution of \([^{188}\text{ReO}_4]^\text{−}\), in an aqueous mixture of sodium chloride (0.9% w/v concentration) and sodium tartrate, was reduced with stannous chloride. This mixture was then reacted with \( \text{H}_2 \text{L}^1(\text{Tyr}^3\text{-Oct}) \) at 100 °C, leading to the formation of \([^{188}\text{ReO}(\text{HL}^1(\text{Tyr}^3\text{-Oct}))_2]^+\) in ca. 67% radiochemical yield.

The compound was characterized by analytical reversed phase HPLC (Figure 4), where \([^{188}\text{ReO}(\text{HL}^1(\text{Tyr}^3\text{-Oct}))_2]^+\) (retention time = 11.6 min, detected using a NaI(Tl)) elutes with a similar retention time to the nonradioactive analogue \([^{nat}\text{ReO}(\text{HL}^1(\text{Tyr}^3\text{-Oct}))_2]^+\) (retention time = 11.3 min, detected at \(\lambda_{220}\) ), where \(^{nat}\text{Re} \) refers to naturally abundant Re isotopes. The small difference in retention times is due to the different configurations of the radioactivity and UV detectors. This elution profile of \([\text{ReO}(\text{HL}^1(\text{Tyr}^3\text{-Oct}))_2]^+\) is distinct from that of the free ligand, \( \text{H}_2 \text{L}^1(\text{Tyr}^3\text{-Oct}) \) that elutes at 9.7 min under the same conditions. Unreacted \(^{188}\text{Re} \) species, presumably \([^{188}\text{ReO}_4]^\text{−}\), elute with the solvent front at 2.1 min (Figure 4).
Preparation of $^{99m}$Tc-Labeled Complexes, $[^{99m}$TcO(HL$^{1-3}$(cRGDfK))$_2]^+$ and $[^{99m}$TcO(HL$^{1-3}$(Tyr$^3$-Oct))$_2]^+$.

The technetium-99m complexes $[^{99m}$TcO(HL$^{1-3}$(cRGDfK))$_2]^+$ and $[^{99m}$TcO(HL$^{1-3}$(Tyr$^3$-Oct))$_2]^+$ were prepared in ca. 60—80% radiochemical yield using mild conditions and relatively simple procedures (Supporting Information, Figures S1 and S2).
radiolabeled 99mTc complexes were characterized by analysis (Table 3). The small difference in the retention times of the rhenium and technetium complexes strongly suggests that the complexes are isostructural (Table 3). The close correlation between the retention times of the rhenium and technetium complexes suggests that the complexes are different systems will exhibit quite different biodistribution in vivo. These new systems warrant further investigation as potential theranostic agents employing an imaging (99mTc) and therapeutic (186/188Re) matched pair for a single targeted agent.

**General Experimental.** All reagents were purchased from standard commercial sources. Nuclear magnetic resonance (NMR) spectra were acquired on either an Agilent 400-MR (1H NMR at 400 MHz and 13C{1H} NMR at 101 MHz) or a Varian FT-NMR 500 spectrometer (1H NMR at 500 MHz and 13C{1H} NMR at 126 MHz) at 298 K. Chemical shifts were referenced to residual solvent peaks and are quoted in ppm relative to TMS.

Fmoc-L-amino acids, Fmoc-D-amino acids, Nvoc-Cl, HATU, DIC, Wang resin, 2-chlorotriyl, Fmoc-Lys(ivDde)—OH, and Fmoc-Cys(Acm)—OH were purchased from standard commercial sources.

Linear protected RGDfK peptide (Arg(Pbf)-Gly(tBoc)-Asp(OrBu)-dPhe-Lys(tBoc)) was synthesized manually using standard Fmoc solid phase peptide synthesis (SPPS) procedures on the 2-chlorotriyl chloride resin. The linear pentapeptide was cleaved from the resin (with retention of protecting groups) using 1% TFA in dichloromethane and 1% DIPEA (6 equiv) at RT for 2 h, then evaporation to dryness.

Comparison of HPLC profiles suggests the rhenium and technetium complexes are isostructural. The complexes described in this manuscript have two ligands coordinated to a single metal ion. It is likely the two different systems will exhibit quite different biodistribution in vivo. These new systems warrant further investigation as potential theranostic agents employing an imaging (99mTc) and therapeutic (186/188Re) matched pair for a single targeted agent.

**General Experimental.** All reagents were purchased from standard commercial sources. Nuclear magnetic resonance (NMR) spectra were acquired on either an Agilent 400-MR (1H NMR at 400 MHz and 13C{1H} NMR at 101 MHz) or a Varian FT-NMR 500 spectrometer (1H NMR at 500 MHz and 13C{1H} NMR at 126 MHz) at 298 K. Chemical shifts were referenced to residual solvent peaks and are quoted in ppm relative to TMS.

Fmoc-L-amino acids, Fmoc-D-amino acids, Nvoc-Cl, HATU, DIC, Wang resin, 2-chlorotriyl, Fmoc-Lys(ivDde)—OH, and Fmoc-Cys(Acm)—OH were purchased from standard commercial sources.

Linear protected RGDfK peptide (Arg(Pbf)-Gly(tBoc)-Asp(OrBu)-dPhe-Lys(tBoc)) was synthesized manually using standard Fmoc solid phase peptide synthesis (SPPS) procedures on the 2-chlorotriyl chloride resin. The linear pentapeptide was cleaved from the resin (with retention of protecting groups) using 1% TFA in dichloromethane and 1% DIPEA (6 equiv) at RT for 2 h, then evaporation to dryness.

**Table 3. RP-HPLC Retention Times (min) for Ligands and [MO(HL)_2]^+ Complexes (M = Re, 99 mTc) L1 (ethyl), L2 (t-butyl), and L3 (phenyl) Compounds**

<table>
<thead>
<tr>
<th>Compound</th>
<th>H2L^−</th>
<th>technetium complex</th>
<th>rhenium complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1^−-cRGDfK</td>
<td>10.0</td>
<td>11.4</td>
<td>11.5</td>
</tr>
<tr>
<td>L2^−-cRGDfK</td>
<td>9.3</td>
<td>10.3</td>
<td>10.9</td>
</tr>
<tr>
<td>L3^−-cRGDfK</td>
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<td>12.0</td>
<td>12.7</td>
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<tr>
<td>L1^−-(Tyr^3-Oct)</td>
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<td>L2^−-(Tyr^3-Oct)</td>
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<tr>
<td>L3^−-(Tyr^3-Oct)</td>
<td>11.0</td>
<td>12.9</td>
<td>13.0</td>
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</tbody>
</table>

*Linear gradient from 0 to 90% Buffer B to A. Buffer A: 0.1% TFA in Milli-Q water. Buffer B: 0.1% TFA in CH3CN.*

**Concluding Remarks.** The new pyridylthiocarbazide ligands (SHYNIC, H3L1^−-3) described here offer a useful alternative to the standard HYNIC system. While HYNIC has proved a very successful and versatile bifunctional ligand for 99mTc coligands are required to complete the coordination sphere of the metal ion and extrapolation to radioactive rhenium isotopes has been challenging. This family of bidentate ligands form stable complexes with the [ReO]3^+ core with two ligands coordinated to a single metal ion. A rhenium complex with a methyl ester functional group has been characterized by X-ray crystallography and features the rhenium ion in a distorted square pyramidal environment with the oxo group in the apical position relative to the pseudo basal plane of two five-membered chelate rings with a N,N'/SS trans configuration about the Re-oxo core. The basic ligands have been decorated with the tumor targeting peptides cyclic-RGD and Tyr^3-Octreotate, and these conjugates form complexes with rhenium to give well-defined single species, [ReO(HL1^−-3)(cRGDfK)^2]_2^- and [ReO(HL1^−-3)(Tyr^3-Octreotate)^2]_2^−, without having to add coligands resulting in the formation of a single structural and geometrical isomer. It is possible to form the rhenium complexes using either standard solution chemistry or “on-resin”, and the latter approach may prove useful in isolating radioactive 188/186Re analogues in high specific activity. These complexes feature two targeting peptides separated by 14 chemical bonds, and there is evidence that molecules containing more than one targeting peptide, sometimes referred to as bivalent, can display enhanced receptor binding due to simultaneous binding to more than one receptor on the surface on any given cell. It is possible to prepare [188ReO(HL1^−-3)(Tyr^3-Oct)]_2^- in ~67% yield from generator produced [188ReO4^-], and improved yields should be possible by optimizing the reaction conditions. The analogous technetium complexes, [TcO((HL1^−-3)(cRGDfK))_2]_2^- and [TcO((HL1^−-3)(Tyr^3-Octreotate))_2]_2^−, were prepared directly from [99mTcO4^-] with tin chloride acting as a reducing agent. Comparison of HPLC profiles suggests the rhenium and technetium complexes are isostructural. The complexes described in this manuscript have two ligands coordinated to a single metal ion, whereas conventional HYNIC systems involve one HYNIC ligand binding to one metal ion. It is likely the two different systems will exhibit quite different biodistribution in vivo. These new systems warrant further investigation as potential theranostic agents employing an imaging (99mTc) and therapeutic (186/188Re) matched pair for a single targeted agent.
in vacuo. A solution of TFA (97.5%) and Milli-Q water (2.5%) was added to the crude material to deprotect the peptide, followed by removal of the trifluoroacetic acid by sparging with a stream of N₂. The peptide was precipitated with diethyl ether, isolated by centrifugation (3 min, 3600 rpm) and dissolved in Milli-Q water (5 mL), and finally purified by semiprep RP-HPLC (Column 1); Gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 40% B to A at 0.1% min⁻¹, UV detection at λ 220 nm with a flow rate of 5 mL min⁻¹.

Linear protected Tyroctreotate peptide, dPhe-Cys(Trt)-Thr(tBu)-Cys(Trt)-Thr- (0.1% TFA in CH₃CN) from 20 to 80% B, over 30 min and UV detection at λ 220 nm with a flow rate of 5 mL min⁻¹.

Analytical reversed phase high performance liquid chromatography (RP-HPLC) was undertaken using an Agilent 1100 Series HPLC system at a flow rate of 1 mL min⁻¹ with both Column 1: A Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm) or Column 2: A Phenomenex Aeras Peptide XB-C18 column (250 mm × 4.6 mm, 3.6 μm). Solvent gradient for analytical analysis were either using System A: Gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 100% B over 25 min and UV detection at λ 214, 254, and 280 nm, System B: Gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 60% B over 30 min and UV detection at λ 214, 220, 254, 280, and 350 nm or System E: Gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 20 to 80% B, over 30 min and UV detection at λ 220, 254, and 350 nm with a flow rate of 1 mL min⁻¹.

Semipreparative reversed phase high performance liquid chromatography (semiprep RP-HPLC) was performed using an Agilent 1200 series preparative HPLC unit with a variable wavelength detector. An automated Agilent 1200 fraction collector collected 0.5–4 mL fractions. Peak separation was achieved using either Column 3: Kinetic C18 100 Å, AXIA column (150 mm × 21.2 mm, 5 μm), Column 4: Phenomenex Synergi Hydro-RP 80 Å (50 mm × 21.2 mm, 4 μm), Column 5: Varian Pursuit XRs C18 100 Å (150 × 21.2 mm, 5 μm) or Column 6: SGE ProteCol C18 120 Å (250 mm × 10 mm, 5 μm). Gradient elution, flow rate, and wavelength detection are compound specific and are detailed under the Experimental Section of a particular compound. Each fraction collected above 400 mA was analyzed using ESI-MS and analytical HPLC.

Analytical HPLC traces of radiolabeled ¹⁸⁶Re compounds were acquired using an Agilent 1200 LC system with in-line UV and gamma detection (Flow-Count, LabLogic). Peak separation was achieved using an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm), with column 1 and system F: Gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 100% B over 20 min and UV detection at λ 220 nm.

Analytical HPLC traces of radiolabeled ⁹⁹ᵐTc compounds were acquired using a Shimadzu 10 AVP UV–visible spectrophotometer (Shimadzu, Kyoto, Japan) and a sodium iodide scintillation detector with two LC-10ATVP solvent delivery systems for solvents A and B. Peak separation was achieved using Column 7: Nacalai Tesque Cosmosol SC18-AR Waters column (4.6 × 150 mm, 5 μm) (Kyoto, Japan) at a flow rate of 1 mL min⁻¹. Gradient elution followed System C: Gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 100% B over 20 min and UV detection at λ 254 nm.

X-ray structure determination and refinement was obtained for [ReO(HL₂(OMe))₃]TFA on an Oxford Diffraction Supernova CCD diffractometer using Cu–Kα radiation, and the temperature during data collection was maintained at 130.0(1) using an Oxford Cryosystems cooling device. The structure was solved by direct methods using SHELXT and refined using least-squares methods using SHELXL.⁷³⁻⁷⁵ Thermal ellipsoid plots were generated using ORTEP-3 integrated within the WINGX suite of programs.⁷⁶ The trifluoroacetate counterion, although recognizable from the difference electron density maps, was badly disordered and could not be modeled satisfactorily. Application of the Squeeze procedure gave a void volume of 272 Å³ containing 127 electrons, consistent with the presence of two trifluoroacetate anions per unit cell.⁷⁷ The charge on the complex is unambiguously (+1) given the presence of the two pyridinium protons which are involved in intramolecular hydrogen bonds and the ethylamino protons which are also involved in hydrogen bonds. The crystallographic data has been deposited in the Cambridge Structural Database (CCDC 1543360).

**Ligand Synthesis.** Note: The designation of H₆L⁻¹⁻⁴ refers to the structure with a carboxylic acid-substituted, pyridylhydrazide with the different thiocarbahydrazide functional groups (Et, tBu, and Ph respectively). Further derivatization of the carboxylate functional group is represented by placing the substituted group at the carboxylic carbon in replacement of the OH group (e.g., H₆L⁻¹⁻³(OMe)), denotes the substitution of a methoxy at the carbonyl carbon to give a methyl ester.

6-[2-Ethylcarbamothioyl]hydrazinyl]-3-pyridinecarboxylic acid, H₆L¹⁻⁴. To a suspension of 6-hydrazino-3-pyridinecarboxylic acid (0.45 g, 2.9 mmol) in anhydrous ethanol (5 mL) was added ethyl isothiocyanate (0.51 mL, 5.8 mmol) under an atmosphere of nitrogen. The suspension was heated to reflux for 16 h then cooled to RT, and the precipitate that formed was collected by filtration, washed with cold ethanol (20 mL), and diethyl ether (20 mL) to give H₂L¹ as a colorless powder (0.67 g, 95%). ¹H NMR [DMSO-d₆, 500 MHz]: δ (ppm) 9.39 (1H, s, NH), 9.01 (1H, s, NH), 8.63 (1H, d, J = 1.7 Hz, pyH), 8.19 (1H, t, J = 4.8 Hz, NH), 8.04 (1H, dd, J = 8.8, 2.2 Hz, pyH'), 6.53 (1H, d, J = 8.8 Hz, pyH'), 3.45 (2H, dt, J = 13.3, 6.8 Hz, CH₂), 1.03 (3H, t, J = 7.1 Hz, CH₃), 13C[¹H] NMR [DMSO-d₆, 125.7 MHz]: δ (ppm) 181.5 (C, NCS), 166.5 (C, CO₂H), 162.0 (C, pyC), 150.4 (C, pyC), 138.8 (C, pyC), 117.8 (C, pyC'), 105.6 (C, pyC'), 38.4 (C, CH₂), 14.6 (C, CH₃); IR: νmax(cm⁻¹) 2981 (s/sh, N=H), 1605 (s/sh, C=C=O), 1539 (s/sh), 1319 (m/sh), 1282 (s/sh), 1245 (s/sh), 782 (s/sh); ESI-MS (+): [M + H⁺]⁺, 100%; RP-HPLC (Column 1, System A): Rₜ (min) 7.2.

6-[2-(tert-Butylcarbamothioyl)hydrazinyl]-3-pyridinecarboxylic acid, H₂L². To a suspension of 6-hydrazino-3-pyridinecarboxylic acid (0.45 g, 2.9 mmol) in anhydrous DMA (5 mL) was added tert-butyl isothiocyanate (0.56 mL, 4.4 mmol) under an atmosphere of nitrogen. The suspension was heated to 85 °C and after 30 min became a yellow mixture, which was heated at 85 °C for a further 3 h. The mixture was concentrated by evaporation under reduced pressure to a volume of approximately 1 mL and cold diethyl ether (15 mL) was added. The suspension was stirred vigorously overnight at RT. The precipitate was collected via filtration and washed with copious amounts of cold diethyl ether to afford an off-white
solid (0.68 g, 86%). 1H NMR [MeOH-d₄, 500 MHz]: δ (ppm) 8.73 (1H, d, J = 2.2 Hz, pyH?), 8.18 (1H, dd, J = 8.7, 2.2 Hz, pyH?), 6.76 (1H, dd, J = 8.7, 0.7 Hz, pyH?), 1.51 (9H, s), 13C{1H} NMR [MeOH-d₄, 125.7 MHz]: δ (ppm) 181.0 (C, NCS), 166.1 (C, CO₂H), 162.0 (C, pyC?), 138.3 (C, pyC?), 117.8 (C, pyC?), 105.6 (C, pyC?), 54.7 (C, -C(=CH₂)), 29.0 (3C, -C(=CH₂)); IR: νmax (cm⁻¹) 1604 (s/sh, C=O), 1533 (s/sh), 1280 (s/sh), 1252 (s/sh), 1133 (m/sh), 1002 (m/sh), 779 (s/sh); HRMS (ESI⁺): m/z = calc'd for C₁₁H₁₄NO₃S 269.1072, found 269.1110 ([M + H⁺]+, 100%); RP-HPLC (Column 1, System A): Rf (min) 10.6.

6-[(Phenylcarbamothioyl)hydroxazinyl]-3-pyridinecarboxylic acid, H₂L₃. To a suspension of 6-hydrazino-3-pyridinecarboxylic acid (0.45 g, 2.9 mmol) in anhydrous dimethylacetamide (DMA) (5 mL) was added phenyl isothiocyanate (0.59 mL, 4.4 mmol) under an atmosphere of nitrogen. The suspension was heated to 65 °C and after 5 min became a yellow solution, which was heated at 65 °C for a further 2 h. The mixture was concentrated by evaporation under reduced pressure to a volume of approximately 1 mL, and cold diethyl ether (15 mL) was added. A precipitate was collected by filtration and washed with copious amounts of cold diethyl ether to afford an off-white powder (0.81 g, 97%). 1H NMR [DMSO-d₆, 400 MHz]: δ (ppm) 9.88 (1H, br s, NH), 9.84 (1H, br s, NH), 9.22 (1H, br s, NH), 8.67 (1H, d, J = 1.9 Hz, pyH?), 8.07 (1H, d, J = 8.4 Hz, pyH?), 7.47 (2H, d, J = 7.2 Hz, ArH?), 7.30 (2H, t, J = 7.2 Hz, ArH?), 7.13 (1H, t, J = 7.2 Hz, ArH?), 6.66 (1H, d, J = 8.8 Hz, pyH?), 13C{1H} NMR [MeOH-d₄, 125.7 MHz]: δ (ppm) 181.3 (C, NCS), 166.4 (C, CO₂H), 161.7 (C, pyC?), 150.3 (C, pyC?), 139.2 (C, pyC?), 138.7 (C, ArC?), 127.9 (2C, ArC?), 125.6 (2C, ArC?), 124.9 (2C, ArC?), 117.8 (C, pyC?), 106.1 (C, pyC?), IR: νmax (cm⁻¹) 1607 (s/sh, C=O), 1596 (s/sh), 1537 (s/sh), 1280 (s/sh), 1254 (s/sh), 1140 (m/sh), 1019 (m/sh), 782 (s/sh); HRMS (ESI⁺): m/z = calc'd for C₁₃H₁₀N₄O₃S 289.0759, found 289.0749 ([M + H⁺]+, 100%); RP-HPLC (Column 1, System A): Rf (min) 9.9.

Methyl 6-Chloropyridine-3-carboxylate, Methyl 6-hydrazinyl-3-pyridinecarboxylate, H₂L₃. The compound was obtained by deoxygenated and dried CH₃OH (20 mL). Sulfuric acid (-0.5 mL) was added dropwise, and the reaction was heated to 50–60 °C for 3 h. The precipitate was collected by filtration as a gray powder (0.13 g, 63%). 1H NMR [CHCl₃-d₆, 500 MHz]: δ (ppm) 8.74 (1H, d, J = 1.7 Hz, pyH?), 8.16 (1H, dd, J = 8.7, 2.2 Hz, pyH?), 6.76 (1H, dd, J = 8.7, 0.3 Hz, pyH?), 3.86 (3H, s, CO₂CH₃), 1.47 (9H, s, (CH₃)₃), 13C{1H} NMR [MeOH-d₄, 125.7 MHz]: δ (ppm) 180.9 (C, NCS), 166.5 (C, CO₂H), 161.4 (C, pyC?), 150.6 (C, pyC?), 140.1 (C, pyC?), 119.8 (C, pyC?), 106.1 (C, pyC?), 53.8 (C, -OCH₃), 52.1 (C, -CH₂), 28.3 (C, -CH₂); IR: νmax (cm⁻¹) 1721 (s/sh, C=O), 1523 (m/sh), 1285 (s/sh), 1250 (s/sh), 1133 (m/sh), 775 (s/sh); ESI-MS (⁺): m/z = calc'd for C₁₁H₁₀N₄O₃S 283.0214, found 283.0240 ([M + H⁺]+, 100%); RP-HPLC (Column 1, System A): Rf (min) 12.9.
was centrifuged (3 min, 3600 rpm) and the supernatant was discarded. The resulting resin was washed with dichloromethane (3 mL), DMF (3 mL), dichloromethane (3 mL), then DMF (3 mL), and again with dichloromethane (3 mL). A positive TNBS assay (2,4,6-trinitrobenzenesulfonic acid) was used to indicate the presence of free lysine residues on the resin.

A solution of NaOrBoc-N-Fmoc-c-Lys (1.7 g, 3.6 mmol) and DIPEA (2.1 mL, 12 mmol) in dichloromethane (15 mL) was added to the resin, which was then shaken at ambient temperature for 2 h. The resin was filtered and the filtrate was discarded. The resulting resin was washed with dichloromethane (3 mL), DMF (3 mL), dichloromethane (3 mL), and finally diethyl ether (3 mL). After several hours of suction drying the resin was weighed and loading determined (0.77 mmol g⁻¹). To the dried resin-bound fBoc-Lys(Fmoc)–OH (0.51 g, 0.39 mmol) was added a DMF/piperidine (80:20 v/v) solution (20 mL), which was manually stirred. After 20 min, the supernatant was removed by filtration through a sintered frit, and the remaining resin was washed with DMF (3 mL). A positive TNBSA assay (2,4,6-trinitrobenzenesulfonyl acid in methanol (5% w/v)) was used to indicate deprotection of the Fmoc protected epsilon (ε) amine. A mixture of HATU (285 mg, 0.75 mmol), H₂L¹ (176 mg, 0.75 mmol), and DIPEA (261 μL, 1.5 mmol) in DMF (4 mL) was added to the resin and reacted for 4 h at ambient temperature. The liquid was drained and the resin was washed successively with dichloromethane (3 mL), then DMF (3 mL), and again with dichloromethane (3 mL). A TFA/H₂O/dichloromethane (18:2:80 v/v) solution was added to half the resin (approximately 0.19 mmol), and the mixture shaken for 1 h and then filtered. The filtrate was concentrated in vacuo and diethyl ether (40 mL) was added to form a suspension, which was centrifuged (3 min, 3600 rpm) and the supernatant was discarded. The crude material was dissolved in H₂O/CH₃CN (90:10 v/v), filtered (Millipore 0.45 μm porous syringe filter), and then purified by semipreparative RP-HPLC (Column 5).

The HPLC system involved a gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 100% B (flow rate of 8 mL min⁻¹) for 30 min. Fractions containing the desired compound were identified by HPLC-MS, and lyophilized to afford a colorless fluffy solid (0.19 g, 27%). ¹H NMR (MeOH-d₄, 600 MHz): δ (ppm) 8.48 (1H, d, J = 2.1 Hz, pyrH), 8.29 (1H, dd, J = 9.1, 2.2 Hz, pyrH), 7.05 (1H, d, J = 9.1 Hz, pyrH), 4.38 (1H, dd, J = 8.9, 5.2 Hz, aCH), 3.70 (3H, s, CO₂CH₃), 3.38 (2H, t, J = 7.2 Hz, εCH₂), 1.97 (3H, s, COCH₃), 1.87–1.84 (1H, m, βCH₂), 1.75–1.69 (1H, m, βCH₂), 1.65–1.61 (2H, m, δCH₂), 1.53 (9H, s, (CH₂)₃), 1.49–1.41 (2H, m, γCH₂); ¹³C{¹H} NMR (MeOH-d₄, 150.9 MHz): δ (ppm) 184.0 (C, NCS), 174.2 (C, -COCH₂), 173.4 (C, -COCH₂), 165.9 (C, CONH), 159.1 (C, pyC), 149.4 (C, pyC), 141.4 (C, pyC), 123.7 (C, pyC), 110.6 (C, pyC), 54.9 (C, -CH₂CH₂), 53.7 (C, αCH₂), 52.7 (C, -CO₂CH₂), 40.7 (C, εCH₂), 32.1 (C, βCH₂), 29.8 (C, δCH₂), 28.9 (3C, -C(CH₂)₃), 24.2 (C, -COCH₂), 22.3 (C, γCH₂); HRMS (ESI⁺): m/z calcd for [C₆H₁₂N₂O₂S]⁺ 453.2284, found 453.2288 [(M + H)⁺, 100%]; RP-HPLC (Column 2, System A): Rₜ (min) 12.5.

H₂L¹(cRGDK). To a solution of 6-[[2-ethylcarbamothioyl]-hydrazinyl]-3-pyridinecarboxylic acid, (H₂L¹) (16 mg, 66 μmol) in DMF (0.5 mL) was added HATU (25 mg, 66 μmol) and DIPEA (23 μL, 0.13 mmol). A solution of cRGDK (10 mg, 17 μmol) in DMF (0.3 mL) was added to the initial mixture and then shaken for 2 h at ambient temperature. Diethyl ether (40 mL) was added and the subsequent suspension centrifuged (3 min, 3600 rpm). The supernatant was discarded and the process repeated. The remaining solid was dissolved in a H₂O/CH₃CN solution (90:10 v/v), filtered (Millipore 0.45 μm porous syringe filter), and then purified by semipreparative RP-HPLC (Column 4). The HPLC system involved a gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 10% B to A, over 65 min (1.3 min⁻¹) and UV detection at λ 220, 254, 275, and 350 nm with a flow rate of 8 mL min⁻¹. Fractions containing the desired compound were identified by HPLC-MS, and lyophilized to afford a colorless solid (10 mg, 80%). ESI-MS (⁺): m/z calcd for [C₆H₁₂N₂O₂S⁺] 826.378, found 826.376, calcd for [C₆H₁₂N₂O₂S]⁺ 826.378, found 826.376; RP-HPLC (Column 2, System B): Rₜ (min) 11.3.

H₂L¹(cRGDK). The same procedure was used as for H₂L¹(cRGDK), except H₂L¹ was replaced with 6-[[2-tert-butylcarbamothioyl]hydrazinyl]-3-pyridinecarboxylic acid, (H₂L¹) (5.3 mg, 19 μmol) and other reagent quantities were adapted accordingly; HATU (6.5 mg, 17 μmol), DIPEA (45 μL, 0.13 mmol), and cRGDK (10 mg, 17 μmol). Semipreparative RP-HPLC purification (Column 4) involved a gradient elution of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 0 to 40% B to A, over 80 min (0.5% min⁻¹) and UV detection at λ 220, 254, 275, and 350 nm with a flow rate of 8 mL min⁻¹. Fractions containing the desired compound were identified by HPLC-MS, and lyophilized to afford a yellowish solid (3.4 mg, 24%). ESI-MS (⁺): m/z calcd for [C₆H₁₂N₂O₂S]⁺ 854.4096, found 854.4097, calcd for [C₆H₁₂N₂O₂S]⁺ 854.4097; RP-HPLC (Column 2, System B): Rₜ (min) 12.9.

H₂L¹(cRGDK). The same procedure was used for H₂L¹(cRGDK), except H₂L¹ was replaced with 6-[[2-phenylcarbamothioyl]hydrazinyl]-3-pyridinecarboxylic acid, (H₂L¹) (5.2 mg, 19 μmol) and other reagent quantities were adapted accordingly; HATU (6.8 mg, 18 μmol), DIPEA (45
µL, 0.13 mmol), and cRGDK (10 mg, 17 µmol). Semi-preparative RP-HPLC purification (Column 4) involved a gradient elution of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA in CH3CN) from 0% to 40% B to A, over 80 min (0.5% min⁻¹) and UV detection at λ 214, 220, and 254 nm with a flow rate of 8 mL min⁻¹. Fractions containing the desired compound were identified by HPLC-MS, consolidated and lyophilized to afford a pale yellow solid (56 mg, 18%). HRMS (⁺) m/z calc’d for [C62H75N14O13S3]⁺ 1319.4800, found 1319.4849, calc’d for [C62H75N14O13S3]⁺ 1319.4849. 

H₄L₁(Tyr³-Oct). The same procedure was used as for H₄L₁(Tyr³-Oct), except H₄L₁ was replaced with H₄L₂ (0.20 g, 0.75 mmol, 3 equiv), and all other reagents were used according to their reported equivalencies. The precipitate after lyophilization was dissolved in CH₃CN/H₂O (8 mL, 50:50 v/v) and filtered (Millipore 0.45 µm porosity syringe filter). The compound was purified by semi-preparative RP-HPLC (Column 5) with an isocratic step gradient system of Buffer A (0.1% TFA in H2O) and Buffer B (0.1% TFA in CH3CN). The elution method involved 28% B for 10 min then 32% B for 40 min (desired peak at 34 min) and UV detection at λ 214, 220, 254, 280, and 350 nm with a flow rate of 8 mL min⁻¹. Fractions containing the desired compound were identified by HPLC-MS, consolidated, and lyophilized to afford a fluffy, colorless compound (18 mg, 5.5%). HRMS (⁺) m/z calc’d for [C62H75N14O13S3]⁺ 1319.4800, found 1319.4849, calc’d for [C62H75N14O13S3]⁺ 1319.4849. 

H₄L₂(Tyr²-Oct). The same procedure was used as for H₄L₁(Tyr³-Oct). 

Synthesis of Rhenium Complexes. Note: Unless specified Re represents ‘rhenium with natural isotope abundance’.

[ReO(HL¹(Ome))₂Cl]. To H₄L₁(Ome) (90 mg, 0.35 mmol) and (Bu₄N)[ReOCl₄] (103 mg, 0.18 mmol) was added anhydrous MeOH (13 mL). The mixture immediately turned deep red and was stirred at room temperature for 4 h. The reaction mixture was filtered, diethyl ether (ca. 15 mL) was added, and the resulting precipitate was collected by filtration to give [ReO(HL¹(Ome))₂Cl] as a dark-red, microcrystalline solid (0.11 g, 84%). ¹H NMR [DMSO-d₆, 400 MHz]: δ (ppm) 8.63 (2H, s, pyH¹), 8.26 (2H, dd, J = 9.3, 1.8 Hz, pyH²), 7.87 (2H, d, J = 9.4 Hz, pyH¹), 7.43 (2H, br, m, NH), 3.89 (6H, s, OCH₃). 

Histidine and Cysteine Challenge Experiments. A 50-fold excess of histidine or cysteine was added to a solution of...
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[ReO(HL\textsuperscript{(OMe)})\textsubscript{3}])\textsuperscript{2+} (1 mg/L) in PBS Buffer (10 mM, pH 7.4, 4 mL) and the mixture was heated to 37 °C. At 2, 4, and 24 h after initiation of the experiment, 10 μL aliquots of the reaction mixture were diluted with 90 μL of Milli-Q water and analyzed using analytical HPLC methods. The HPLC traces showed little or no decomposition (<5%) of the rhenium complex at all the time-points.

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HRMS (ESI +) m/z calc’d for [ReC72H102N26O17S2]^+ 617.8968, found 617.9939; RP-HPLC (Column 2, System A): Rf (min) 11.0.

[ReO(HL2(cRGDK))]+: The same procedure was used as for [ReO(HL(cRGDK))]3+, except H2L1(cRGDK) was replaced with H2L2(cRGDK) (8.0 mg, 9.4 μmol) in MeOH (250 μL); (tBuN)2[ReOCl4] (2.7 mg, 4.7 μmol) in MeOH (500 μL). The compound was purified by semipreparative RP-HPLC purification (Column 4) involved gradient elution of BuOH (0.1% TFA in H2O) and BuOH (0.1% TFA in CH3CN) from 0 to 20% over 20 min (1% min−1), then 20 to 60% B to A over 40 min (0.5% min−1) and UV detection at 214, 220, 254, 280, and 350 nm with a flow rate of 8 mL min−1. Fractions containing the desired compound were identified by HPLC-MS, consolidated, and lyophilized to a white solid (2.0 mg, ∼42%, assuming [ReO(HL2(cRGDK))]3+CF3CO2). HRMS (ESI+) m/z calc’d for [ReC72H102N26O17S2]^+CF3CO2 636.5847, found 636.5837; RP-HPLC (Column 2, System A): Rf (min) 12.5.

[ReO(HL3(cRGDK))]+: The same procedure was used as for [ReO(HL(cRGDK))]3+, except H2L1(cRGDK) was replaced with H2L3(cRGDK) (4.0 mg, 4.6 μmol) in MeOH (200 μL), and other reagent quantities were adapted accordingly. (tBuN)2[ReOCl4] (1.3 mg, 2.3 μmol) in MeOH (200 μL). The HPLC system (Column 6) involved a linear gradient elution of BuOH (0.1% TFA in H2O) and BuOH (0.1% TFA in CH3CN) from 0 to 20% over 20 min (1% min−1), then 20 to 60% B to A over 40 min (0.5% min−1) and UV detection at 214, 220, 254, 280, and 350 nm with a flow rate of 8 mL min−1. Fractions containing the desired compound were identified by HPLC-MS, consolidated, and lyophilized to afford a white solid (2.0 mg, ∼42%, assuming [ReO(HL3(cRGDK))]3+CF3CO2). HRMS (ESI+) m/z calc’d for [ReC80H102N26O17S2]^+ 933.6548, found 933.6548; RP-HPLC (Column 2, System A): Rf (min) 16.2.

[ReO(HL3(Tyr3-Oct))]+: Preloaded, resin-bound (Wang) ligand, H2L1(Tyr3-Oct) (approximately 6.0 μmol), was swelled in CH2Cl2 and drained twice. Anhydrous DMF (2 × 10 mL) was added to the resin the mixture was stirred and drained. DMF (1 mL) was added to the washed resin and (tBuN)2[ReOCl4] (2.1 mg, 2.9 μmol) in DMF (50 μL) was added to the resin. The resin mixture was reacted for 2 h at RT. The solution was drained and the resin was washed with copious amounts of DMF (5 × 10 mL), CH2Cl2 (3 × 10 mL), and diethyl ether (10 mL). The crude material was cleaved off the resin by addition of TFA/CH2Cl2/TIPS/H2O (50:46:1.3 v/v/v) (20 mL). The mixture was shaken for 1.5 h and filtered and the filtrate sparged with a stream of N2 until the red mixture had reduced to approximately 0.5 mL. Diethyl ether (2 × 10 mL) was added to the crude material, and the resulting precipitate was collected via centrifugation (3 min, 3600 rpm). The compound was purified by semipreparative RP-HPLC purification (Column 6) by gradient elution of BuOH (0.1% TFA in H2O) and BuOH (0.1% TFA in CH3CN) from 10 to 80% B to A, over 70 min (1.0% min−1) and UV detection at λ 214, 220, and 254 nm, with a flow rate of 4 mL min−1. Fractions containing the desired compound were consolidated and lyophilized to afford a red, fluffy material (5.0 mg, ∼58%, assuming [ReO(HL3(Tyr3-Oct))2]CF3CO2). ESI-MS (+) m/z calc’d for [ReC76H110N26O17S2]3+ 1372.0024, found 1372.0024; RP-HPLC (Column 2, System A): Rf (min) 16.2.
mg mL\(^{-1}\) in a separate evacuated vial, and a 0.5 mL aliquot was taken from both solutions and mixed together. The solution was added to [\(^{99m}\)TcO\(_4\)]\(^-\) (0.1 mL in 0.9% saline, 108 MBq). The conjugated peptide, H\(_2\)L\(^{1-3}\)(cRGDK) or H\(_2\)L\(^{1-3}\)(Tyr\(^3\)-Oct), was dissolved in degassed Milli-Q water (1 mg mL\(^{-1}\)), and 100 \(\mu\)L of this mixture was added to the techiuon solution. The sample was neutralized with NaHCO\(_3\) (pH 6.5, approximately 55 \(\mu\)L) then filtered or allowed to react without neutralizing at ambient temperature for 30 to 120 min. The samples were centrifuged with a Heraeus Labofuge 6000 centrifuge at 5000 rpm (Heraeus, Hanau, Germany). Radiochemical yields were evaluated by reverse-phase high-performance liquid chromatography (Column 7, System C). Stability Studies in Human Serum. Human blood samples were centrifuged with a Heraeus Labofuge 6000 centrifuge at 3000g for 10 min (Heraeus, Hanau, Germany). Radioactivity readings for stability studies were taken with a Capintec CRC-35R dose calibrator (Capintec, New Jersey, USA) and were measured in MBq. Centrifugation of radioactive compounds was undertaken using an Eppendorf 5415 D \(\gamma\) counter, which measured the radioactive decay of each sample in counts per minute (cpm).

For stability studies, blood from a healthy male (20 mL) was centrifuged (10 min, 3000 rpm) to separate blood plasma and red blood cells. The plasma was transferred to a separate vial and the red blood cells were discarded. An aliquot of plasma (0.6 mL) was added to labeled compound, [\(^{99m}\)Tc(HL\(_2\)(Tyr\(^3\)-Oct))\(_2\)]\(^+\) (0.15 mL), the radioactivity was monitored and the mixture was then incubated at 37 °C. Aliquots (0.1 mL) of the mixture were removed from heating at 10 min and after 2 h. Acetonitrile (0.1 mL) was added to the serum/tracer mix to precipitate serum proteins. The suspension was shaken for 5 min and then centrifuged (5 min, 13 200 rpm). The radioactivity of the supernatant and pellet was recorded. The supernatant (20 \(\mu\)L) was analyzed by analytical RP-HPLC (Column 7, System C) for UV and radioactivity analysis and the pellet was washed with acetonitrile (3 \(\times\) 0.1 mL), and radioactivity levels were again recorded (radioactivity levels of the pellet were negligible).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.7b01247.

Representative RP-HPLC chromatograms (PDF)

**Accession Codes**

CCDC 1543360 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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