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PAPER

Investigation of isomer formation upon coordination of bifunctional histidine analogues with ^{99m}Tc/Re(CO)$_3$†‡

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Histidine is a convenient tridentate chelator used in the synthesis of technetium-99m radiopharmaceuticals, as it can be pendantly attached to a biomolecule for molecular imaging applications. Once coordinated, it forms a neutral complex that is capable of forming diastereomers at the alpha amine of the histidine. This is demonstrated through the synthesis and characterization of four different histidine chelators; three small molecule chelators, which consist of a benzylated histidine at the alpha amine, and one modified dipeptide, containing a phenylalanine derivative at the C-terminus and a histidine at the N-terminus. Upon rhenium coordination, two products are observed, each having the desired exact mass of the metal-containing species. The two products have been characterized through LC-MS, ^{1}H, gCOSY, NOESY and ROESY NMR experiments, and the relative stereochemistry determined. The implications of diastereomer formation when using this chelation system for creating molecular imaging agents is also discussed.

Introduction

Technetium-99m is the most common radionuclide used in diagnostic nuclear medicine; incorporated in more than 80% of radiopharmaceuticals today.[1] Previously, the majority of technetium was utilized in the +5 oxidation state. The $[Tc{=}O]^{3+}$ core forms complexes with square pyramidal geometry upon treatment with various tetradentate ligand sets.[2–5] However, the presence of an unprotected side adjacent to the oxo ligand left these complexes open to protonation, which could lead to decomposition.[6] The use of technetium in the +1 oxidation state was pioneered by Davison, Jones *et al.*,[7] and was developed for use in radiopharmaceuticals by Alberto *et al.*[8–12] These octahedral Re/Tc tricarbonyl complexes, when coordinated with a tridentate chelator, are completely protected against ligand attack.[6] Another advantage to this core is its small size and lipophilicity, which allows for easy incorporation into the ligand structure, therefore having little effect on biological activity.[6,13] These octahedral complexes are generally used with tridentate chelators as they tend to form a more stable complex and have a higher *in vivo* stability than bidentate chelators.[14] As well, they tend to react much faster, making them ideal for radiolabelling as the time required for synthesis can be decreased.[14]

N^{α}-Substituted histidine is a tridentate chelator capable of coordinating Re/^{99m}Tc tricarbonyl.[15–17] Histidine, as a chelator, forms complexes with many desirable properties, such as high biological stability and specific activity, as well as the ability to coordinate rapidly and quantitatively at low concentrations.[2,18,19] This chelator has found use in the labelling of many different biomolecules including neurotensin analogues, which target pancreatic carcinoma;[15,16] bombesin analogues, which target gastrin-releasing peptide receptors;[20] and cyclic RGD peptide systems, which target the $\alpha_v\beta_3$ integrin, over-expressed in angiogenic vessels and malignant tumours.[21]

Once histidine is coordinated to the Re/Tc core, a neutral metal complex is created, which has the potential to produce diastereomers. The issue of isomer formation has previously been reported for Re/Tc chelators containing various amine-based ligand sets.[22–24] However, diastereomer formation for biomolecules containing the N^{α}-substituted histidine chelator has not been described. Work has been done in our lab with a cyclic RGD peptide system containing an N^{α}-substituted histidine chelator (Fig. 1). When coordinated with Re/Tc tricarbonyl, it was found that two products were formed. These products had the same molecular weight and, therefore, were thought to be isomers at the alpha amine of the histidine.

Diastereomer formation is a significant concern in drug development as the presence of an unwanted isomer can cause side effects, toxicity and result in differing pharmacokinetic properties from that of the desired isomer. For this reason, there often is increased scrutiny during regulatory approval for drugs containing diastereomers. For example, the Food and Drug

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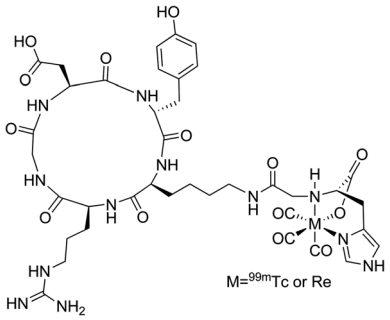


Fig. 1 Cyclic RGD peptide system containing an N^{α}-substituted histidine chelator.

Administration (FDA) in the United States has indicated that, "diastereomers therefore should, with the rare exception of cases where *in vivo* interconversion occurs, be treated as separate drugs and developed accordingly".[25]

There are examples reported of N^{τ}-derivatized histidine,[19,26] as well as the related 1,2,3-triazole analogues,[26,27] which avoid isomer formation by leaving the N^{α} position as a primary amine. As such, these metal complexes show promise for radiopharmaceutical development. However, as the issue of diastereomer formation for N^{α}-substituted histidine complexes has not been previously addressed in the literature, the focus of this paper is the investigation of the existence and nature of the products formed upon coordination of this chelator with the $M(CO)_3^+$ core. This was done through coordination of simplified chelation models with rhenium followed by thorough analysis by NMR spectroscopy, allowing for determination of the stereochemistry of the resultant diastereomers.

Results and discussion

Four different histidine chelators (Fig. 2) were synthesized and characterized both prior to and following coordination with

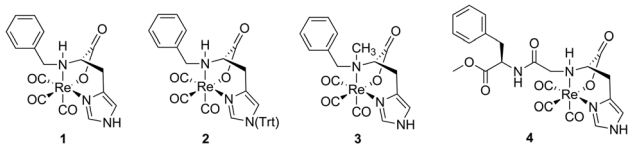
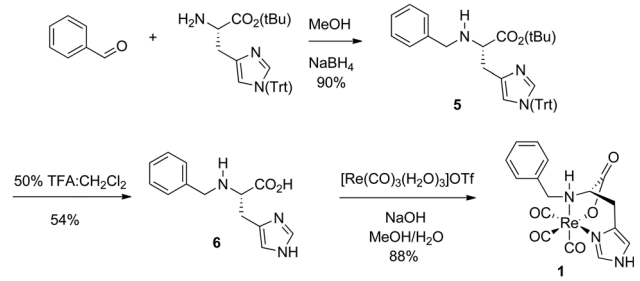


Fig. 2 Simplified histidine chelation models used for investigation of diastereomer formation.



Scheme 1 Synthesis and coordination of chelator **6**.

rhenium tricarbonyl in order to determine the nature of the isomers being formed. The first was a small molecule histidine chelator synthesized by reductive amination, **1**. This chelator was then modified with a protected imidazole **2** in order to eliminate the possibility that the observed isomers were pros-tele (π–τ) linkage isomers of the imidazole ring. The third chelator developed was a derivative of **1** with the alpha amine on the histidine methylated, **3**, in order to determine the role of the secondary amine in isomer formation. Finally, a modified dipeptide **4** was also synthesized by solution-phase techniques, in order to compare the role of histidine in isomer formation between the small molecule chelators and those within a peptide-like system.

N^{α}-Benzyl-L-histidine

As a model system, a small molecule chelator consisting of a benzylated histidine was prepared. This was done by reductive amination of H-His(Trt)-O(tBu) and benzaldehyde with sodium borohydride to form chelator **6** (Scheme 1).[28] Chelator **6** could then be coordinated with $[Re(CO)_3(H_2O)_3]OTf$[29] in the presence of sodium hydroxide and methanol. This formed the final coordinated product **1**, which precipitated out of solution and was easily recovered by centrifugation.

Analytical LC-MS showed that two products were formed in approximately a 2 : 1 ratio upon coordination (Fig. 3a). These products were believed to be diastereomers at the alpha amine of the histidine, as both products had the theoretical coordinated mass of 515.9/517.9 *m/z* ($NaC_{16}H_{14}O_5N_3{}^{185/187}Re$) displaying the characteristic rhenium 185/187 isotopic signature. The major isomer was isolated (Fig. 3b) by recrystallization in acetonitrile, allowing for further experimentation.

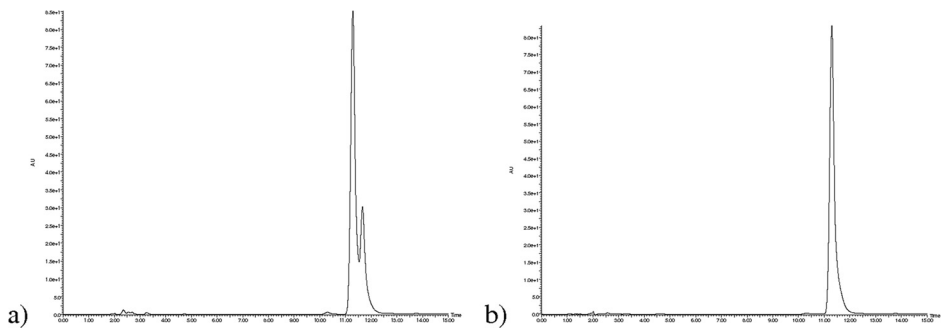


Fig. 3 Analytical HPLC trace, UV absorbance detected from 210–800 nm (RP-C_{18} 4.6 × 250 mm, 5 μm), of (a) the mixture of isomers of **1**, and (b) the purified major isomer **1a**.

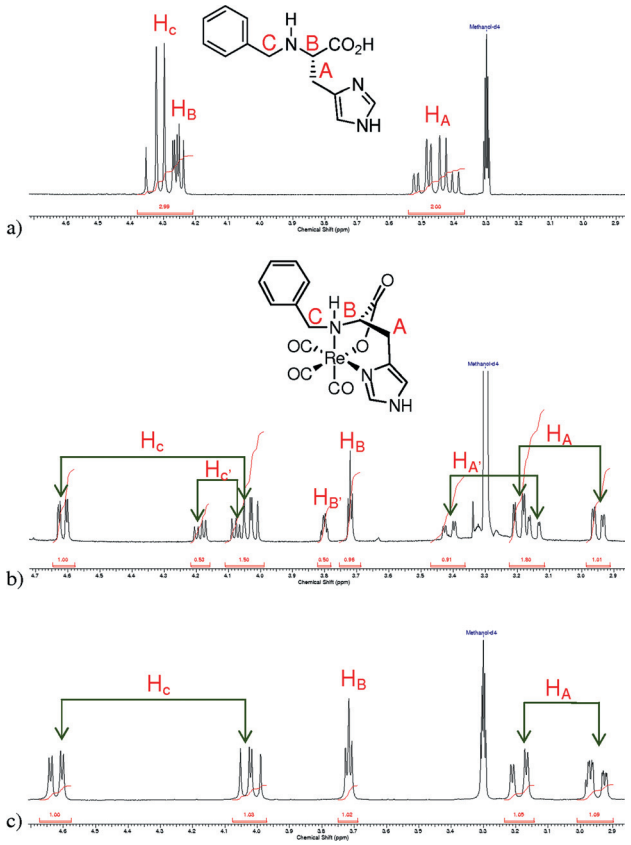


Fig. 4 Methylene region of the ^{1}H NMR (400 MHz, MeOH-d_4) of (a) the uncoordinated product **6**, (b) the coordinated mixture of **1**, and (c) the pure major isomer **1a**.

^{1}H NMR spectra of the purified product before and after coordination (Fig. 4) was used to confirm the presence of isomers. When comparing the two NMR spectra, it was quite evident that $Re(CO)_3^+$ incorporation was successful as a large change in the splitting and chemical shift of the methylene protons was observed. Fig. 4b shows that, upon coordination of $Re(CO)_3^+$, all peaks were doubled, which indicated that there were two diastereomers formed.

The major isomer was successfully isolated from the mixture and ^{1}H (Fig. 4c), ^{13}C, gCOSY, and NOESY NMR studies were performed. Through the NOESY experiment (Fig. 5), the structure of the major isomer was determined based on the through-space correlations of the protons. Of particular interest was the interaction of the protons on carbon C as well as the amine proton with the protons on carbon A. There is a correlation that can be seen between the amine proton and one of the A protons (solid circle on Fig. 5), while no correlation is seen between the C protons and the A protons (dashed circle on Fig. 5). This leads to the conclusion that the major isomer has the stereochemistry indicated as **1a** (Fig. 6).

Stability studies

In order to determine the stability of the complex, the purified major isomer **1a** was dissolved in MeOH under either neutral, acidic (0.1 M HCl) or basic (0.1 M NaOH) conditions and monitored by HPLC to see if the isomers interconvert in solution (Fig. 6).

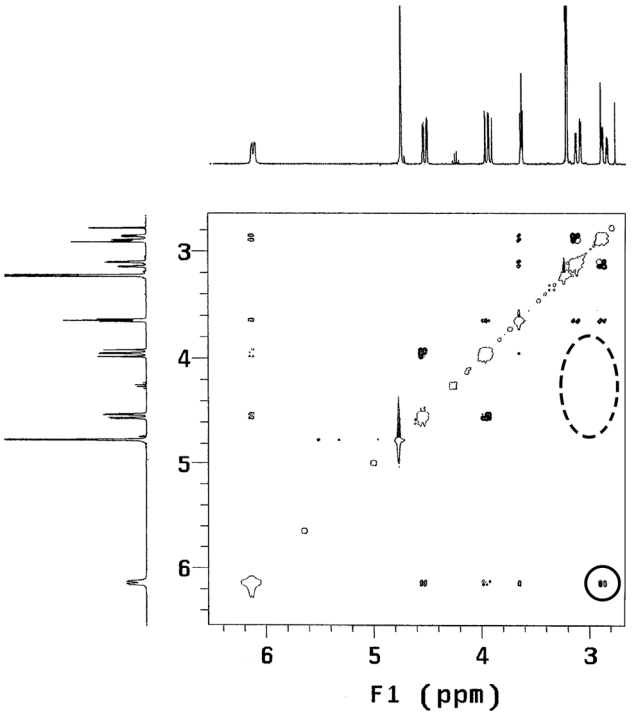


Fig. 5 NOESY spectrum (400 MHz, MeOH-d_4) of the pure major isomer of **1** showing correlation between one proton on carbon A and the amine proton (solid circle) and the lack of correlation between protons on carbons A and C (dashed circle).

In neutral solution no interconversion between isomers was detected at room temperature after 48 h. However, upon heating to approximately 35 °C for 24 h, the isomers interconverted to their former ratio of approximately 2 : 1. Under acidic conditions (0.1 M HCl) there was no interconversion detected after 48 h. Upon heating, **1a** decomposed, rather than interconverting to the minor isomer. When **1a** was put into 0.1 M NaOH it immediately interconverted to the previous ratio. This ratio remained unchanged after stirring at room temperature for 48 h, followed by heating for 48 h. Since the interconversion occurred under both neutral and basic conditions, it can be concluded that the isomer formation is not due to epimerization at the alpha carbon upon addition of base during metal coordination.

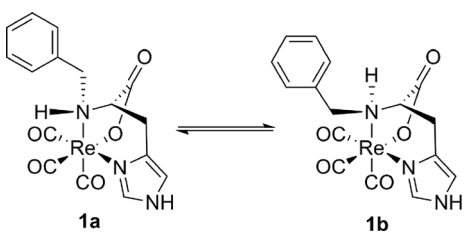


Fig. 6 Stereochemistry of the major isomer **1a** as determined by gCOSY and NOESY experiments and interconversion of the major isomer **1a** to the minor isomer **1b**.

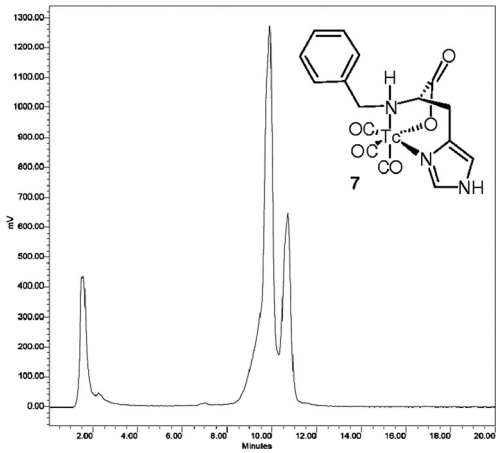


Fig. 7 Analytical HPLC gamma trace (C_{18} 4.6 × 150 mm, 5 μm) of ^{99m}Tc labelled **7**.

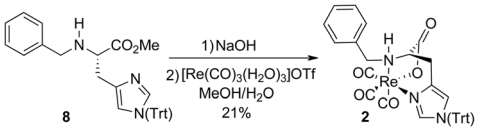
Technetium-99m labelling of N^{α}-benzyl-L-histidine

The small molecule chelator **6** was labelled with technetium-99m to give **7** (Fig. 7). Addition of pertechnetate to a commercially available Isolink kit[30] formed the $^{99m}Tc(CO)_3(H_2O)_3^+$ starting material. This reduced Tc-99m was then added to a solution of **6** to form the radiolabelled histidine derivative **7**. The analytical HPLC gamma trace revealed two peaks at the same retention time as the rhenium analogue; these two peaks also appear in the same ratio as seen when **6** is coordinated with rhenium (Fig. 7).

From this, it could be seen that **6** was successfully labelled with $^{99m}Tc(CO)_3^+$ and formed a mixture of two isomers in a similar fashion to that seen upon rhenium coordination.

Histidine linkage isomers

It was recently reported in the literature that histidine can form pros-tele (π–τ) linkage isomers with palladium and ruthenium, in a square planar arrangement, where the coordination can be to either of the imidazole nitrogens on the histidine.[31,32] In order to ensure that the observed isomers were, in fact isomers at the secondary amine, a protected version of **6** was synthesized (Scheme 2). It was prepared in the same manner as **6**, with the exception that the histidine contained a methyl ester instead of a *tert*-butyl ester, which is an orthogonal protecting group to the trityl group present on the imidazole. This allowed for selective deprotection of the methyl ester prior to coordination, while leaving the trityl group intact. This experimental design would eliminate the possibility that the observed isomers were pros-tele (π–τ) linkage isomers, given that only the unprotected imidazole nitrogen is able to coordinate to the rhenium.



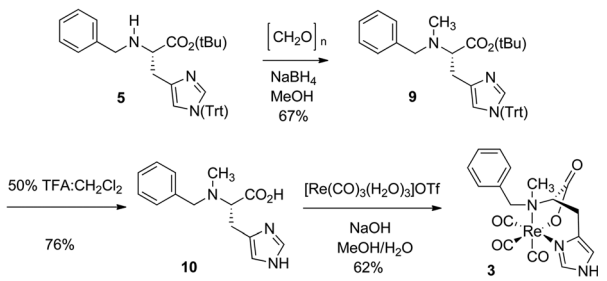
Scheme 2 Coordination of **8** with $Re(CO)_3^+$ to form **2**, a modification of **1** with a trityl protected imidazole to demonstrate that the isomers are formed at the secondary amine, as opposed to linkage isomers of the imidazole.

Chelator **8** was synthesized by the same method as was previously used for **5**, with the only difference being the protecting group on the histidine. Once purified by preparative TLC, a one-pot deprotection and subsequent coordination using $[Re(CO)_3(H_2O)_3]OTf$ was performed in a mixture of methanol and water at reflux. Analysis by LC-MS again showed two peaks, both with the same mass of 759.2/761.2 *m/z*, which matches the theoretical mass ($NaC_{35}H_{28}N_3O_5{}^{185/187}Re$) of complex **2**. As the imidazole was still trityl protected, this shows that the two products formed upon coordination are not linkage isomers of the histidine.

Methylation of N^{α}-benzyl-L-histidine

In order to determine the role of the secondary amine in the formation of diastereomers, another chelation model, where **6** is methylated at the alpha amine, was developed. This chelator was prepared starting from **5** (Scheme 3). Through treatment with paraformaldehyde, the alpha amine of the histidine was methylated, giving **9**. Following TFA deprotection and rhenium coordination, the desired complex **3** was obtained in a 62% yield.

Through LC-MS analysis of **3**, two products were observed to have formed, as was seen with **1**. Although the peaks were not fully resolved, it could be seen that both products had the same molecular weight and rhenium signature. However, it appeared as though one diastereomer was much more predominant than in the non-methylated version **1**. This was most easily seen in the ^{1}H NMR of the mixture of isomers, where, by integration, there was approximately a 5 : 1 ratio (Fig. 8). Although separation of the isomers was not possible, further characterization including gCOSY and NOESY spectra were performed on the mixture (see



Scheme 3 Synthesis of methylated complex **3**.

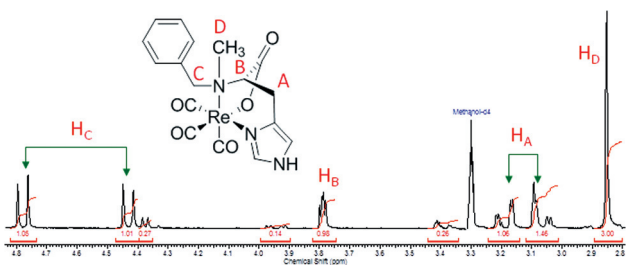


Fig. 8 Methylene region of ^{1}H NMR (400 MHz, MeOH-d_4) of **3**. Isomers can still be seen even when the secondary amine is methylated; however, one isomer is much more predominant.

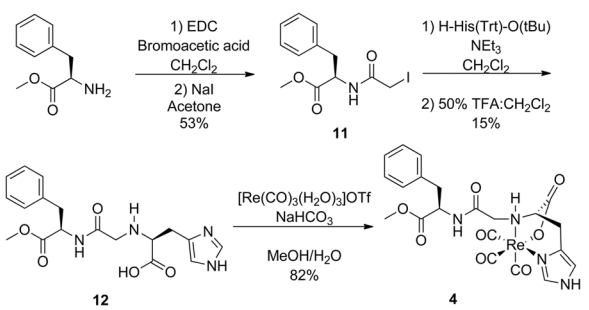
ESI†). The NOESY spectrum confirmed the identical major isomer that was formed previously for **1**, as determined by the lack of a through-space correlation between the protons on carbons A and C. From this, it can be seen that the same diastereomers are still formed when the alpha amine is methylated. Therefore, isomer formation is not due to the fact that the amine in **1** is secondary. This also leads to the possibility of controlling isomer formation through substitution at the amine in order to obtain a predominant product.

The histidine chelator is generally reported in the literature as forming a single product upon coordination. As the retention times of the isomers of **1** and **3** are so close by LC-MS, two peaks could only be seen when using certain HPLC columns, whereas for others only one peak could be seen. The column used when studying this chelation system is a potential reason as to why a single product is commonly reported for the histidine chelator when conjugated to a biomolecule.[17]

N^{α}-Histidinyl-acetyl-phenylalanine-OMe

In order to compare the formation of coordination isomers between a small molecule chelator and a peptide-like chelator, a modified dipeptide was prepared by solution-phase synthesis (Scheme 4).

A methyl ester protected phenylalanine was used as the first amino acid in this dipeptide. The coupling of the phenylalanine to bromoacetic acid was attempted using three different coupling reagents: (i) diisopropylcarbodiimide (DIC), (ii) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and (iii) dicyclohexylcarbodiimide (DCC). It was found that coupling using DIC gave a mixture of the bromide and chloride analogues, with the bromide being the major component; while coupling with EDC gave the same mixture, but with the chloride analogue being the major component. Coupling using DCC gave approximately a 50 : 50 mixture of the two. The mixture prepared using DIC could not be purified to eliminate the diisopropyl urea side product, and DCC was not ideal to work with. For these reasons, EDC was chosen as the preferred coupling reagent for the reaction. After purification of the bromide–chloride substituted mixture, a Finkelstein reaction was performed to exchange the mixture of chloride and bromide leaving groups for iodine to make **11**. The iodo derivative then underwent a substitution reaction with H-His(Trt)-O(tBu) as in the synthesis of **5**, followed by TFA treatment to afford trityl deprotection. With the methyl ester protecting group still intact, **12** was then purified by preparative HPLC.



Scheme 4 Synthesis of dipeptide complex **4**.

Given that the dipeptide contains a base-labile methyl ester protecting group, the coordination could not be done under the same conditions as those used for **1** and **3**. Therefore, coordination was performed by substituting $NaHCO_3$ for NaOH (Scheme 4). The rhenium product **4** precipitated out of solution and was collected by centrifugation. By LC-MS it was clear that two products were again formed upon coordination, each with the same mass ($C_{21}H_{22}N_4O_8{}^{185/187}Re$, 644.1/646.1 *m/z*) and the expected isotopic rhenium signature. The isomers were separated by preparative HPLC, and NMR studies including ^1H, gCOSY and ROESY spectra were performed. Through the ROESY spectrum (Fig. 9), the same through-space correlation between the protons on carbon A and the amine proton could be seen. As well, there was a lack of correlation between the protons on carbons A and C. Therefore, it can be seen that diastereomers also form in a peptide-like system with the same predominant isomer as in **1** and **3**.

Conclusions

Histidine is well suited as a tridentate chelator for the radiolabelling of peptides by coordination of rhenium or technetium-99m. However, in this work a new challenge has been presented in that multiple products are formed upon metal complexation. Two products can be seen in both small molecule and peptide-like systems. Through protecting group manipulation, it was determined that the isomers were not linkage isomers of the imidazole, but rather isomers at the alpha amine. When the secondary amine is methylated, the isomers are still present, yet the ratio is more favoured towards one isomer than in the non-methylated version. From gCOSY, NOESY and ROESY experiments, the stereochemistry of the major and minor isomers of **1**, **3** and **4** were determined (Fig. 10).

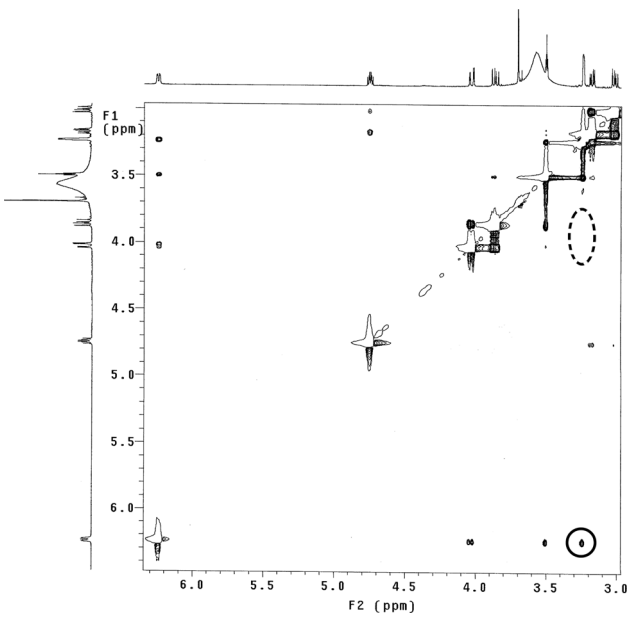
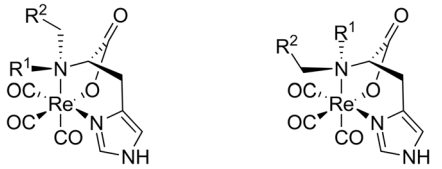


Fig. 9 ROESY spectrum (600 MHz, DMF-d_7) of the pure major isomer of **4** showing correlation between the protons on carbon A and the amine proton (solid circle) and the lack of correlation between protons on carbons A and C (dashed circle).



1: R^1 = H R^2 = Ph
3: R^1 = CH_3 R^2 = Ph
4: R^1 = H R^2 = $CONHC(CH_2Ph)COOCH_3$

Fig. 10 Stereochemistry of the diastereomers of **1**, **3** and **4**.

Histidine is a chelator that can be attached to biomolecules for use in radiopharmaceuticals; an example of such a system is the cyclic RGD peptide. The formation of diastereomers in any radiopharmaceutical is a major concern as regulatory approval of drugs is more difficult when isomers are involved. Therefore, it is critical that the formation of diastereomers is addressed when dealing with this chelation system for the coordination of $^{99m}Tc/Re(CO)_3$.

Experimental

Materials and equipment

All chemicals were purchased from Sigma-Aldrich, Novabiochem, Peptides International and Chem-Impex and were used without further purification unless indicated. Isolink kits were a generous gift from Mallinckrodt Medical. Dry CH_2Cl_2 was prepared by distillation from CaH under argon. For LC-MS, a Waters, Inc. system was used, consisting of a Waters 2998 Photodiode Array Detector and a Waters 2767 Sample Manager. For analytical LC-MS studies, a Sunfire RP-C18 4.6 × 250 mm, 5 μm column and a Waters Symmetry RP-C18 4.6 × 150 mm, 5 Å column were used. For preparative LC-MS work, a Sunfire RP-C18 19 × 150 mm, 5 μm column was used. In both cases, absorbance was detected at wavelengths of 220 and 254 nm. A gradient solvent system consisting of CH_3CN + 0.1% of TFA (solvent A) and H_2O + 0.1% of TFA (solvent B) was used. Silicycle Silica Gel 60 (230–400 mesh) was used for flash column chromatography purification. EMD Silica Gel 60 F_{254} plates were used for analytical TLC, and EMD Silica Gel 60 F_{254}, 2 mm glass plates were used for preparative TLC. Varian Mercury 400 and Varian INOVA 400 and 600 NMR spectrometers were used for ^1H, ^{13}C, gCOSY, NOESY and ROESY NMR studies, with TMS as the internal standard. For electron impact (EI) mass spectra a Finnigan MAT 8400 mass spectrometer was used and for electro-spray ionization (ESI) mass spectra a Micromass Quattro Micro API mass spectrometer was used.

N^{α}-Benzyl-L-histidine(Trt)-O(tBu) (5)

The HCl salt of H-His(Trt)-O(tBu) was extracted using 0.1 M Na_2CO_3 aqueous solution and CH_2Cl_2 to give the neutral histidine (1.23 g, 2.71 mmol), which was subsequently dissolved in 20 mL methanol followed by the addition of 1.05 equivalents of benzaldehyde (287 μL, 2.85 mmol). This mixture was stirred at room temperature and reaction completion was followed by TLC. Formation of the imine was complete after 2 h, at which point 1.05 equivalents $NaBH_4$ (108 mg, 2.85 mmol) was added. This was allowed to stir at room temperature for 18 h. The methanol was removed with a rotary evaporator and the resulting oil was extracted from water with CH_2Cl_2 and then washed with de-ionized water (3 × 5 mL). The organic layers were filtered through cotton, and the solvent was removed on a rotary evaporator. This resulted in a 90% yield of **5** (1.33 g, 2.44 mmol). HRMS: *m/z* calculated for $C_{36}H_{38}N_3O_2$, 544.2964; observed $[M + H]^+$ 544.2965. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, δ): 7.35 (1H, d, imidazole, $^5J_{H-H}$ = 1.4 Hz), 7.11–7.31 (20H, m, ar), 6.65 (1H, d, imidazole, $^5J_{H-H}$ = 1.2 Hz), 3.83 (1H, d, PhCH_2N, $^2J_{H-H}$ = 12.9 Hz), 3.65 (1H, d, PhCH_2N, $^3J_{H-H}$ = 12.9 Hz), 3.50 (1H, dd, N$CH$$CO_2(CH_3)_3$, $^5J_{H-H}$ = 1.4 Hz, $^2J_{H-H}$ = 5.9 Hz), 2.97 (1H, dd, NHCHCH_2, $^3J_{H-H}$ = 5.7 Hz, $^2J_{H-H}$ = 14.5 Hz), 2.83 (1H, dd, NHCHCH_2, $^3J_{H-H}$ = 7.2 Hz, $^2J_{H-H}$ = 14.5 Hz), 1.41 (9H, s, $CO_2C(CH_3)_3$). ^{13}C-NMR spectrum (100 MHz, $CDCl_3$, δ): 173.61 (CHC(O)O), 142.39 (ar), 140.06 (ar), 138.22 (imidazole), 137.36 (ar), 129.65 (ar), 128.12 (ar), 128.09 (ar), 127.86 (ar), 127.83 (ar), 126.68 (ar), 119.15 (imidazole), 80.62 (NCPh$_3$), 74.96 ($CO_2C(CH_3)_3$), 61.12 (N$CH$$CO_2(CH_3)_3$), 51.81 (Ph$CH_2$N), 32.19 (NHCH$CH_2$), 28.03 ($CO_2C(CH_3)_3$).

N^{α}-Benzyl-L-histidine (6)

Purified **5** (1.33 g, 2.44 mmol), was then deprotected in CH_2Cl_2 (6 mL) with trifluoroacetic acid (TFA) (6 mL). Two equivalents of triethylsilane (866 μL, 5.42 mmol) was added as a scavenger for the trityl protecting group. This was stirred at room temperature for 4 h. The solution was then concentrated on a rotary evaporator and the trityl by-product was removed by filtration. No further purification was necessary. This resulted in 323 mg (54%) of **6**. HRMS: *m/z* calculated for $C_{13}H_{16}O_2N_3$, 246.1243; observed $[M + H]^+$ 246.1247. ^{1}H-NMR spectrum (400 MHz, MeOH-d_4, δ): 8.83 (1H, s, imidazole), 7.43–7.53 (6H, m, ar and imidazole), 4.24–4.35 (3H, m, PhCH_2N and N$CH$$CO_2$H), 3.50 (1H, dd, NHCH$CH_2$, $^3J_{H-H}$ = 5.5 Hz, $^2J_{H-H}$ = 15.6 Hz), 3.42 (1H, dd, NHCHCH_2, $^3J_{H-H}$ = 7.8 Hz, $^2J_{H-H}$ = 15.6 Hz). ^{13}C-NMR spectrum (100 MHz, MeOH-d_4, δ): 171.12 (CHC(O)O), 135.26 (ar), 132.45 (imidazole), 131.19 (ar), 130.67 (ar), 130.20 (ar), 118.97 (ar), 111.37 (imidazole), 60.83 (N$CH$$CO_2$H), 51.73 (Ph$CH_2$N), 26.32 (NHCH$CH_2$).

Rhenium(I) tricarbonyl-N^{α}-benzyl-L-histidine (1)

Chelator **6** (323 mg, 1.31 mmol) was coordinated with 1.2 equivalents of $[Re(CO)_3(H_2O)_3]OTf$ (15.7 mL of a 0.1 M solution, 1.57 mmol) and 5 equivalents of NaOH (1.31 mL of a 5 M solution, 6.55 mmol) in a solution of methanol (3 mL) and water (6 mL). This solution was stirred at 80 °C for 2 h and was then centrifuged in order to separate the precipitated product. The crude solid was washed with water several times followed by centrifugation, resulting in 600 mg (88%) of **1**. HRMS: *m/z* calculated for $NaC_{16}H_{14}O_5N_3{}^{185/187}Re$, 536.0361/538.0389; observed $[M + Na]^+$ 536.0385/538.0333. ^{1}H-NMR spectrum of **1a** and **1b** (denoted by ′) (600 MHz, MeOH-d_4, δ): 8.06 (1H, s,

ReN*CH*NH), 8.05 (0.5H, s, ReN*CH*NH′), 7.34–7.47 (5H, m, ar), 7.10 (0.5H, s, ReNCHNH*CH*′), 7.00 (1H, s, ReNCHNH*CH*), 6.74 (0.5H, d, PhCH$_2$*NH*′, $^3J_{H-H}$ = 4.4 Hz), 6.22 (1H, dd, PhCH$_2$*NH*, $^3J_{H-H}$ = 3.1 Hz, $^3J_{H-H}$ = 10.5 Hz), 4.62 (1H, dd, Ph*CH*$_2$NH, $^3J_{H-H}$ = 4.0 Hz, $^2J_{H-H}$ = 14.5 Hz), 4.19 (0.5H, dd, Ph*CH*$_2$NH′, $^3J_{H-H}$ = 6.6 Hz, $^2J_{H-H}$ = 14.1 Hz), 4.08 (0.5H, dd, Ph*CH*$_2$NH′, $^3J_{H-H}$ = 7.9 Hz, $^2J_{H-H}$ = 14.1 Hz), 4.03 (1H, dd, Ph*CH*$_2$NH, $^3J_{H-H}$ = 11.0 Hz, $^2J_{H-H}$ = 14.1 Hz), 3.80 (0.5H, dd, NH*CH*CH$_2$′, $^3J_{H-H}$ = 4.4 Hz, $^3J_{H-H}$ = 7.9 Hz), 3.72 (1H, dd, NH*CH*CH$_2$, $^3J_{H-H}$ = 4.0 Hz), 3.42 (0.5H, dd, NHCH*CH*$_2$′, $^3J_{H-H}$ = 4.8 Hz, $^2J_{H-H}$ = 18.5 Hz), 3.21 (1H, dd, NHCH*CH*$_2$, $^3J_{H-H}$ = 3.5 Hz, $^2J_{H-H}$ = 17.1 Hz), 3.16 (0.5H, dd, NHCH*CH*$_2$′, $^3J_{H-H}$ = 2.6 Hz, $^2J_{H-H}$ = 18.0 Hz), 2.96 (1H, dd, NHCH*CH*$_2$, $^3J_{H-H}$ = 4.0 Hz, $^2J_{H-H}$ = 17.1 Hz). ^{13}C-NMR spectrum of **1a** (100 MHz, MeOH-d$_4$, δ): 183.95 (CH*C*(O)O), 141.26 (ar), 137.57 (imidazole), 135.00 (ar), 130.30 (ar), 130.08 (ar), 129.61 (ar), 116.7 (imidazole), 64.08 (N*CH*CO$_2$), 58.52 (Ph*CH*$_2$N), 28.77 (NHCH*CH*$_2$).

^{99m}Technetium(I) tricarbonyl-N^{α}-benzyl-L-histidine (7)

To an Isolink kit containing sodium tartrate, sodium tetraborate, sodium carbonate, and sodium boranocarbonate, 13.71 mCi (0.5 mL) of [^{99m}Tc]-pertechnetate was added and the syringe rinsed with 0.5 mL water. This was stirred at 75 °C for 30 min, then 0.7 mg of **6** was dissolved in 0.5 mL of the solution and stirred at 90 °C for 1 h. HPLC analysis showed retention time of 9.8 and 10.8 min for **7** at a linear gradient of 20–40% solvent A in B for 15 min. HPLC analysis of **1a** showed retention time of 10.8 min for the same gradient system.

N^{α}-Benzyl-L-histidine(Trt)-OMe (8)

H-His(Trt)-OMe·HCl was neutralized by stirring at room temperature in CH$_2$Cl$_2$ along with 1 equivalent NEt$_3$ for approximately 20 min. This was then extracted with water and the organic layers were concentrated and dried under vacuum to yield 144 mg of neutralized starting material. The same synthetic procedure was used as with **6**; however, a preparative TLC plate was run as a final purification step in a solution of 95% CH$_2$Cl$_2$, 4% MeOH, and 1% NEt$_3$. This yielded 47 mg (27%) of **8**. ESI + MS: *m/z* calculated for C$_{33}$H$_{33}$N$_3$O$_2$, 503.3; observed [M + H]$^+$ 503.9. ^{1}H-NMR spectrum (400 MHz, CDCl$_3$, δ): 7.31 (1H, s, imidazole), 7.04–7.26 (20H, m, ar), 6.52 (1H, s, imidazole), 3.78 (1H, d, Ph*CH*$_2$NH, $^2J_{H-H}$ = 13.3 Hz), 3.56–3.62 (5H, m, Ph*CH*$_2$NH, NH*CH*CO$_2$Me, and CO$_2$*Me*), 2.88 (2H, dd, NHCH*CH*$_2$, $^3J_{H-H}$ = 5.9 Hz).

Rhenium(I) tricarbonyl-N^{α}-benzyl-L-histidine(Trt) (2)

The methyl ester protected **8** (47 mg, 0.094 mmol) was dissolved in MeOH (6 mL) and H$_2$O (3 mL) and added 5 equivalents (94 μL of a 5 M solution, 0.47 mmol) of NaOH, this was stirred at room temperature for 1 h. To this solution was added 1.2 equivalents of [Re(CO)$_3$(H$_2$O)$_3$]OTf (1.13 mL of a 0.1 M solution, 0.11 mmol) and the mixture was stirred at reflux for 2 h. The solution was then lyophilized to yield a white powder. After dissolving the powder in acetonitrile, the product precipitated out of solution with the addition of water. After centrifugation, the liquid was decanted off and the remaining solid was washed with water, giving 15 mg (21%) of **2**. HRMS: *m/z* calculated for NaC$_{35}$H$_{28}$N$_3$O$_5$$^{185/187}$Re, 778.1457/780.1485; observed [M + Na]$^+$ 778.1475/780.1521. ^{1}H-NMR spectrum of isomers of **2** (minor isomer denoted by′) (400 MHz, CDCl$_3$, δ): 7.60 (1.5H, s, imidazole and imidazole′), 6.95–7.27 (27H, m, ar), 4.48 (1H, d, Ph*CH*$_2$NH, $^2J_{H-H}$ = 14.1 Hz), 4.16 (0.4H, d, Ph*CH*$_2$NH′, $^2J_{H-H}$ = 13.3 Hz), 3.94 (1H, d, Ph*CH*$_2$NH, $^2J_{H-H}$ = 14.1 Hz), 3.84 (0.4H, d, Ph*CH*$_2$NH′, $^2J_{H-H}$ = 13.3 Hz), 3.69 (1.3H, m, NH*CH*C(O)), 3.32 (0.4H, dd, NHCH*CH*$_2$′, $^3J_{H-H}$ = 3.5 Hz, $^2J_{H-H}$ = 18.0 Hz), 3.16 (1H, dd, NHCH*CH*$_2$, $^3J_{H-H}$ = 3.9 Hz, $^2J_{H-H}$ = 18.4 Hz), 3.02 (0.4H, dd, NHCH*CH*$_2$′, $^3J_{H-H}$ = 3.9 Hz, $^2J_{H-H}$ = 10.6 Hz), 2.60 (1H, dd, NHCH*CH*$_2$, $^3J_{H-H}$ = 3.9 Hz, $^2J_{H-H}$ = 18.0 Hz).

N^{α}-Benzyl(methyl)-L-histidine(Trt)-O(tBu) (9)

The HCl salt of H-His(Trt)-O(tBu) was extracted using 0.1 M Na$_2$CO$_3$ aqueous solution and CH$_2$Cl$_2$ to give the neutral histidine. The neutral histidine (935.4 mg, 2.06 mmol) was dissolved in 20 mL methanol to which 1.05 equivalents of benzaldehyde (219 μL, 2.17 mmol) was added. This was stirred at room temperature and the reaction progress was followed by TLC. The reaction was complete after 2 h, at which point 1.05 equivalents NaBH$_4$ (81.9 mg, 2.17 mmol) was added. This was allowed to stir at room temperature for 18 h followed by the addition of 1 equivalent of powder paraformaldehyde (185 mg, 2.06 mmol). Upon complete dissolution (approximately 18 h), 1.05 equivalents of NaBH$_4$ (81.9 mg, 2.17 mmol) was added. After allowing to react overnight, the solution was concentrated with a rotary evaporator and the resulting oil was extracted from water with CH$_2$Cl$_2$ and then washed with de-ionized water (3 × 5 mL). The organic layers were filtered through cotton, and concentrated on a rotary evaporator. The resulting product was purified using flash column chromatography with an eluent of 98 : 1 : 1 CH$_2$Cl$_2$: MeOH : NEt$_3$, resulting in a 67% yield of **9** (772 mg, 1.38 mmol). HRMS: *m/z* calculated for C$_{37}$H$_{39}$N$_3$O$_2$, 557.3042; observed [M + H]$^+$ 557.3032. ^{1}H-NMR spectrum (400 MHz, CHCl$_3$, δ): 7.15–7.40 (21H, m, imidazole and ar), 6.67 (1H, d, imidazole, $^5J_{H-H}$ = 1.2 Hz), 3.81 (1H, d, Ph*CH*$_2$N, $^2J_{H-H}$ = 13.7 Hz), 3.71 (1H, d, N(CH$_3$)*CH*CO$_2$(CH$_3$)$_3$, $^2J_{H-H}$ = 7.2 Hz), 3.67 (1H, d, Ph*CH*$_2$N, $^3J_{H-H}$ = 13.7 Hz), 3.09 (1H, dd, N(CH$_3$)CH*CH*$_2$, $^3J_{H-H}$ = 8.0 Hz, $^2J_{H-H}$ = 14.7 Hz), 2.93 (1H, dd, N(CH$_3$)CH*CH*$_2$, $^3J_{H-H}$ = 7.0 Hz, $^2J_{H-H}$ = 14.7 Hz), 2.28 (3H, s, CH$_2$N(*CH*$_3$)CH), 1.47 (9H, s, CO$_2$C(*CH*$_3$)$_3$).

N^{α}-Benzyl(methyl)-L-histidine (10)

Chelator **9** (772 mg, 1.38 mmol) was then deprotected with CH$_2$Cl$_2$ (6 mL) and TFA (6 mL). Two equivalents of triethylsilane (446 μL, 2.76 mmol) were added as a scavenger for the trityl group. After stirring for 4 h at room temperature, the solution was concentrated on a rotary evaporator, at which point the oil was dissolved in acetonitrile. With the addition of water the trityl by-product precipitated out of solution and was filtered off and the filtrate was lyophilized to give the final product. No further purification was necessary. This resulted in 272 mg

(76%) of **10**. HRMS: *m/z* calculated for $C_{14}H_{17}N_3O_2$, 259.1321; observed $[M + H]^+$ 259.1329. ^{1}H-NMR spectrum (400 MHz, MeOH-d_4, δ): 8.85 (1H, s, imidazole), 7.07–7.55 (6H, m, NCH_2Ph and imidazole), 4.52 (1H, d, NCH_2Ph, $^2J_{H-H}$ = 12.7 Hz), 4.40 (2H, m, NCH_2Ph and $NCHCO_2H$), 3.61 (1H, dd, $NCHCH_2$, $^3J_{H-H}$ = 3.7 Hz, $^2J_{H-H}$ = 14.9 Hz), 3.51 (1H, dd, $NCHCH_2$, $^3J_{H-H}$ = 9.8 Hz, $^2J_{H-H}$ = 14.9 Hz), 2.92 (3H, s, $PhCH_2N(CH_3)$).

Rhenium(I) tricarbonyl-N^{α}-benzyl(methyl)-L-histidine (3)

The histidine chelator **10** (272 mg, 1.05 mmol) was coordinated with 1.2 equivalents of $[Re(CO)_3(H_2O)_3]OTf$ (12.6 mL of a 0.1 M solution, 1.26 mmol) and 5 equivalents of NaOH (1.05 mL of a 5 M solution, 5.25 mmol) in a solution of methanol (3 mL) and water (6 mL). This was stirred at 80 °C for 2 h. The coordinated product precipitated and was collected by centrifugation and washed with water. This resulted in 342 mg (62%) of **3**. HRMS: *m/z* calculated for $NaC_{17}H_{16}O_5N_3{}^{185/187}Re$, 550.0518/552.0546; observed $[M + Na]^+$ 550.0524/552.0539. ^{1}H-NMR spectrum of isomers of **3** (minor isomer denoted by ′) (400 MHz, MeOH-d_4, δ): 8.14 (0.2H, s, imidazole′), 8.11 (1H, s, imidazole), 7.39–7.50 (5H, m, ar), 7.18 (0.2H, s, imidazole′), 7.05 (1H, s, imidazole), 4.78 (1H, d, $PhCH_2N(Me)$, $^2J_{H-H}$ = 13.5 Hz), 4.43 (1H, d, $PhCH_2N(Me)$, $^2J_{H-H}$ = 13.5 Hz), 4.38 (0.2H, d, $PhCH_2N(Me)′$, $^2J_{H-H}$ = 7.0 Hz), 3.94 (0.2H, dd, $N(Me)CHCH_2′$, $^3J_{H-H}$ = 4.9 Hz, $^2J_{H-H}$ = 15.6 Hz), 3.79 (1H, dd, $N(Me)CHCH_2$, $^3J_{H-H}$ = 3.3 Hz, $^3J_{H-H}$ = 4.9 Hz), 3.38 (0.2H, dd, $N(Me)CHCH_2′$, $^3J_{H-H}$ = 4.7 Hz, $^2J_{H-H}$ = 18.6 Hz), 3.18 (1H, dd, $N(Me)CHCH_2$, $^3J_{H-H}$ = 3.1 Hz, $^2J_{H-H}$ = 18.8 Hz), 3.07 (1H, dd, $N(Me)CHCH_2$, $^3J_{H-H}$ = 4.8 Hz, $^2J_{H-H}$ = 18.6 Hz), 2.85 (3H, s, $N(Me)CHCH_2$). ^{13}C-NMR Spectrum (100 MHz, MeOH-d_4, δ): 183.71 ($CHC(O)O$), 141.46 (ar), 133.85 (imidazole), 133.54 (ar), 132.02 (ar), 130.43 (ar), 129.74 (ar), 116.59 (imidazole), 73.06 ($NCHCO_2$), 63.30 ($PhCH_2N$), 45.65 ($PhCH_2N(CH_3)$), 26.11 ($NHCHCH_2$).

N-Iodocarbonyl-*O*-methyl phenylalanine (11)

H-Phe-OMe·HCl (520 mg, 2.41 mmol) was neutralized by stirring in CH_2Cl_2 with 1 equivalent of triethylamine (336 µL, 2.41 mmol) at room temperature for approximately 30 min. 1.1 equivalents of bromoacetic acid (37.1 mg, 2.65 mmol) was then pre-activated with 1.1 equivalents of EDC (411 mg, 2.65 mmol) in CH_2Cl_2 for approximately 5 min. This solution was added to the reaction mixture and allowed to stir at room temperature overnight. The crude solution was concentrated on a rotary evaporator. This was then purified using flash column chromatography using an eluent system of 99% CH_2Cl_2 and 1% MeOH. The column yielded 330 mg of crude **11**. This was dissolved in acetone and 1.1 equivalents of NaI (213 mg, 1.41 mmol) was added. The solution was refluxed overnight. The solid NaCl could then be filtered off, and the resulting filtrate concentrated on a rotary evaporator and dried under high vacuum, to yield 236 mg (0.68 mmol, 53%) of **11**. HRMS: *m/z* calculated for $C_{12}H_{14}INO_3$, 347.0018; observed $[M + H]^+$, 347.0022. ^{1}H-NMR spectrum (400 MHz, MeOH-d_4, δ): 7.20–7.29 (5H, m, $CHCH_2Ph$), 4.63 (1H, dd, $PhCH_2CH$, $^3J_{H-H}$ = 5.5 Hz, $^3J_{H-H}$ = 8.6 Hz), 3.69 (5H, m, OCH_3 and $C(O)CH_2I$), 3.14 (1H, dd, $PhCH_2CH$, $^3J_{H-H}$ = 5.5 Hz, $^2J_{H-H}$ = 14.1 Hz), 2.97 (1H, dd, $PhCH_2CH$, $^3J_{H-H}$ = 8.6 Hz, $^2J_{H-H}$ = 14.1 Hz).

N^{α}-Histidinyl-acetyl-phenylalanine (12)

From the previous step, **11** (236 mg, 0.68 mmol) was used without further purification. To this, 1.1 equivalents of neutralized H-His(Trt)-O(tBu) (340.5 mg, 0.75 mmol) was added in CH_2Cl_2 and refluxed for 48 h. The solution was then concentrated on a rotary evaporator and dried on high vacuum, which yielded 529 mg of the crude protected **12**. The crude material was then purified by flash column chromatography using an eluent system of 92% CH_2Cl_2, 7% MeOH and 1% NEt_3. This yielded 250 mg (0.37 mmol, 55%) of pure trityl protected **12**. The trityl containing **12** (220 mg, 0.33 mmol) was then deprotected using a 50 : 50 mixture of CH_2Cl_2 and TFA and 1 equivalent (52.2 µL, 0.33 mmol) of triethylsilane. This was stirred at room temperature for approximately 4 h, at which point it could be concentrated on a rotary evaporator by azeotroping with excess CH_2Cl_2. It was dried on high vacuum to yield 18.5 mg (0.05 mmol, 15%) of **12**. HRMS: *m/z* calculated for $C_{18}H_{23}N_4O_5$, 375.1668; observed $[M + H]^+$ 375.1671. ^{1}H-NMR spectrum (400 MHz, MeOH-d_4, δ): 8.84 (1H, s, imidazole), 7.42 (1H, s, imidazole), 7.18–7.29 (5H, m, ar), 4.76 (1H, dd, $NCHCO_2H$, $^3J_{H-H}$ = 5.1 Hz, $^3J_{H-H}$ = 8.6 Hz), 4.05 (1H, dd, $PhCH_2CH$, $^3J_{H-H}$ = 5.9 Hz), 3.92 (1H, d, $PhCH_2CH$, $^2J_{H-H}$ = 16.0 Hz), 3.80 (1H, d, $PhCH_2CH$, $^2J_{H-H}$ = 16.0 Hz), 3.70 (3H, s, $MeOC(O)$), 3.35 (2H, d, $C(O)CH_2NH$, $^2J_{H-H}$ = 6.3 Hz), 3.18 (1H, dd, CO_2HCHCH_2, $^3J_{H-H}$ = 5.1 Hz, $^2J_{H-H}$ = 13.7 Hz), 2.98 (1H, dd, CO_2HCHCH_2, $^3J_{H-H}$ = 8.6 Hz, $^2J_{H-H}$ = 13.7 Hz).

Rhenium(I) tricarbonyl-N^{α}-histidinyl-acetyl-phenylalanine (4)

The purified **12** (18.5 mg, 0.05 mmol) was coordinated with 1.2 equivalents of $[Re(CO)_3(H_2O)_3]OTf$ (600 µL of a 0.1 M solution, 0.06 mmol) and 5 equivalents of $NaHCO_3$ (21.2 mg, 0.25 mmol) in a mixture of methanol (3 mL) and water (6 mL). This was stirred at 80 °C for 2 h. The precipitate was collected by centrifugation and washed with water. This resulted in 26.4 mg (82%) of **4**. HRMS: *m/z* calculated for $C_{21}H_{22}N_4O_8{}^{185/187}Re$, 643.0968/645.0995; observed $[M + H]^+$ 643.1015/645.0984. ^{1}H-NMR spectrum (600 MHz, DMF-d_7, δ): 8.82 (1H, d, $MeOC(O)CHNH$, $^3J_{H-H}$ = 8.2 Hz), 8.27 (1H, t, imidazole, $^4J_{H-H}$ = 1.2 Hz), 7.24–7.35 (5H, m, ar), 7.21 (1H, d, imidazole, $^4J_{H-H}$ = 1.2 Hz), 6.23 (1H, dd, $C(O)CH_2NHRe$, $^3J_{H-H}$ = 2.9 Hz, $^3J_{H-H}$ = 10.5 Hz), 4.74 (1H, m, $MeOC(O)CHNH$), 4.01 (1H, dd, $C(O)CH_2NHRe$, $^3J_{H-H}$ = 3.5 Hz, $^2J_{H-H}$ = 15.8 Hz), 3.86 (1H, dd, $C(O)CH_2NHRe$, $^3J_{H-H}$ = 10.5 Hz, $^2J_{H-H}$ = 15.8 Hz), 3.69 (3H, s, $MeOC(O)$), 3.49 (1H, t, $NHCH(O)CH_2$, $^3J_{H-H}$ = 4.1 Hz), 3.23 (2H, d, $NHCH(O)CH_2$, $^3J_{H-H}$ = 2.3 Hz), 3.16 (1H, dd, $PhCH_2CH$, $^3J_{H-H}$ = 5.9 Hz, $^2J_{H-H}$ = 14.1 Hz), 3.01 (1H, dd, $PhCH_2CH$, $^3J_{H-H}$ = 9.4 Hz, $^2J_{H-H}$ = 14.1 Hz).

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