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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 pyridylthiosemicarbazide 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}3+ 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 \( \left( t_{1/2} = 6.01 \text{ h}, E_{\text{max}} = 141 \text{ keV} \right) \). 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. 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 isotopes of technetium that are of potential use in targeted radiotherapeutics, rhenium-186 \( \left( t_{1/2} = 89.3 \text{ h}, E_{\text{max}} = 1.07 \text{ MeV} \right) \) and rhenium-188 \( \left( t_{1/2} = 16.9 \text{ h}, E_{\text{max}} = 2.12 \text{ MeV} \right) \). The similar coordination chemistry of technetium and rhenium offers the possibility of using their radioisotopes as an imaging \( \left( 99m\text{Tc} \right) \) and therapeutic \( \left( 186/188\text{Re} \right) \) matched pair using a single targeted ligand to form essentially isostructural complexes.

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 \( \alpha_v\beta_3 \) 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. 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.

Tumor targeting technetium based imaging agents can be prepared using 6-hydrazinonicotinamide (HYNIC) derivatives conjugated to targeting molecules as ligands to form...
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 $\text{Re}^{5+}$ cores while retaining the bioconjugation possibilities well established for HYNIC.44,45 A preliminary communication reported the structural characterization of a $\text{Re}^{5}$-oxo complex featuring two pyridylphenylthiocarbazide (SHYNIC) ligands (Figure 1).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 $\{\text{M}O\}^{3+}$ 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 $\text{HL}_{1}{L}^{4}$, and Their Ester Derivatives, $\text{HL}_{1}{L}^{2}$(OMe) and $\{\text{ReO\}}^{3+}$ Complexes. Synthesis of 6-hydrazinonicotinic acid (HYNIC), 2, required treatment of 6-chloronicotinic acid (1) with aqueous hydrazine.17 Ligands $\text{HL}_{1}{L}^{2}$ to $\text{HL}_{4}$ were prepared by reaction of either the ethyl, tert-butyl, phenyl, or nitrophenyl isothiocyanate with 6-hydrazinonicotinic acid (1) in anhydrous $\text{N},\text{N}$-dimethylacetamide (DMA) (Scheme 1).

The rhenium complexes of the methyl ester derivatives of $\text{HL}_{1}{L}^{1–3}$ complexes, $\{\text{ReO}(\text{HL}_{1}{L}^{1–3}(\text{OMe})_{2})\}_{2}^{+}$, can be prepared by reaction of the either trans-$\{\text{ReOCl}_{2}(\text{PPh}_{3})_{2}\}_{2}$ or $[\text{Bu}_{3}\text{N}]_{2}\{\text{ReOCl}_{4}\}_{2}$ with the two equivalents of ligand (Scheme 2). The IR spectra for the three complexes, $\{\text{ReO}(\text{HL}_{1}{L}^{1–3}(\text{OMe})_{2})\}_{2}^{+}$, display medium intensity bands at $\nu 960–963$ cm$^{-1}$ characteristic of Re=O stretches.48 Bands, which occur between $\nu 1553$ 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 $^1\text{H}$ 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 pyridyl CH protons for $\{\text{ReO}(\text{HL}_{1}{L}^{1}(\text{OMe})_{2})\}_{2}^{+}$, 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 gives rise to singlets at $\delta 3.89$ (DMSO-$d_{6}$), 3.86 ($\text{CHCl}_{3}$-$d$), and 3.92 (DMSO-$d_{6}$) for complexes $\{\text{ReO}(\text{HL}_{1}{L}^{1–3}(\text{OMe})_{2})\}_{2}^{+}$ respectively. The $\{\text{ReO}(\text{HL}_{1}{L}^{1}(\text{OMe})_{2})_{2}^{+}$ 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 $\{\text{ReO}(\text{HL}_{1}{L}^{1}(\text{OMe})_{2})_{2}\}_{2}\text{CF}_{3}\text{CO}_{2}$ 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, $P\text{i}$, and the rhenium ion is in a distorted square pyramidal environment with the o xo group in the apical position relative to the pseudo basal plane of two five-membered chelate rings. Each thiocarbacontadazide functional group is doubly deprotonated and serves as a

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$^{5}$-oxo complex featuring two SHYNIC ligands.44

Inorganic Chemistry
of multiple bond character. The Re−S bond distances in rhenium complexes with thiosemicarbazonato ligands were assigned using HSQC and HMBC techniques. The selective formation of the \( \text{Re}^{V}\)-monooxo complexes and consistent bonding effects a strong “trans effect”, although steric requirements may also play some role. Protonation of the pyridyl nitrogen atom in each ligand results in the hydrogen bonded centrosymmetric dimer (Figure 2b) with the two overall monocationic complex. The two pyridinium protons remaining 

### Scheme 1. Synthesis of Ligands \( \text{H}_2\text{L}^1−\text{H}_2\text{L}^3 \) and Their Methyl Ester Derivatives \( \text{H}_2\text{L}^1(\text{OMe})−\text{H}_2\text{L}^3(\text{OMe}) \)

![Scheme 1](image1)

### Scheme 2. Synthesis of Rhenium Complexes \([\text{ReO}(\text{HL}^{1−3})(\text{OMe}))_2]^+\)

![Scheme 2](image2)

The potential of the substituted pyridylthiosemicarbazide (SHYNIC) ligands \( \text{H}_2\text{L}^1−\text{L}^3 \) to be modified with amino acids using standard solid phase peptide synthesis techniques was first accomplished by attaching \( \text{i-Lys} \) to \( \text{H}_2\text{L}^1 \) to give \( \text{H}_2\text{L}^1(\text{Lys}) \). The doubly N-protected lysine derivative, \( \text{N}^\alpha\text{Fmoc}-\text{i-Lys} \), was immobilized on chlorotrityl resin, and the \( \text{N}^\alpha\text{Fmoc} \) group was removed by treatment with piperidine. The ligand, \( \text{H}_2\text{L}^1 \), was added to the resin in a mixture of DMF followed by the coupling agent HATU (HATU = \text{1-H-[1,2,3-triazolo[4,5-b]-pyridinium-3-oxido}]) hexafluorophosphate) in the presence of \( \text{N,N-dio} \)

### Scheme 3. Analysis of \([\text{ReO}(\text{HL}^{1−3})(\text{OMe}))_2]^+ \) complex by electrospray ionization mass spectrometry (ESI-MS) reveals the expected peaks. Analysis by \( ^1\text{H} \) NMR shows the singlet attributed to the aromatic proton on the pyridine ring (py\( ^H \)) shifts upon coordination to the metal center from \( \delta \) 8.48 in \( \text{H}_2\text{L}^1(\text{Lys}) \) to 8.58 ppm in \([\text{ReO}(\text{HL}^{1−3})(\text{OMe}))_2]^+ \). The four downfield \( ^1\text{C} \) (\( ^1\text{H} \)) NMR signals in \([\text{ReO}(\text{HL}^{1−3})(\text{OMe}))_2]^+ \) are used as HSQC and HMBC techniques.

### Synthesis of Peptide-Conjugated Ligands, \( \text{H}_2\text{L}^1−\text{L}^3(\text{cRGDFK})_n \) and \( \text{ReO}(\text{HL}^{1−3}(\text{cRGDFK}))_n \)

The ligand, \( \text{H}_2\text{L}^1(\text{Lys}) \), was immobilized on chlorotrityl resin, and the \( \text{N}^\alpha\text{Fmoc} \) group was removed by treatment with piperidine. The ligand, \( \text{H}_2\text{L}^1 \), was added to the resin in a mixture of DMF followed by the coupling agent HATU (HATU = \text{1-H-[1,2,3-triazolo[4,5-b]-pyridinium-3-oxido}]) hexafluorophosphate) in the presence of \( \text{N,N-di} \)

### Inorganic Chemistry

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DMF on the solid phase, followed by cleavage from the resin and cyclization using a small modification of published procedures. The ligands, H₂L₁−₃, were conjugated to cRGDFK using standard peptide coupling conditions (HATU, DIPEA) to give H₂L₁−₃(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, H₂L₁(cRGDfK), resulted in a higher isolated yield (80%) than H₂L₂(cRGDfK) (24%) and H₂L₃(cRGDfK) (55%). Analysis of aqueous solutions of H₂L₁−₃(cRGDfK) by RP-HPLC revealed no degradation over a 24 h.

The {ReO}₃⁺ complexes of H₂L₁−₃(cRGDfK) were prepared by adding [ReOCl₄]⁻ in DMF at ambient temperature followed by cleavage from the resin and cyclization using a small modification of published procedures. The ligands, H₂L₁−₃, were conjugated to cRGDFK using standard peptide coupling conditions (HATU, DIPEA) to give H₂L₁−₃(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, H₂L₁(cRGDfK), resulted in a higher isolated yield (80%) than H₂L₂(cRGDfK) (24%) and H₂L₃(cRGDfK) (55%). Analysis of aqueous solutions of H₂L₁−₃(cRGDfK) by RP-HPLC revealed no degradation over a 24 h.

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**Table 1. Selected Bond Lengths (Å) and Angles (deg) for the Rhenium Complex for [ReO(HL₁(OMe))₂]CF₃CO₂⁻**

<table>
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<tr>
<th>Bond Lengths</th>
<th>Bond Angles</th>
<th>Torsion Angles</th>
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<tr>
<td>Re−O(1)</td>
<td>1.679(3)</td>
<td>Re−N(1)</td>
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<td>N(1)—C(1)</td>
<td>1.350(6)</td>
<td>Re−N(1)</td>
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<td>C(6)—N(3)</td>
<td>1.343(6)</td>
<td>Re−N(5)</td>
</tr>
<tr>
<td>C(14)—N(7)</td>
<td>1.359(6)</td>
<td>N(5)—C(9)</td>
</tr>
<tr>
<td>N(5)—C(9)</td>
<td>1.356(6)</td>
<td>C(6)—S(1)</td>
</tr>
<tr>
<td>Bond Angles</td>
<td>O(1)—Re—S(1)</td>
<td>115.44(11)</td>
</tr>
<tr>
<td>N(1)—Re—S(2)</td>
<td>116.75(11)</td>
<td>N(5)—Re—S(2)</td>
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<tr>
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<tr>
<td>O(1)—Re—N(5)</td>
<td>102.61(15)</td>
<td>N(1)—Re—S(2)</td>
</tr>
<tr>
<td>N(1)—Re—N(5)</td>
<td>155.64(15)</td>
<td>C(1)—N(1)—Re</td>
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<td>S(1)—Re—S(2)</td>
<td>127.80(4)</td>
<td>C(9)—N(5)—Re</td>
</tr>
<tr>
<td>Torsion Angles</td>
<td>N(4)—C(1)—N(1)—Re</td>
<td>169.5(3)</td>
</tr>
<tr>
<td>N(8)—C(9)—N(5)—Re</td>
<td>173.3(3)</td>
<td>N(2)—C(6)—N(3)—C(7)</td>
</tr>
<tr>
<td>N(4)—C(1)—N(1)—N(2)</td>
<td>−10.5(6)</td>
<td>N(2)—C(6)—S(1)—Re</td>
</tr>
<tr>
<td>N(8)—C(9)—N(5)—N(6)</td>
<td>−7.8(6)</td>
<td>N(6)—C(14)—S(2)—Re</td>
</tr>
<tr>
<td>C(2)—C(1)—N(1)—N(2)</td>
<td>167.1(4)</td>
<td>N(3)—C(6)—S(1)—Re</td>
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<tr>
<td>C(10)—C(9)—N(5)—N(6)</td>
<td>168.4(4)</td>
<td>N(7)—C(14)—S(2)—Re</td>
</tr>
</tbody>
</table>

**Figure 2.** (a) ORTEP representation of [ReO(HL₁(OMe))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.
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 \([\text{ReO}(\text{HL}^1(\text{cRGDFK}))_2]^+\), \([\text{ReO}(\text{HL}^2(\text{cRGDFK}))_2]^+\), and \([\text{ReO}(\text{HL}^3(\text{cRGDFK}))_2]^+\) 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 \(\text{HL}^1\) to Tyr3-octreotate was complicated by degradation of the pyridylthiocarbazide (SHYNIC) ligands (HL1L1), respectively. The loss of H2S 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 H2S from the ligands (Scheme 5).

As conventional off-resin oxidative cyclization methodologies were inadequate for this synthesis, \(\text{HL}^1\) (Tyr3-Oct) was prepared entirely on solid support, where intramolecular oxidation/cyclization preceded bioconjugation of \(\text{HL}^1\). 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 (HL1L1) was

<table>
<thead>
<tr>
<th>Table 2. Summary of Crystal Data and Structure Refinement for ([\text{ReO}(\text{HL}^1(\text{OMe}))_2]\text{CF}_3\text{CO}_2]^+)</th>
</tr>
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<tbody>
<tr>
<td><strong>Data Collection</strong></td>
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<td>-----------------------------------------------</td>
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<tr>
<td><strong>data collection</strong></td>
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<td><strong>Z</strong></td>
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<tr>
<td><strong>V (Å(^3))</strong></td>
</tr>
<tr>
<td><strong>D(_{calc.}) (Mg m(^{-3}))</strong></td>
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<td><strong>(000)</strong></td>
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*Crystals were grown from a concentrated solution of the complex in methanol.
reacted at the deprotected d-phenylalnine N-terminus. Cleavage and deprotection of remaining protecting groups are achieved by treatment with trifluoracetic acid (Scheme 6). The rhenium complexes of \( \text{H}_2\text{L}_1^{3-} \text{(Tyr3-Oct)} \) could be prepared on-resin or in-solution by treatment with \([\text{Bu}_4\text{N}]^{-}\text{[ReOCl}_4]^{-}\) in methanol (Scheme 6). The on-resin approach is potentially of interest in producing radioactive complexes in high specific activity as unreacted \([\text{ReO}_4]^{-}\) 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 \([\text{ReO-HL}_1^{3-}(\text{Tyr3-Oct}))_2]^+\) complexes showing signals in the ESI-MS that could be attributed to the 3+ molecular ion with expected rhenium isotope peak patterns (Figure 3).

Preparation of \([^{188}\text{ReO-HL}_1^{3-}(\text{Tyr3-Oct}))_2]^+\). Preliminary radiolabeling of \( \text{H}_2\text{L}_1^{3-}(\text{Tyr3-Oct}) \) with radioactive \(^{188}\text{Re} \) was performed using generator-produced \([^{188}\text{ReO}_4]^{-}\). A solution of \([^{188}\text{ReO}_4]^{-}\), 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 \( \text{H}_2\text{L}_1^{3-}(\text{Tyr3-Oct}) \) at 100 °C, leading to the formation of \([^{188}\text{ReO-HL}_1^{3-}(\text{Tyr3-Oct}))_2]^+\) in ca. 67% radiochemical yield. The compound was characterized by analytical reversed phased HPLC (Figure 4), where \([^{188}\text{ReO-HL}_1^{3-}(\text{Tyr3-Oct}))_2]^+\) (retention time = 11.6 min, detected using a NaI(Tl)) elutes with a similar retention time to the nonradioactive analogue \([\text{natReO-HL}_1^{3-}(\text{Tyr3-Oct}))_2]^+\) (retention time = 11.3 min, detected at \( \lambda_{220} \)), where \( \text{natRe} \) 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 \([\text{ReO-HL}_1^{3-}(\text{Tyr3-Oct}))_2]^+\) is distinct from that of the free ligand, \( \text{H}_2\text{L}_1^{3-}(\text{Tyr3-Oct}) \) that elutes at 9.7 min under the same conditions. Unreacted \(^{188}\text{Re} \) species, presumably \([^{188}\text{ReO}_4]^{-}\), 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).

Inorganic Chemistry

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times between the traces for $^{99m}$Tc and Re complexes is, in part, due to the detector configurations but could also reflect the difference in polarity between the oxorhenium(V) and oxotechnetium(V) cores.

The stability of $[^{99m}$TcO(HL$^3$(Tyr$^3$-Oct))$_2]$ was assessed by incubation in human plasma at 37 °C. The complex was stable for at least 2 h with only small amounts of degradation products (<5%) evident that, based on their retention times in analytical HPLC, are most likely due to degradation of the peptide.

## CONCLUDING REMARKS

The new pyridylthiocarbazide ligands (SHYNIC, H$_2$L$^1$-3) described here offer a useful alternative to the standard HYNIC system. While HYNIC has proved a very successful and versatile bifunctional ligand for $^{99m}$Tc coligands are required to complete the coordination sphere of the metal ion and extrapolation to radioative 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 o xo group in the apical position relative to the pseudo basal plane of two five-membered chelate rings with a N,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((HL$^1$-3)(cRGDfK))$_2$] and [ReO((HL$^1$-3)(Tyr$^3$-octreotate))$_2$], 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 $^{188/186}$Re 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 $[^{188}$ReO(HL$^3$(Tyr$^3$-Oct))]$_2^-$ in ~67% yield from generator produced $[^{188}$ReO$_2^-$], and improved yields should be possible by optimizing the reaction conditions. The analogous technetium complexes, [TcO((HL$^1$-3)(cRGDfK))]$_2$ and [TcO((HL$^1$-3)(Tyr$^3$-octreotate))$_2$], were prepared directly from $[^{99m}$TcO$_2^-$] with tin chloride acting as a reducing agent. Comparison of HPLC profiles suggests the rhenium and technetium complexes are isostuctural. 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 $^{99m}$Tc and therapeutic $^{188/186}$Re 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-amin acids, Fmoc-D-amin acids, Nvoc-Cl, HATU, DIC, Wang resin, 2-chlorotrityl, Fmoc-Lys(iVdDe)−OH, and Fmoc-Cys(Ac)m−OH were purchased from standard commercial sources.

Linear protected RGDfK peptide (Arg(Pbf)-Gly(tBoc)-Asp(OtBu)-dPhe-Lys(tBoc)) was synthesized manually using standard Fmoc solid phase peptide synthesis (SPPS) procedures on the 2-chlorotrityl chloride resin. The linear pentapeptide was cleaved from the resin (with retention of protecting groups) using 1% TFA in dichloromethane and shaking for 40 min. The mixture was filtered and the filtrate was reduced in volume to afford crude linear product. Cyclization involved reacting the crude material in a mixture of dichloromethane (1 mg mL$^{-1}$), HATU (0.9 equiv), and DIPEA (6 equiv) at RT for 2 h, then evaporation to dryness for at least 2 h with only small amounts of degradation products (<5%) evident that, based on their retention times in analytical HPLC, are most likely due to degradation of the peptide.

### Table 3. RP-HPLC Retention Times (min) for Ligands and [MO(HL)$_3$] Complexes (M = Re, $^{99m}$Tc) Complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>H$_2$L$^1$</th>
<th>rhenium complex</th>
<th>technetium complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>L$^1$-cRGDK</td>
<td>10.0</td>
<td>11.4</td>
<td>11.5</td>
</tr>
<tr>
<td>L$^2$-cRGDK</td>
<td>9.3</td>
<td>10.3</td>
<td>10.9</td>
</tr>
<tr>
<td>L$^3$-cRGDK</td>
<td>10.9</td>
<td>12.0</td>
<td>12.7</td>
</tr>
<tr>
<td>L$^1$-(Tyr$^3$-Oct)</td>
<td>10.1</td>
<td>11.4</td>
<td>11.9</td>
</tr>
<tr>
<td>L$^2$-(Tyr$^3$-Oct)</td>
<td>10.9</td>
<td>11.7</td>
<td>12.1</td>
</tr>
<tr>
<td>L$^3$-(Tyr$^3$-Oct)</td>
<td>11.0</td>
<td>12.9</td>
<td>13.0</td>
</tr>
</tbody>
</table>

$^a$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 CH$_3$CN.

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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 semi-prep 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, over 40 min (1.0% min⁻¹), UV detection at λ 220 nm with a flow rate of 5 mL min⁻¹.

Linear protected Tyr-octreotide peptide, dPhe-Cys(Trt)-Tyr(tBu)-dTrp(tBoc)-Lys(tBoc)-Thr(tBu)-Cys(Trt)-Thr-(tBu)-OH, was synthesized using standard automated Fmoc SPPS procedures on a 2-chlorotrityl chloride or Wang resin unless otherwise specified.

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 either Column 1: A Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5.0 μm) or Column 2: A Phenomenex Aerus Peptide XB-C18 column (250 mm × 4.6 mm, 3.6 μm). Solvent gradients for analytical analyses 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 variable wavelength detector. An automated Agilent 1200 fraction collector collected 0.5–4 mL fractions. Peak separation was achieved using either Column 3: Kinetex 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 Puris XR 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 mAU was analyzed using ESI-MS and analytical HPLC.

Analytical HPLC traces of radiolabeled ⁹⁹ᵐTc 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 ¹⁸⁸Re 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(HL1(OMe))₂]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 SHELX.5,7 Thermal ellipsoid plots were generated using ORTEP-3 integrated within the WINGX suite of programs.6 The trifluoroacetic 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 ethalamino 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 

H₃L¹⁻⁴ refers to the structure with a carboxylic acid-substituted, pyridylhydrazine with the different thioacarbodiaziridine functional groups (Et, tBu, and Ph respectively). Further derivatization of the carboxylic functional group is represented by placing the substituted group at the carboxylic carbon in replacement of the OH group (e.g., H₃L²⁻⁴(OMe), denotes the substitution of a methoxy at the carboxyl carbon to give a methyl ester.

6-[2-Ethylcarbamothioyl]hydrazinyl]-3-pyridinecarboxylic acid, H₃L¹. 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 at 85 °C for 3 h. A suspension of 6-hydrazino-3-pyridinecarboxylic acid, H₂, was added. The suspension was stirred vigorously overnight at 85 °C and filtered, washed with cold ethanol (20 mL), and diethyl ether (20 mL) to give H₃L¹ 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, pyF₂), 8.19 (1H, t, j = 4.8 Hz, NH), 8.04 (1H, dd, j = 8.2, 2.2 Hz, pyF₂), 6.53 (1H, d, j = 8.8 Hz, pyF₂), 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, pyC₁), 150.4 (C, pyC₁), 138.8 (C, pyC₁), 117.8 (C, pyC₁), 105.6 (C, pyC₁), 38.4 (C, CH₂), 146.4 (C, CH₃); IR: νmax (cm⁻¹) 2981 (s/sh, N–H), 1605 (s/sh, C=O), 1539 (s/sh), 1319 (m/sh), 1282 (s/sh), 1245 (s/sh), 782 (s/sh); ESI-MS (*): m/z calcd for C₄H₄N₂O₂S 241.0749, found 241.0817 {[M + H]⁺, 100%}; RP-HPLC (Column 1, System A): Rₚ (min) 7.2.

6-[(2-Tert-Butylcarbamothioyl)hydrazinyl]-3-pyridinecarboxylic acid, H₃L². 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 [H] NMR [MeOH-d₄, 125.7 MHz]: δ (ppm) 181.0 (C, NCS), 166.1 (C, CO₂H), 162.0 (C, pyC), 150.4 (C, pyC), 138.8 (C, pyC), 117.8 (C, pyC), 105.6 (C, pyC), 54.7 (C, C-C(CH₃)₂), 29.0 (3C, C-C(CH₃)₂); IR: vₘₐₓ (em⁻¹) 1604 (s), 1280 (s), 1133 (m), 1002 (m), 779 (s) (sh); HRMS (ESI⁺): m/z calc’d for C₂₁H₂₃N₅O₅S 369.1329, found 369.1347 ([M + H⁺], 100%); RP-HPLC (Column 1, System A): Rₜ (min) 9.9.

Methyl 6-Chloropypyridine-3-carboxylate, 3. A suspension of 6-chloronicotinic acid (5.0 g, 32 mmol) in methanol (100 mL) was cooled to 0 °C, and H₂SO₄ (0.5 mL) was added dropwise. The mixture was heated at 60 °C for 8 h. The crude reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate and washed with sat. NaHCO₃. The organic layer was dried over MgSO₄, filtered, and concentrated in vacuo. The residue obtained was dissolved in ethyl acetate and washed with sat. NaCl. The organic layer was collected and dried over Na₂SO₄, filtered, and concentrated to afford a chalky, yellow powder (0.08 g, 81%). 1H NMR [CHCl₃-d₆, 600 MHz]: δ (ppm) 8.95 (1H, d, J = 2.4 Hz, pyH), 8.21 (1H, dd, J = 8.3, 2.4 Hz, pyH), 7.40 (1H, d, J = 8.3 Hz, pyH), 3.92 (3H, s, OCH₃); 13C [H] NMR [CHCl₃-d₆, 151 MHz]: δ (ppm) 165.0 (C, CO₂Me), 155.7 (C, pyC), 151.1 (C, pyC), 139.7 (C, pyC), 125.1 (C, pyC), 124.3 (C, pyC), 52.7 (C, OCH₃); HRMS (ESI⁻): m/z calc’d for C₁₂H₈NO₃S, 216.0792, found 216.0792 ([M - H⁻], 100%); RP-HPLC (Column 2, System A): Rₜ (min) 9.6.

Methyl 6-(2-Ethylcarbamothioyl)hydrazinyl]-3-pyridinecarboxylate, H₄L₃(OMe). The compound 6-(2-ethylcarbamothioyl)hydrazinyl]-3-pyridinecarboxylate, 4, (approximately 0.06 g, 0.67 mmol), was added to anhydrous ethanol (8 mL) and heated until entirely dissolved. Ethyl isothiocyanate (60 μL, 0.67 mmol) was added to the solution and stirred at reflux for 3 h. The solution was cooled to RT. A stream of N₂ was blown over the solution, and the resulting precipitate was collected by filtration and washed with copious amounts of diethyl ether to afford a colorless crystalline solid (0.05 g, 51%). 1H NMR [DMSO-d₆, 400 MHz]: δ (ppm) 9.91 (1H, s, -NHNH₂), 9.10 (1H, s, -NHNHC₅), 8.66 (1H, d, J = 1.8 Hz, pyH), 8.21 (1H, br, J = 6.5 Hz, -NHC₅H₄CH₂), 8.06 (1H, dd, J = 8.8, 2.1 Hz, pyH), 6.55 (1H, d, J = 8.8 Hz, pyH), 3.80 (3H, s, OCH₃), 3.45 (2H, quin., J = 6.6 Hz, CH₂CH₂), 1.03 (3H, t, J = 7.1 Hz, CH₂CH₂); 13C [H] NMR [DMSO-d₆, 151 MHz]: δ (ppm) 181.3 (C, NCS), 165.4 (C, CO₂Me), 162.2 (C, pyC), 150.2 (C, pyC), 138.5 (C, pyC), 1167. (C, pyC), 105.9 (C, pyC), 51.9 (C, CH₃), 38.4 (C, CH₃), 14.6 (C, CH₃); IR: vₘₐₓ (em⁻¹) 3143 (m), 2977 (m), 1710 (s, sh, C=O), 1533 (m, sh), 1295 (s, sh), 1254 (s, sh), 1129 (m, sh), 781 (m, sh); HRMS (ESI⁻): m/z calc’d for C₃₁H₂₃N₅O₅S, 525.0916, found 525.0938 ([M + H⁻], 100%); RP-HPLC (Column 2, System A): Rₜ (min) 9.2.

Methyl 6-(2-Tert-Butylcarbamothioyl)hydrazinyl]-3-pyridinecarboxylate, H₄L₃(OMe). The compound 6-(2-tert-butylcarbamothioyl)hydrazinyl]-3-pyridinecarboxylic acid, (H₄L₃)0.2 (0.74 mmol), was added to 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. Addition of aqueous NaOH (0.1 M) caused a precipitate to form (pH 7–8). 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 [H] NMR [CHCl₃-d₆, 125.7 MHz]: δ (ppm) 180.9 (C, NCS), 166.5 (C, CO₂CH₃), 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, -CH₃H₄), 28.7 (3C, -C(CH₃)₃); IR: vₘₐₓ (em⁻¹) 1721 (s, sh, C=O), 1523 (m, sh), 1285 (m, sh), 1250 (s, sh), 1133 (m, sh), 775 (m, sh); ESI-MS⁺: m/z calc’d for C₁₂H₂₃N₃O₃S, 283.1229, found 283.1240 ([M + H⁺], 100%); RP-HPLC (Column 2, System A): Rₜ (min) 12.9.

Methyl 6-(2-Phenylcarbamothioyl)hydrazinyl]-3-pyridinecarboxylate, H₄L₃(OMe). The procedure and reagents were used as for the synthesis of H₄L₃(OMe), except using phenyl isothiocyanate (45 μL, 0.38 mmol) and methyl 6-hyrazinyl-3-pyridinecarboxylic acid (63 mg, 0.38 mmol) to afford a white, microcrystalline solid (0.08 g, 71%). 1H NMR [CH₃OH-d₆, 400 MHz]: δ (ppm) 8.77 (1H, s, pyH), 8.19 (1H, d, J = 8.8 Hz, pyH), 7.51 (2H, d, J = 8.1 Hz, ArH),...
was centrifuged (3 min, 3600 rpm) and the supernatant was discarded and the remaining yellow oil was suction drying the resin was weighed and loading determined (0.1% TFA in H2O) and BuOH (0.51 g, 0.39 mmol) was added a DMF/piperidine (80:20 v/v) solution was added to half the resin finally diethyl ether (3 mL). After several hours of suction drying the resin was weighed and loading determined (0.77 mmol g−1) was added to the resin, which was then shaken for 2 h 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 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-trinitrobenzensulfonyl 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), H2L (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 pore filter 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 50% 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, consolidated, 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, pyH2), 8.29 (1H, d, J = 9.1, 2.2 Hz, pyH4), 7.05 (1H, d, J = 9.1 Hz, pyH6), 4.38 (1H, d, J = 8.9, 5.2 Hz, aCH), 3.70 (3H, s, CO2CH3), 3.38 (2H, t, J = 7.2 Hz, eCH2), 1.97 (3H, s, CO2CH3), 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, CH2Ph), 1.49–1.41 (2H, m, γCH2); 13C{1H} NMR [MeOH-d4, 150.9 MHz]: δ (ppm) 184.0 (C, NCS), 174.2 (C, -COCH3), 173.4 (C, -CO2CH3), 165.9 (C, CONH), 159.1 (C, pyC6), 149.4 (C, pyC5), 141.4 (C, pyC4), 123.7 (C, pyC3), 110.6 (C, pyC6), 54.9 (C, -CH2CH3), 53.7 (C, αCH2), 52.7 (C, -CO2CH3), 40.7 (C, sCH3), 32.1 (C, βCH2), 29.8 (C, δCH2), 28.9 (3C, -CH(CH3)2), 24.2 (C, -COCH3), 22.3 (C, γCH2); HRMS (ESI+) m/z calc'd for C36H52N13O8S4 453.2284, found 453.2288 [{M + H}]+, 100%; RP-HPLC (Column 2, System A): Rf (min) 12.5.

H2L1(cRGDK). A solution of 6-[2-(ethylcarbamothioyl)-hydroxazinyl]-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 (90:10 v/v) solution, filtered (Millipore 0.45 μm pore 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 50% 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, consolidated, and lyophilized to afford a colorless solid (10 mg, 80%). ESI-MS (+) m/z calc'd for [C38H57N13O8S2]+ 427.7083, found 427.7086; RP-HPLC (Column 2, System A): Rf (min) 11.3.

H2L1(cRGDK). The same procedure was used as for H2L1(cRGDK), except H2L1 was replaced with 6-[2-(tert-butylcarbamothioyl)hydroxazinyl]-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). 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 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, consolidated, and lyophilized to afford a colorless solid (10 mg, 80%). ESI-MS (+) m/z calc'd for [C36H52N13O8S]+ 413.693, found 413.692; RP-HPLC (Column 2, System A): Rf (min) 12.9.

H2L1(cRGDK). The same procedure was used as for H2L1(cRGDK), except H2L1 was replaced with 6-[2-(phenylcarbamothioyl)hydroxazinyl]-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 calcd for [C₉H₃N₅O₄S]⁺ 1271.4800, found 1271.4760, calcd for [C₉H₃N₅O₄S]²⁺ 2542.9620, found 2542.9521; RP-HPLC (Column 2, System A): Rₚ (min) 13.0.

H₂L₁(Tyr⁻Oct). The same procedure was used as for H₂L₁(Tyr⁻Oct), except H₂L₁ was replaced with H₂L₂ (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 semipreparative 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 yellow solid (14 mg, 4.2%). HRMS (⁺) m/z calcd for [C₉H₃N₅O₄S]⁺ 1319.4800, found 1319.4849, calcd for [C₉H₃N₅O₄S]²⁺ 2638.9619, found 2638.9627; RP-HPLC (Column 2, System A): Rₚ (min) 14.1.

H₂L₂(Tyr⁻Oct). The same procedure was used as for H₂L₁(Tyr⁻Oct), except H₂L₁ was replaced with H₂L₂ (0.22 g, 0.75 mmol, 3 equiv). The precipitate after lyophilization was dissolved in CH₃CN/H₂O (10 mL, 50:50 v/v) and filtered (Millipore 0.45 µm porosity syringe filter). The compound was purified by semipreparative RP-HPLC (Column 5) with a linear gradient system of Buffer A (0.1% TFA in H₂O) and Buffer B (0.1% TFA in CH₃CN) from 20% to 50% B to A over 60 min (0.5% min⁻¹) (or isocratic elution at 29% Buffer B) and UV detection at λ 254 nm with a flow rate of 7 mL min⁻¹. Fractions containing the desired compound were identified by HPLC-MS, consolidated, and lyophilized to afford a yellow solid (14 mg, 4.2%). HRMS (⁺) m/z calcd for [C₉H₃N₅O₄S]⁺ 1319.4800, found 1319.4849, calcd for [C₉H₃N₅O₄S]²⁺ 2638.9619, found 2638.9627; RP-HPLC (Column 2, System A): Rₚ (min) 14.1.

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

[ReO(HL₁(Omε))₂Cl]. To H₂L₁(Omε) (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 then filtered, diethyl ether (ca. 15 mL) was added, and the resulting precipitate was collected by filtration to give [ReO(HL₁(Omε))₂Cl] as a dark-red, microcrystalline solid (0.11 g, 84%). ᵃ¹H NMR [DMSO-d₆, 400 MHz]: δ (ppm) 8.63 (2H, s, pyH²), 8.26 (2H, dd, J = 9.3, 1.8 Hz, pyH¹), 7.87 (2H, d, J = 9.4 Hz, pyH²), 7.43 (2H, br m, NH), 3.89 (6H, s, OCH₃), 3.47 (4H, qd, J = 13.6, 7.0 Hz, CH₂CH₃), 1.16 (6H, t, J = 7.5 Hz, CH₂CH₃), 13C{¹H} NMR [CH₃CN-d₃, 150.9 MHz]: δ (ppm) 173.7 (2C, CO₂H), 165.1 (2C, N=CC₂), 125.6 (2C, pyC), 140.5 (2C, pyC), 139.9 (2C, pyC), 123.9 (2C, pyC), 122.0 (2C, pyC), 52.3 (2C, CO₂CH₂), 42.6 (2C, CH₂CH₃), 14.5 (2C, CH₂CH₃); IR: vₘₙ₈ (cm⁻¹) 1553 (s/sh, C=O), 1287 (s/sh, C=O), 963 (s/sh, Re=O), 764 (m/sh); HRMS (ESI): m/z calcd for ReC₉H₆N₅O₄S₂ 709.0916, found 709.0898; RP-HPLC (Column 2, System A): Rₚ (min) 14.8.

Histidine and Cysteine Challenge Experiments. A 50-fold excess of histidine or cysteine was added to a solution of
[ReO(\(\text{HL}^+\text{(OMe)})_2\))]\(^+\) (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.

[ReO(\(\text{HL}^+(\text{OMe})_2\))]\(^+\) \(\text{BPh}_4\). To a stirred suspension of trans-[ReOCl\(_2\)\{PPh\(_3\)\}_2] (0.15 g, 0.17 mmol) in MeOH (10 mL) was added H\(\text{L}^2\)\{(OMe)\}_2\] (0.10 g, 0.35 mmol) and 2 drops of Et\_3N. The mixture was heated at reflux for 16 h, then filtered, and diethyl ether was added to the filtrate. The resulting precipitate was collected by filtration, washed with cold diethyl ether, and then dissolved in MeOH. Addition of excess Na\(\text{BPh}_4\) resulted in the precipitation of a red-brown precipitate that was collected, and washed with hot hexane (10 mL) to afford [ReO(\(\text{HL}^+(\text{OMe})_2\))]\(^+\) \(\text{BPh}_4\) (72 mg, 39%). \(^1^H\) NMR [DMSO-\(d_6\): δ (ppm) 8.10 (2H, d, J = 9.5 Hz, py\(_\text{H}\)) 7.75 (2H, d, J = 9.4 Hz, py\(_\text{H}\)), 7.62 (8H, m, Ar\_7), 7.33 (2H, m, py\(_\text{H}\)), 6.93 (8H, t, J = 7.1 Hz, Ar\_10), 6.72 (4H, t, J = 7.1 Hz, Ar\_9), 3.86 (6H, s, CO\(_\text{CH}_3\)), 1.39 (18H, s, (CH\(_2\))\_18). \(^{13}\text{C}NMR [\text{DMSO-\(d_6\): δ (ppm)} 164.2 (2C), 145.5 (2C), 142.2 (2C, py\(_\text{H}\)), 135.8 – 135.6 ppm (8C, Ar\_C)).

The mixture was heated at reflux for 2 h, and then cooled to ambient temperature for 2 h. The mixture was then diluted with MeOH (5 mL). The resulting precipitate was collected by filtration, washed with cold diethyl ether, and then dissolved in MeOH. Addition of excess Na\(\text{BPh}_4\) resulted in the precipitation of a red-brown precipitate that was collected, washed with hot hexane (10 mL) to afford [ReO(\(\text{HL}^+(\text{OMe})_2\))]\(^+\) \(\text{BPh}_4\) (72 mg, 39%). \(^1^H\) NMR [DMSO-\(d_6\): δ (ppm) 8.10 (2H, d, J = 9.5 Hz, py\(_\text{H}\)) 7.75 (2H, d, J = 9.4 Hz, py\(_\text{H}\)), 7.62 (8H, m, Ar\_7), 7.33 (2H, m, py\(_\text{H}\)), 6.93 (8H, t, J = 7.1 Hz, Ar\_10), 6.72 (4H, t, J = 7.1 Hz, Ar\_9), 3.86 (6H, s, CO\(_\text{CH}_3\)), 1.39 (18H, s, (CH\(_2\))\_18). \(^{13}\text{C}NMR [\text{DMSO-\(d_6\): δ (ppm)} 164.2 (2C), 145.5 (2C), 142.2 (2C, py\(_\text{H}\)), 135.8 – 135.6 ppm (8C, Ar\_C)).

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\[\text{ReO(HL}^\text{1}(\text{cRGDK}))_2\text{CF}_2\text{CO}_2\]. HRMS (ESI\textsuperscript{+}) \textit{m/z} calc'd for [\text{ReC}_{116}\text{H}_{148}\text{N}_{28}\text{O}_{27}\text{S}_6]^2+ 1371.9470, found 1371.9470; RP-HPLC (Column 2, System A): \(R_f\) (min) 1.29.

\[\text{ReO(HL}^\text{2}(\text{cRGDK}))_2\text{]. The same procedure was used as for [ReO(HL\textsuperscript{1}(\text{cRGDK}))\textsubscript{2}]\textsuperscript{2+}, except H\textsubscript{2}L\textsubscript{1}(cRGDK) was replaced with H\textsubscript{2}L\textsubscript{2}(cRGDK) (4.0 mg, 4.6 \mu mol) in MeOH (200 \mu L) and other reagent quantities were adjusted accordingly. \[\text{ReO(HL}^\text{3}(\text{Tyr}^3\text{-Oct}))_2\text{]. A solution of H\textsubscript{2}L\textsubscript{1}(\text{Tyr}^3\text{-Oct}) (9.0 mg, 7.1 \mu mol) in MeOH (50 \mu L) was added to a mixture of [\text{Bu}_4\text{N}]\text{ReOCl}_4 (2.1 mg, 2.9 \mu mol) in DMF (50 \mu L) and diethyl ether (2 \times 10 \text{ mL minutes}^{-1}). Fractons containing the desired compound were identified by HPLC-MS, consolidated, and lyophilized to afford a red, fluffy material (5.0 mg, \sim 58\%, assuming [ReO(HL\textsuperscript{3}(\text{Tyr}^3\text{-Oct}))\textsubscript{2}]\textsuperscript{2+}). ESI-MS \textit{m/z} calc'd for [\text{ReC}_{121}\text{H}_{148}\text{N}_{28}\text{O}_{27}\text{S}_6]^2+, 1419.9470, found 1419.9271, \textit{m/z} calc'd for [\text{ReC}_{121}\text{H}_{148}\text{N}_{28}\text{O}_{27}\text{S}_6]^3+, 946.9673, found 946.9660; RP-HPLC (Column 2, System A): \(R_f\) (min) 16.2.

\[\text{Synthesis of L}^{\text{188}}\text{ReO(HL}^\text{1}(\text{Tyr}^3\text{-Oct}))\text{J]. Rheinmos-188 was produced from an ITG \textsuperscript{188}Re\textsuperscript{188}Re generator (ITG Isotope Technologies, Garching, Germany). Generator-produced \textsuperscript{188}ReO\textsuperscript{2+} (86 MBq) \textsuperscript{188}ReO\textsuperscript{2+} in aqueous sodium chloride solution (200 \mu L, 0.9% w/v) was added to a sealed, N\textsubscript{2}-purged vial containing sodium tartrate (0.2 mg) and stannous chloride (0.1 mg) in water (400 \mu L), followed by heating at 80 °C for 30 min. An aliquot of this solution (50 \mu L) was added to a separate sealed, N\textsubscript{2}-purged vial containing H\textsubscript{2}L\textsubscript{1}(\text{Tyr}^3\text{-Oct}) (100 \mu g) dissolved in water (100 \mu L). The reaction was left for 30 min at ambient temperature, after which an aliquot was removed for HPLC analysis, revealing that there was no reaction between \textsuperscript{188}Re and peptide. The reaction vial was then heated at 100 °C for 1 h, after which an aliquot was analyzed by HPLC (column 1, system F). Radiochemical yield: 67\%, retention time of [\textsuperscript{188}ReO(HL\textsuperscript{1}(\text{Tyr}^3\text{-Oct}))\textsubscript{2}]\textsuperscript{2+} = 11.6 min, compared to retention time of [\textsuperscript{188}ReO(HL\textsuperscript{1}(\text{Tyr}^3\text{-Oct}))\textsubscript{2}]\textsuperscript{3+} = 11.3 min and H\textsubscript{2}L\textsubscript{1}(\text{Tyr}^3\text{-Oct}) = 9.7 min.

\[\text{Synthesis of Technetium-99m Complexes. Sodium pertechnetate, Na}^{99m}\text{TcO}_4^-\text{(1000 MBq), was eluted from a Gentech 99 Mo}\textsuperscript{99m}\text{Tc sterile generator (Austin Health: Nuclear Medicine and Centre for PET, Australia, via ANSTO Health) as a 1.0 mL saline solution (0.9% v/v). A solution of SnCl\textsubscript{2} in 0.1 M HCl (0.5 mg mL\textsuperscript{-1}) was prepared and purged with nitrogen. Disodium tartrate dihydrate was dissolved in water (1}
mg mL⁻¹) in a separate evacuated vial, and a 0.5 mL aliquot was taken from both solutions and mixed together. To the solution was added [⁹⁹mTcO₄]⁻ (0.1 mL in 0.9% saline, 108 MBq). The conjugated peptide, H₂L¹⁻³c(RGDfK) or H₂L¹⁻³(Tyr³-Oct), was dissolved in degassed Milli-Q water (1 mg mL⁻¹), and 100 μL of this mixture was added to the test solution. The sample was neutralized with NaHCO₃ (pH 6.5, approximately 55 μL) then filtered or allowed to react without neutralizing at ambient temperature for 30 to 120 min. The samples were filtered (Supelco, Iso-disc Filter, 4 mm x 0.45 μm). 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 3000 g for 10 min (Heraeus, Hanau, Germany). Radioactivity readings for serum 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 centrifuge (Eppendorf, Hamburg, Germany). Partition coefficient data were collected with a PerkinElmer, Wizard 1470 (PerkinElmer, Massachusetts, USA) automatic γ counter, which measured the radioactive decay of each sample in counts per minute (cpm).

For serum 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, [⁹⁹mTc(β₂(Tyr³-Oct))₁₀]⁻ (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 0 min and then centrifuged (5 min, 13 200 rpm). The radioactivity of the supernatant and pellet was measured the radioactive decay of each sample in counts per minute (cpm). For serum 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, [⁹⁹mTc(β₂(Tyr³-Oct))₁₀]⁻ (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 then centrifuged (5 min, 13 200 rpm). The radioactivity of the supernatant and pellet was measured. The supernatant (20 μL) was analyzed by analytical RP-HPLC (Column 7, System C) for UV and radioactivity analysis and the pellet was washed with acetonitrile (3 × 0.1 mL), and radioactivity levels were again recorded (radioactivity levels of the pellet were negligible).

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