# $N^{\varepsilon}$ Functionalization of Metal and Organic Protected L-Histidine for a Highly Efficient, Direct Labeling of Biomolecules with $[Tc(OH_2)_3(CO)_3]^+$

**Jae Kyoung Pak, Paul Benny, Bernhard Spingler, Kirstin Ortner, and Roger Alberto*[a]**

**Abstract:** Two different pathways for the introduction of an acetyl group at $N^{\varepsilon}$ in a $N^{\alpha}$, $N^{\delta}$, and -COO protected histidine to afford $N^{\varepsilon}$-($CH_2COOH$)-histidine derivative **7b** are presented. The purpose of this study is the coupling of **7b** to amino groups in bioactive molecules such as peptides. After full deprotection of such a bioconjugate, histidine provides three coordination sites which efficiently coordinate to $[^{99m}Tc(OH_2)_3(CO)_3]^+$ or $[Re(OH_2)_3(CO)_3]^+$ in a facial geometry. This allows the development of novel radiopharmaceuticals. Selective derivatization at the $N^{\varepsilon}$ position has conveniently been achieved by concomitant protection of $N^{\alpha}$ and $N^{\delta}$ with a carbonyl group forming a six-membered urea. Cyclic urea ring opening with Fm-OH, coupling of phenylalanine as a model to **7b** through its primary amine and removing of all protecting groups in one step gave a histidine derivative of phenylalanine which could be labeled at $10^{-5}$ M with $^{99m}$Tc in very high yield and even in about 50% yield at $10^{-6}$ M. The X-ray structure of a complex with $[Re(CO)_3]^+$ in which anilin is coupled to **7b** confirms the facial arrangement of histidine. A second pathway applies directly the $[Re(CO)_3]^+$ moiety as a protecting group. This is one of the rare examples in which a metal fragment is used as a protecting group for organic functionalities. The coordination to histidine protects the $N^{\alpha}$, $N^{\delta}$ and COO group in one single step, subsequent alkylation with $BrCH_2COOH(R)$ at $N^{\varepsilon}$, coupling to phenylalanine and oxidative deprotection of $[Re(CO)_3]^+$ to $[ReO_4]^-$ gave the corresponding bioconjugate in which histidine is coupled to phenylalanine through an acetylamide at $N^{\varepsilon}$. Both methods offer convenient pathways to introduce histidine in a biomolecule under retention of its three coordination sites. The procedures are adaptable to any biomolecule with pendant amines and allow the development of novel radiopharmaceuticals or inversed peptides.

**Keywords:** bioorganometallic chemistry · histidine · radiopharmaceuticals · rhenium · technetium

## Introduction

The labeling of biologically active molecules with $^{99m}$Tc for radiopharmaceutical purposes is a field of intense research.[1–4] The commercially available perfusion agents for radioimaging have to be complemented by labeled vectors which will allow a more precise targeting of various receptors expressed in higher density on for example cancer cells. So far, a few compounds are in pre-clinical trials[5–9] but none has found commercial application so far. Many chemical and biological difficulties have to be overcome. Chemically, the targeting vector has to be i) derivatized with an appropriate chelator for $^{99m}$Tc, ii) should be labeled at high specific activity (low vector concentration) and finally retain its physicochemical properties and its affinity towards the corresponding receptor. For routine use, the labeling process must be performed preferentially in one single step. Different procedures are available from literature and for peptides in particular the hynic approach seems to be promising although it suffers from the lack of a clearly defined compound which is required for clinical approval.[10–15]

A valuable extention of these "classical" approaches is the use of bioorganometallic compounds, the versatility of which has recently been highlighted by Metzler-Nolte.[16] In that context, we recently presented the one pot synthesis of the organometallic aqua ion $[^{99m}Tc(OH_2)_3(CO)_3]^+$ and showed the versatility of using this complex fragment for the labeling of various biomolecules and peptides in particular.[17–23] One of the major advantages of the carbonyl approach is the availability of a well defined complex with very high specific activity only depending from the ligand type.[24] Naturally occurring bidentate ligands such as N-terminal histidines in peptide chains can efficiently be labeled with $[^{99m}Tc(OH_2)_3(CO)_3]^+$. An improvement in respect of specific activity was the introduction of a terminal histidine through

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Supporting information for this article is available on the WWW under http://www.chemeurj.org or from the author: Ortep plots and atom labeling schemes for non-hydrogen atoms of **6a** and **11**. X-ray crystallographic tables of complexes **3**, **6a**, and **11**.

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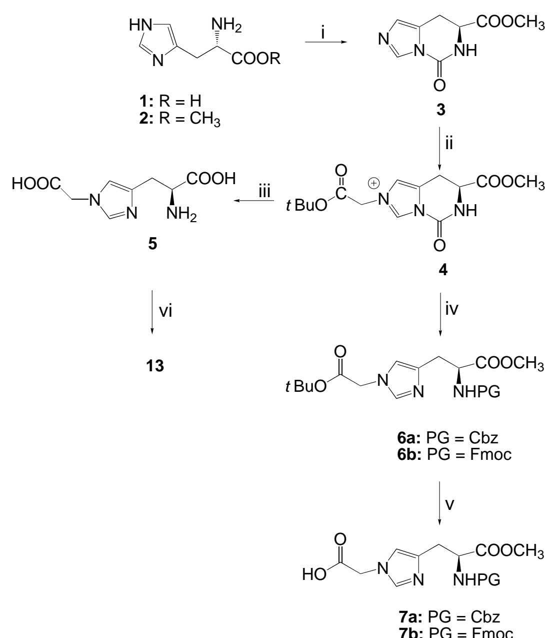
the $\alpha$-amino group which allowed labeling at low ligand concentration.[25] The relatively high lipophilicity of this type of bifunctional chelator related to the difficulty of its synthesis prompted us to seek for a differently derivatized histidine which would allow its introduction into any peptide with a minimum of synthetic work and a maximum of labeling efficiency. Since the complex $[^{99m}Tc(his)(CO)_3]$ is hydrophilic it is appropriate to derivatize the histidine at the $\varepsilon$-nitrogen in the imidazole ring. This functionalization leaves the highly efficient tripodal coordination site untouched but still allows the coupling to amine or carboxylic groups in biomolecules. Finally, since the synthesis of $[^{99m}Tc(his)(CO)_3]$ can be performed in one single step from $[^{99m}TcO_4]^-$, histidine also fulfils the requirement for a one pot labeling procedure without affecting the ligand.

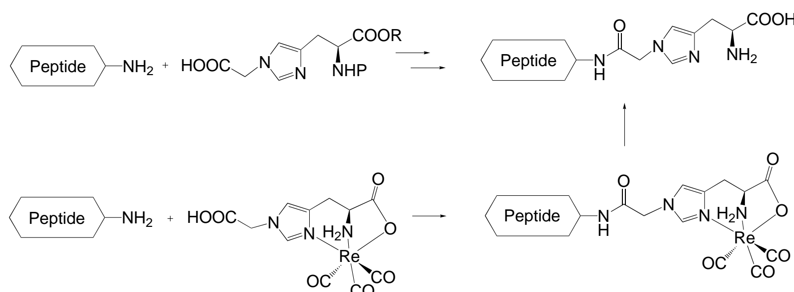
We want to present in this paper two different strategies for the synthesis of $N^\varepsilon$ derivatized tripodal histidine, its convenient coupling to an amino acid and its highly efficient labeling with $^{99m}$Tc. One strategy employs the $[Re(CO)_3]^+$ moiety as a novel organometallic protecting group for the three functionalities, carboxylic acid, primary and aromatic $N^\delta$ amine in histidine. Alkylation at $N^\varepsilon$ in $[Re(his)(CO)_3]$[26] can be performed in parallel to a procedure very recently described for $[Mo(his)(CO)_3]^-$.[27–29] After coupling to an amino acid or a peptide, the organometallic protecting group is released by mild oxidation. Alternatively, the functionalities in histidine were protected stepwise by organic groups. The simultaneous protection of $N^\delta$ and $N^\alpha$ in one step by formation of a cyclic urea ring is very convenient and is used to introduce an active coupling group in $N^\varepsilon$. The two methods are shown in Scheme 1.

## Results and Discussion

The coupling of histidine **1** through a carboxyl functionality introduced at $N^\varepsilon$ to peptides is a multi-step procedure. The acetyl linker attached to $N^\varepsilon$ has to be selectively deprotected after the alkylation. Deprotection, activation and coupling to peptides or amino acids should not affect the other protecting groups as well. Deprotection of the coordinating functionalities after the coupling process must occur under conditions typically encountered in peptide synthesis to be generally useful. Due to potential cross reactions, the protecting groups for $N^\delta$, $N^\alpha$ and the carboxylate had to be introduced prior to $N^\varepsilon$ derivatization. A major difficulty arose from the selective protection of $N^\varepsilon$ and $N^\delta$ since typical protecting agents tend to react with both. In general, mixtures of $N^\varepsilon$ and $N^\delta$ derivatives are very difficult to separate even if the desired $N^\varepsilon$-derivative represents the main product. In addition, $N^\alpha$ can also be very competitive along such a derivatization. The key step of our method to bypass these problems is represented by the ring closure and the formation of a six-membered urea ring in histidine which allows simultaneous and exclusive protection of $N^\alpha$ and $N^\delta$ in histidine methyl ester. Subsequently, $N^\varepsilon$ was alkylated to give the desired derivative of histidine. Amine and carboxylate groups can be introduced in that way, we focus in this paper only on the latter. These groups can then be coupled with the C- or the N-terminus of a peptide through amide formation. The approach is straightforward but demands a multi-step synthesis. The full reaction scheme for the introduction of a alkyl carboxylate at $N^\varepsilon$ is given in Scheme 2.




Scheme 2. i) **2**, $Im_2CO$, DMF, 70 °C, 6 h, 61 %; ii) bromoacetic *tert*-butyl ester, $CH_3CN$, reflux, 24 h; iii) 6 N HCl, reflux, 48 h; iv) **6a**: BnOH, THF, reflux, 16 h, 62 % (from **3** in two steps); **6b**: Fm-OH, $CH_3CN$, RT, 14 h, 55 % (from **3** in two steps); v) TFA/$CH_2Cl_2$ 1:1, RT, 2.5 h; vi) $[Et_4N]_2[ReBr_3(CO)_3]$, $H_2O$, RT, 21 h, 68 % (from **3** in three steps).




Scheme 1. Introduction of tripodal, $N^\varepsilon$ functionalized histidine into a biomolecule for labeling with $[^{99m}Tc(CO)_3]^+$.

An approach to protect $N^\alpha$ and $N^\delta$ in one step is the reaction of L-histidine methyl ester **2** with *N,N'*-carbonyldiimidazole in DMF which led to compound **3** by formation of a six-membered urea ring comprising the $N^\alpha$ and $N^\delta$ protected amino acid in very good yield.[30] The structure of compound **3** could be confirmed by an X-ray structure and the molecule is depicted in Figure 1.[31]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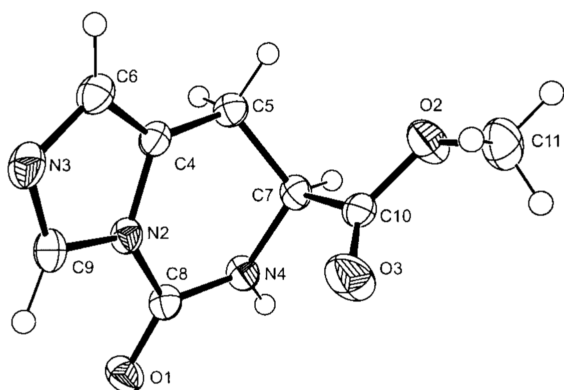



Figure 1. ORTEP plot of **3**, ellipsoids drawn at 50% probability.

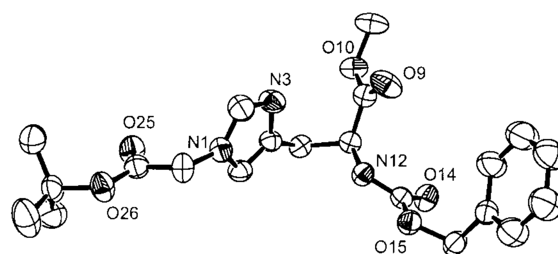


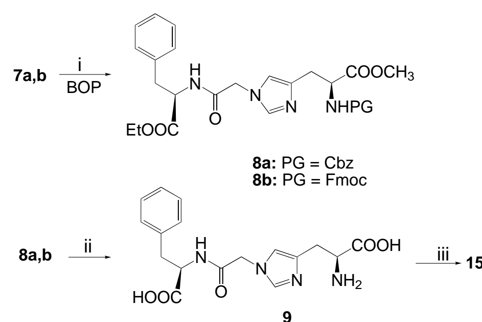

Figure 2. ORTEP plot of **6a**, ellipsoids drawn at 50% probability, showing one of the two molecules in the asymmetric unit.

Alkylation of the urea compound **3** with bromoacetic *tert*-butyl ester in refluxing acetonitrile for 24 h provided the quaternary ammonium salt **4** in almost quantitative yield. According to $^1H$ NMR spectroscopy, the crude product **4** was clean enough to proceed to the next step without further purification.

Starting from compound **4**, several options are feasible to achieve a histidine derivative of a biomolecule. Refluxing **4** in 6 M HCl for 48 h led to the fully deprotected compound **5** which can then be used for introduction into a biomolecule with the "$[Re(CO)_3]^+$" moiety as a protecting group, as discussed later in this text. Optionally, the urea ring can be opened by refluxing **4** in neat methanol or ethanol. If subsequent coupling with a biomolecule is attempted, the resulting carbamates will not be very convenient since their cleavage to give the amines requires drastic conditions. We have therefore chosen other alcohols such as benzyl alcohol or fluorenol (Fm-OH) which would result in a Cbz or a Fmoc group easier to cleave. In neat benzyl alcohol, many side products were found and only the reaction in acetonitrile or THF gave a clear product. The $N^\alpha$-Cbz protected compound **6a** was obtained from the reaction of benzyl alcohol in refluxing THF in the presence of $NEt_3$ overnight in an overall yield of 62% relative to **3**. Compound **6b** was prepared from acetonitrile since Fm-OH is poorly soluble in THF. Unexpectedly, the reaction with Fm-OH required much milder conditions than the reaction with any other alcohol. We observed that reflux conditions or even 40 °C were sufficient to cause spontaneous deprotection of the once formed Fmoc group. Thus, the ring opening reaction was performed at RT for 14 h to give a 55% yield of **6b** relative to **3**. To obtain a Boc protecting group, the reaction was performed with *tert*-butanol; however even after 4 d the yield was less than 20%. The X-ray structure of **6a** could be elucidated and is shown in Figure 2.[32]

Compounds **6a** and **6b** are fully protected with groups each of which can independently be removed to receive a variety of histidine derivatives. These can be employed in further derivatizations for biomolecule coupling through $N^\alpha$, $N^\delta$, or the carboxyl group of histidine. A free acetyl group at $N^\varepsilon$ histidine is required for attaching **6a** or **6b** to the N-terminus of a peptide after standard activation. Correspondingly, the *tert*-butyl ester group in **6a** and **6b** were hydrolyzed to the carboxylic group by reaction with $TFA/CH_2Cl_2$ 1:1 for 2.5 h to afford compounds **7a** and **7b** which can now be coupled to a free amino group in the biomolecules. Deprotection of $N^\alpha$ and hydrolysis of the remaining ester will then give the histidine derivatives retaining its tripodal mode of coordination. To probe this process with an amino acid, we have chosen L-phenylalanine ethyl ester (H-Phe-OEt). The reaction of **7a** or **7b** with H-Phe-OEt in the presence of BOP as a coupling reagent and $NEt_3$ as an organic base gave the compounds **8a** and **8b** in 90 and 74% yield as white solids. The reaction was strongly influenced by the pH of the solution and it proved to be important to keep it neutral by repeatedly adding $NEt_3$ during the reaction.

To enable the coordination of the *fac*-$[Re(OH_2)_3(CO)_3]^+$[33] complex to tripodal histidine in **8a** or **8b** (or in any other derivatized biomolecule) or for labeling with the $^{99(m)}Tc$ homologue $[^{99(m)}Tc(OH_2)_3(CO)_3]^+$,[34] full deprotection is required. In case of **8a** which contains benzyloxycarbamate (Cbz) as a protecting group, a two-step procedure was employed to produce **9** (Scheme 3). In a first step, Cbz was removed by hydrogenation with $H_2$ and Pd/C under acidic




Scheme 3. i) H-Phe-OEt, BOP, $Et_3N$, $CH_2Cl_2$, RT, 1.5–2.5 d, 74–90%; ii) deprotection: Cbz: $H_2$, Pd/C, $MeOH/H_2O/AcOH$ (7:2:1), RT, 2 h; Fmoc: piperidine, RT, 30 min; ester: 0.5 M $LiOH_{aq}$/MeOH (1:2), RT, overnight; iii) $[Et_4N]_2[ReBr_3(CO)_3]$, $H_2O$, RT, 19 h, 40–50%.

conditions. Clean deprotection occurred with a yield of 75–80% in $MeOH/H_2O/AcOH$ (7:2:1). Subsequent and quantitative deprotection of the ester groups was performed by alkaline hydrolysis in aqueous 0.5 M LiOH at RT overnight. A much more convenient one step deprotection is possible for **8b**, containing Fmoc as a protecting group. Stirring these compounds in piperidine for 40 min at RT was sufficient to remove all the protecting groups and to produce **9** in one step and in quantitative yield. Thus, for convenience the applica-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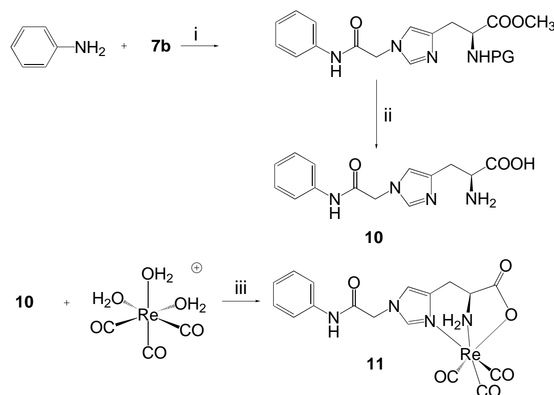
tion of **7b** for the derivatization of biomolecules with tripodal histidine is highly favoured.

This fully deprotected derivative **9** was treated with $[Re(OH_2)_3(CO)_3]^+$ to yield the corresponding neutral complex, that is, [(**9**)Re$(CO)_3$] **15**. Beside the coupling to H-Phe-OEt, we linked **7b** to a variety of other model compounds containing an amino group in order to get suitable crystals to confirm the structure of a rhenium complex by X-ray structure analysis. Compound **7b** was coupled to aniline to yield the corresponding anilide **10** after deprotection with piperidine (Scheme 4). Reaction of **10** with $[Re(OH_2)_3(CO)_3]^+$ in water or methanol gave the rhenium




Scheme 4. i) BOP, $Et_3N$, $CH_2Cl_2$/DMF, RT, 2 d; ii) piperidine, RT, 30 min; iii) $[Et_4N]_2[ReBr_3(CO)_3]$, $H_2O$, RT, overnight, 75 %.

complex [(**10**)Re$(CO)_3$] **11**, its structure could finally be elucidated,[35] and an ORTEP presentation is given in Figure 3.

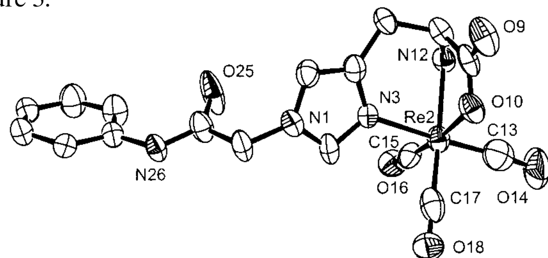
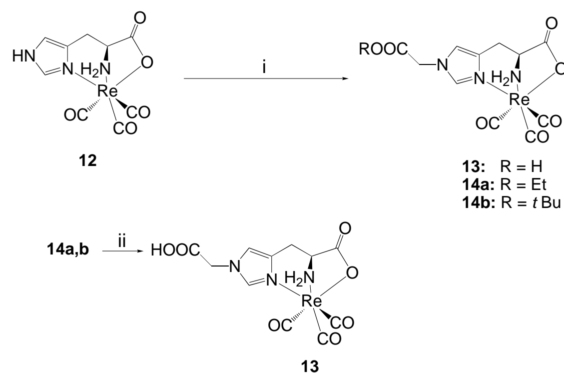



Figure 3. ORTEP plot of **11**, ellipsoids drawn at 50 % probability, showing one of the two molecules in the asymmetric unit.

The molecular structure of **11** shows the tripodal histidine coordination to the $[Re(CO)_3]^+$ fragment occurring through $N^\delta$, $N^\alpha$ and the carboxylate. The complex is neutral and the primary amino group is not deprotonated. The anilide group, which is a model for a biomolecule, points away from the metal complex. Bond lengths and angles are within the expected range and comparable to the isoelectronic Mo complex $[Mo(his)(CO)_3]^-$.[28]

The method discussed with organic protecting groups is convenient to introduce tripodal histidine in biomolecules. Deprotection occurs under mild conditions and is compatible with sensitive biomolecules. As a valid alternative to the pure organic procedure, we have introduced the organometallic moiety $[Re(CO)_3]^+$ as a protecting motif which occupies the three reactive sites in histidine at the same time. This significantly reduces the number of steps for derivatization and coupling. The coordination of $[Re(CO)_3]^+$ with L-histidine gives quantitatively well characterized **12**[26, 36] in which $N^\alpha$, $N^\delta$, and the carboxylate are all protected in one single step. The alkylation of **12** with ethyl bromoacetate or *tert*-butyl bromoacetate in the presence of $Cs_2CO_3$ directly produced the alkylated complex **14a** or **14b**, respectively, as single products in very good yield (Scheme 5). A similar reaction has recently been described for an organometallic




Scheme 5. i) Ethyl/*tert*-butyl bromoacetate, $Cs_2CO_3$, $CH_3CN$, 35 °C, 1.5 h, 90 %; ii) deprotection: ethyl ester: 0.5 M LiOH/MeOH (1:2), RT, overnight; *tert*-butyl ester: TFA/$CH_2Cl_2$ 1:1, RT, 2 h.

Mo complex with the purpose of attaching targeting molecules to the new carboxylate but not for protecting histidine.[37] It is imperative to note that alkylation at $N^\alpha$ or $N^\delta$ was not observed at all during the alkylation reaction, illustrating the high kinetic and thermodynamic stability of $[Re(CO)_3]^+$ in the histidine complex. With a labile complex, it might be expected that alkylation also occurs at the other amines in **1** when in equilibrium they are shortly released from the protecting metal.

The ester hydrolysis in **14a,b** to the corresponding carboxylic acid **13** was performed under different conditions depending on the type of ester. The ethyl ester of **14a** was deprotected by treatment with LiOH in MeOH/$H_2O$ mixture and the *tert*-butyl ester of **14b** was removed under acidic conditions with trifluoroacetic acid in methylene chloride. With each method, **13** was produced in high yield which can now be coupled to a biomolecule by standard techniques. Phenylalanine was chosen as a model compound and coupled with **13** to produce **15** (Scheme 6). The $[Re(CO)_3]^+$ acts as a versatile protecting group for **1** during the whole process. Although the use of metal cations or complex fragments as protecting groups is known in organometallic chemistry, examples with Werner-type ligands are rare and only a limited number of examples have been described at all.[38, 39] In principle, a metal center could protect six functionalities the same time. To successfully apply such a procedure, the complex fragment must be robust, otherwise dynamic decomplexation/complexation would not allow selective derivatization at non coordinated sites. The prerequisite limits the type of metal centers for this purpose essentially to $d^6$ systems as is the case with the $[Re(CO)_3]^+$ moiety.

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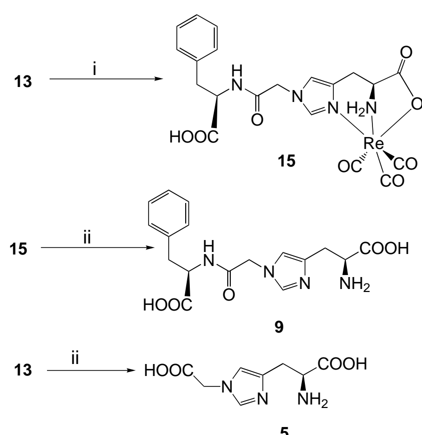



Scheme 6. i) H-Phe-OEt, BOP, $Et_3N$, $CH_2Cl_2$/DMF, RT, overnight, 75%; ester deprotection: 0.5 M LiOH/MeOH (1:2), RT, overnight, quantitative; ii) 10–30 equiv $H_2O_2$, $H_2O$, HCl (TFA or AcOH), 50 °C, 4–48 h.

Instead of alkylating **12**, ligand **5** can also directly be coordinated to the $[Re(CO)_3]^+$ moiety. Compound **5** was obtained analytically pure after reacting **4** in refluxing 6 N HCl for 2 d. Further reaction with $[Et_4N]_2[ReBr_3(CO)_3]$ in water afforded the complex **13** in 68% overall yield relative to **3** in three steps (Scheme 2).

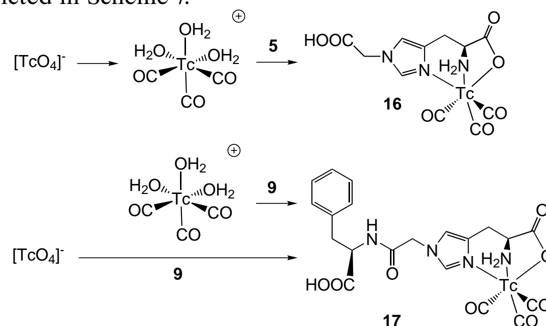
For subsequent labeling of a histidine derivatized biomolecule with $[^{99m}Tc(OH_2)_3(CO)_3]^+$, the protecting $[Re(CO)_3]^+$ moiety must be removed to receive the final bioconjugate. Due to the high kinetic and thermodynamic stability of $[Re(his)(CO)_3]$, simple decomplexation at low pH is not sufficient since recomplexation readily takes place.

The $[Re(CO)_3]^+$ moiety must be "deactivated" by oxidation with $H_2O_2$ under acidic conditions to yield $[ReO_4]^-$ and provide the unprotected derivatized biomolecule. Experimentally, the disappearance of the rhenium complex and the appearance of $[ReO_4]^-$ can conveniently be followed in the HPLC trace at 250 nm wavelength.

Complexes **13** and **15** were examined as simple models for more complicated biomolecules to assess the conditions required for the complete oxidative removal of $[Re(CO)_3]^+$. Conditions with variable equivalents of $H_2O_2$ relative to rhenium and different pH values were screened with three acids, HCl, TFA, and acetic acid. It was found that the $H_2O_2$ concentration (10, 20, or 30 equivalents) gradually improved the conversion of **13** or **15** to $[ReO_4]^-$. The pH (0.5, 1, or 3) of the solution greatly affected the reactivity of the rhenium complex towards oxidation. 20 or 30 equivalents of $H_2O_2$ relative to Re at a pH of 0.5 with HCl were sufficient to completely remove the Re after 8 h at 50 °C. At higher pH values, the reaction proceeds with significantly slower conversion rate to $[ReO_4]^-$. Similar results were also obtained with trifluoroacetic acid whereas the reaction with acetic acid and $H_2O_2$ showed only very slow oxidation. A low pH value is crucial for a reasonable conversion rate and suggests that oxidation occurs only when the $[Re(CO)_3]^+$ moiety is fully or partially decomplexed from (protonated) histidine ligand but not directly in closed shell $[Re(his)(CO)_3]^+$.

For the derivatization of a biomolecule with **5**, the use of $[Re(CO)_3]^+$ as a protecting group is versatile and the method shorter than the fully organic approach discussed in the first part. The only limitation for the choice of the biomolecule is its sensitivity towards oxidative conditions. When the peptide contains for example methionine, the organic method is favorable since oxidative removal of rhenium would lead to oxidation of the thio ether group. For many radiopharmaceutically interesting molecules such as central nervous system receptor ligands, this sensitivity does not apply and the method using an organometallic protecting group is much more straightforward.

The tripodal histidine ligand is one of the most efficient chelating system for the $[^{99m}Tc(CO)_3]^+$ moiety, a crucial prerequisite for the preparation of radiopharmaceuticals with the highest possible specific activity. If high biomolecule concentrations are required for complete labeling, the $^{99m}$Tc radiopharmaceutical has to be separated from unlabeled, cold material to prevent blocking of the receptors with non-labeled compounds. Ideally, the concentration of the derivatized biomolecule is equal to $^{99m}$Tc. Since the concentration of $^{99m}$Tc is very low ($10^{-7}$–$10^{-6}$ M), this is hardly achieved for kinetic reasons but histidine allows complete labeling at concentrations near stochiometry.[24] To assess this behaviour for the derivatized histidines, we have performed labeling studies under various, radiopharmaceutically useful conditions. Two labeling procedures are possible. One is a two-step procedure consisting in initial preparation of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ and subsequent coordination to the compounds **5** and **9** to yield **16** and **17**, respectively. Clinically more relevant, the labeling is performed directly in one single step in one single vial from $[^{99m}TcO_4]^-$ in the presence of for example **9** and the reducing/CO transfer reagent $Na_2[H_3BCO_2]$. The two procedures are depicted in Scheme 7.




Scheme 7. One- and two-step labeling of a histidine derivatized amino acid.

The complex $[^{99m}Tc(OH_2)_3(CO)_3]^+$ was prepared as described in the literature[17, 40] or from commercially available Isolink kits (available from Mallinckrodt). After buffering with phosphate buffer of pH 7.4, an aliquot of a standard solution of the ligands **5** or **9** was added in a final concentration of $10^{-4}$, $10^{-5}$ or $10^{-6}$ M, in general in a total volume of 1 mL. For each reaction, only the $^{99m}$Tc complexes **16** and **17** were obtained. The two different synthetic routes (the organic or the organometallic protection pathway) gave similar labeling yields illustrating that compound **15** did not decompose during the oxidative deprotection process. Slightly lower yields found for the labeling of ligands obtained from the oxidation process are likely to be due to lower effective

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concentrations. For characterization of the $^{99m}$Tc compounds, the retention times were compared to the fully characterized rhenium analogues and proved to be the same. Labeling yields under different conditions are summarized in Table 1.

Table 1. Labeling of Ligand **5** and **9** with $[^{99m}Tc(CO)_3]^+$.

| Method | No. of ligand | $c$ [M] | $T$ [°C] | $t$ [min] | Yield [%] |
|---|---|---|---|---|---|
| two-step labeling | **5** | $10^{-4}$ | 70 | 30 | quantitative |
| | **5** | $10^{-5}$ | 70 | 30 | 91[a] |
| | **5** | $10^{-6}$ | 70 | 30 | 20[b] |
| | **9** | $10^{-4}$ | 90 | 30 | quantitative[c] |
| | **9** | $5 \times 10^{-5}$ | 90 | 30 | quantitative |
| | **9** | $10^{-5}$ | 90 | 30 | quantitative[d] |
| | **9** | $10^{-6}$ | 90 | 30 | 41[e] |
| one-pot labeling | **9** | $10^{-4}$ | 90 | 20 | 94 |
| | **9** | $5 \times 10^{-5}$ | 90 | 20 | 96 |
| | **9** | $10^{-5}$ | 90 | 20 | 34[e] |

[a] The labeling was done quantitatively in 1 h. Ligand **5** from the oxidation, at 75 °C for 30 min yield was 85 %. [b] The labeling reached 64 % yield after 1.5 h. [c] Ligand **9** from the oxidation showed 88 % yield at 90 °C for 30 min. [d] Ligand **9** from the oxidation showed 73 % yield at 90 °C for 30 min. [e] The labeling reached more than 65 % yield after 1.5 h.

At a ligand concentration of $10^{-4}$ M both **5** and **9** were quantitatively labeled at 70 °C after 30 min to provide the products **16** and **17**. The yields relative to $[^{99m}Tc(OH_2)_3(CO)_3]^+$ are better than 95 %, the difference counts for $[^{99m}TcO_4]^-$ small amounts of which were sometimes present after the synthesis of $[^{99m}Tc(OH_2)_3(CO)_3]^+$. In case of ligand **5**, labeling was completed even at RT after 30 min at this concentration. To afford complete complex formation, $10^{-5}$ M solutions required longer reaction times or higher temperatures and gave exclusively **16** and **17**. In case of complex **17**, quantitative yield was achieved at 90 °C within 30 min. Since the concentration of the ligand is reduced by a factor of 10 in this pseudo first-order kinetics, higher temperature must compensate low concentration. At $10^{-6}$ M (corresponds to 1 nmol of biomolecule), the concentrations of $^{99m}$Tc and ligand become comparable and the kinetic law switches to second order slowing down the rate further. The labeling reaction took longer and the yields at 70 °C were 64 % for **5** and 69 % for **9** after 90 min, respectively. At this concentration the oxidation of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ by traces of $O_2$ becomes a major side reaction but still significant amounts of labeled compounds were produced.

The one-pot reaction from $[^{99m}TcO_4]^-$ in the presence of **9** to compound **17** was investigated according to Scheme 7. The $[^{99m}TcO_4]^-$ generator eluate was now added to a vial containing the compounds required for the preparation of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ and, in addition, a solution of ligand **9** to yield a final ligand in the concentration of $10^{-4}$, $5 \times 10^{-5}$ and $10^{-5}$ M, respectively. At 90 °C, for both concentrations $10^{-4}$ M and $5 \times 10^{-5}$ M, almost quantitative formation of **17** was found within 20 min. No side products were observed by radioactive HPLC analysis. At $10^{-5}$ M the yield was 67 % after 90 min. Although heating at 95 °C was continued for much longer time, no significant formation of side products was observed proving the high thermal stability of the product **17**. The one-pot reaction under reductive conditions did not affect the peptide bond in **9** and no reduction or hydrolysis occurred. This demonstrates the versatility of this one-pot process not only for the model compounds presented herein but also for other biologically active polypeptides or other receptor binding molecules. The conditions employed in the one-pot reaction corresponds exactly to what is required for routine application, the quantitative formation of one single structurally determined species at very high dilution. This product is principally ready to be injected without purification or separation from excess cold material or byproducts.

## Conclusion

We have presented in this report two convenient methods for the introduction of a tripodal $N^{\varepsilon}$ derivatized histidine into model peptides. The organic method requires more steps but can also be applied to biomolecules susceptible to oxidation whereas the method using an organometallic moiety as protecting group is straightforward but limited to biomolecules which are not very sensitive towards oxidation. Both methods end up with the same compounds in which an acetyl group at the imidazole ring can be coupled to an amino group of any biomolecule. The functionality on the pendant arm attached to $N^{\varepsilon}$ is variable and primary amines as well as hydroxy groups can be introduced. After deprotection, the histidines can be labeled with $^{99m}$Tc to yield one single radiopharmaceutical of very high specific activity without purification. A full one-step procedure is possible without preparing precursors or intermediates. Finally due to the high hydrophilicity of the attached histidine complex, this method is in particular suitable for hydrophilic biomolecules such as most of the biologically active peptides. Currently, we are studying a number of these peptides for their application as novel radiopharmaceuticals.

## Experimental Section

**General techniques**: All chemicals were purchased at highest commercial quality and used without further purification, unless stated otherwise. All the reactions were performed under nitrogen or argon and monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Merck silica gel aluminium plates (60 F254) using UV light as visualizing agent. Silica gel (particle size 0.040–0.063 mm) was used for flash column chromatography. NMR spectra were recorded on Bruker, DRX-500 (500 MHz) or Varian Gemini-2000 (300 MHz) instruments. Mass spectra were recorded on a Merck, M-8000 LC/3DQMS under electrospray ionization(ESI) condition. Yields refer to chromatographically and spectroscopically homogeneous materials.

Crystal data and experimental details are listed in the Supporting Information. Suitable crystals were covered with Paratone N oil, mounted on top of a glass fibre and immediately transferred to a Stoe IPDS diffractometer. Data was collected at 183(2) K using graphite-monochromated $Mo_{K\alpha}$ radiation ($\lambda = 0.71073$ Å). Data was corrected for Lorentz and polarisation effects as well as for absorption (numerical, but not for **3**). Structures were solved with direct methods using SHELXS-97[41] or SIR97[42] and were refined by full-matrix least-squares methods on $F^2$ with SHELXL-97.[43] In case of the organic structures **3** and **6a**, the configuration of the histidine was set to match with the configuration of the used L-histidine. The asymmetric units of **6a** and **11** contain two molecules each. In both cases with the exception of a torsion angle, the corresponding pair of molecules possesses a practical identical geometry.

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$[NEt_4]_2[ReBr_3(CO)_3]$,[33] *fac*-$[Re(his)(CO)_3]$,[36] and *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$[17] were prepared as previously reported.

**5-Oxo-5,6,7,8-tetrahydroimidazo[1,5-*c*]pyrimidine-7-carboxylic methyl ester (3)**: The compound was prepared according to literature with slight modification.[30] $Im_2CO$ (1.88g, 11.61 mmol) was added at RT to a solution of L-histidine methyl ester (2.73 g, 11.28 mmol) in DMF (80 mL). The reaction mixture was heated to 70 °C for 6 h, then cooled down to RT and poured slowly to 1M $NaHCO_3$ aqueous solution (250 mL). Some solid precipitated from the aqueous layer which was extracted with $CH_2Cl_2$. During extraction the precipitate dissolved completely in $CH_2Cl_2$. The combined organic extracts were dried over $Na_2SO_4$, concentrated under reduced pressure, and purified by flash column chromatography to afford **3** as white solid (1.35 g, 61 %). $R_f = 0.2$ (EtOAc); $^1H$ NMR (500 MHz, $CD_3CN$, 25 °C): $\delta = 8.01$ (s, 1H; $CH_{im}$), 6.77 (s, 1H; $CH_{im}$), 6.61 (brs, 1H; NH), 4.37–4.34 (m, 1H; CHCO), 3.67 (s, 3H; $OCH_3$), 3.25–3.23 (m, 2H; $CH_2CH$); $^{13}C$ NMR (500 MHz, $CD_3CN$, 25 °C): $\delta = 172.1$, 149.2, 135.4, 126.9, 125.9, 53.6, 53.5, 23.7; MS (ESI): *m/z* (%): 195.73 (100) [$M^+$], 167.8 (35), 135.8 (24); elemental analysis calcd (%) for $C_8H_9N_3O_3$ (195.18): C 49.23, H 4.62, N 21.54; found: C 49.32, H 4.77, N 21.24. Crystals suitable for X-ray structure analysis were obtained by slow evaporation from EtOAc.

**2-(2-*tert*-Butoxy-2-oxoethyl)-7-methoxycarbonyl-5-oxo-5,6,7,8-tetrahydroimidazo[1,5-*c*]pyrimidine-2-ium bromide (4)**: Bromoacetic *tert*-butyl ester (0.57 mL, 3.86 mmol) was added to a solution of **3** (250 mg, 1.28 mmol) in $CH_3CN$ (25 mL). The reaction mixture was heated under reflux for 24 h, then cooled to RT and concentrated in vacuo. The residue was washed with $Et_2O$ (2 × 10 mL) and THF (2 × 5 mL) and dried in vacuo to afford **4** as a white sticky solid which was used in the next step without any further purification. $^1H$ NMR (300 MHz, $D_2O$, 20 °C): $\delta = 9.39$ (s, 1H; $CH_{im}$), 7.40 (s, 1H; $CH_{im}$), 5.05 (s, 2H; $CH_2N_{im}$), 4.78–4.61 (m, 1H; CHCO), 3.63 (s, 3H; $OCH_3$), 3.42–3.39 (m, 2H; $CH_2CH$), 1.39 (s, 9H; *t*Bu); MS (ESI): *m/z* (%): 309.40 (13) [$M^+ - HBr$], 253.80 (100) [$M^+ - HBr - (CH_2{=}C(CH_3)_2)$].

**Methyl *N*-[(benzyloxy)carbonyl]-1-(2-*tert*-butoxy-2-oxoethyl) histidinate (6a)**: DIPEA (0.52 mL, 3.01 mmol) and BnOH (2.1 mL, 20.08 mmol) were added to a solution of crude **4** (390 mg) in THF (50 mL). After 16 h of refluxing, the reaction solution was cooled down to room temperature, concentrated under reduced pressure, and purified by flash column chromatography to afford **6a** as white solid (260 mg, 62 % from **3**). $R_f = 0.15$ ($CH_2Cl_2$/MeOH 45:1); $^1H$ NMR (500 MHz, $CD_3CN$, 25 °C): $\delta = 7.39$–7.32 (m, 6H; 5 × $CH_{ph}$, $CH_{im}$), 6.78 (s, 1H; $CH_{im}$), 6.66 (brd, $J = 7.8$ Hz, 1H; NH), 5.06 (s, 2H; $CH_2$-Bn), 4.58 (s, 2H; $CH_2N_{im}$), 4.42 (q, $J = 2.6$ Hz, 1H; CHCO), 3.62 (s, 3H; $OCH_3$), 2.96 (t, $J = 5.26$ Hz, 2H; $CH_2CH$); $^{13}C$ NMR (500 MHz, $CD_3CN$, 25 °C): $\delta = 173.2$, 168.3, 157.0, 139.1, 138.2, 137.8, 129.5, 129.0, 128.9, 119.2, 83.2, 67.2, 66.9, 55.2, 52.7, 49.3, 30.2, 28.2; MS (ESI): *m/z* (%): 417.53 (100) [$M^+$]; elemental analysis calcd (%) for $C_{21}H_{27}N_3O_6$ (417.48): C 60.43, H 6.47, N 10.07; found: C 60.43, H 6.57, N 9.97. Crystals suitable for X-ray structure analysis were obtained by vapor diffusion of 1-hexene into EtOAc.

**Methyl 1-(2-*tert*-butoxy-2-oxoethyl)-*N*-(9*H*-fluoren-9-ylmethoxycarbonyl) histidinate (6b)**: Crude **4** (400 mg), Fm-OH (543 mg, 2.77 mmol), and DIPEA (0.24 mL, 1.39 mmol) were dissolved in acetonitrile (50 mL). After 16 h at RT the reaction solution was neutralized by adding 1N HCl solution and concentrated under reduced pressure. The residue was dissolved in $CH_2Cl_2$ (80 mL) and extracted with water (50 mL) and 1N HCl solution (50 mL). The organic layer was dried over $Na_2SO_4$, concentrated in vacuo and purified by flash column chromatography afforded **6b** as a colorless oil (233 mg, 55 % from **3**). $R_f = 0.1$ ($CH_2Cl_2$/MeOH 60:1); $^1H$ NMR (300 MHz, $CD_3CN$, 20 °C): $\delta = 7.83$ (d, $J = 7.63$ Hz, 2H; $CH_{fluoren}$), 7.64 (d, $J = 7.66$ Hz, 2H; $CH_{fluoren}$), 7.44–7.31 (m, 5H; $CH_{im}$, 4 × $CH_{fluoren}$), 6.78 (s, 1H; $CH_{im}$), 6.63 (brd, $J = 7.83$ Hz, 1H; NH), 4.56 (s, 2H; $CH_2N_{im}$), 4.44–4.34 (m, 3H; CHCO, $CH_2$-fluoren), 4.23 (t, $J = 7.0$ Hz, 1H; CH-fluoren), 3.61 (s, 3H; $OCH_3$), 2.94 (m, 2H; $CH_2CH$), 1.43 (s, 9H; *t*Bu); $^{13}C$ NMR (300 MHz, $CD_3CN$, 20 °C): $\delta = 173.5$, 168.6, 157.2, 145.4, 142.4, 139.3, 138.1, 128.9, 128.3, 126.3, 121.2, 119.4, 83.3, 67.3, 55.3, 52.8, 49.3, 48.1, 30.3, 28.2; MS (ESI): *m/z* (%): 506.40 (100) [$M^+$+H]; elemental analysis calcd (%) for $C_{28}H_{31}N_3O_3$+0.5$H_2O$ (514.59): C 65.37, H 6.22, N 8.17; found: C 65.14, H 6.40, N 7.96.

**Methyl *N*-[(benzyloxy)carbonyl]-1-{2-[(1-ethoxycarbonyl-2-phenylethyl)-amino]-2-oxoethyl} histidinate (8a)**: A solution of **6a** (140 mg, 0.34 mmol) in $CH_2Cl_2$/TFA (2:2 mL) was stirred for 2.5 h at RT. The solvent was removed under reduced pressure and dried more in vacuo. The residue, the crude compound **7a** was dissolved in $CH_2Cl_2$ (10 mL) and neutralized by adding $Et_3N$ dropwise. BOP (148 mg, 0.34 mmol) and $Et_3N$ (46 µL, 0.34 mmol) were added to the reaction mixture. After 45 min, a solution of phenylalanine ethyl ester (84.6 mg, 0.37 mmol) and $Et_3N$ (51 µL, 0.37 mmol) in $CH_2Cl_2$ (10 mL) was added slowly by a syringe. The reaction mixture was stirred for an additional 2.5 d at room temperature. The solution was diluted with $CH_2Cl_2$ (30 mL) and extracted with 1N HCl solution (20 mL), 1N $NaHCO_3$ (20 mL), brine (20 mL). The organic layer was dried over $Na_2SO_4$, concentrated under reduced pressure and purified by flash column chromatography to afford **8a** as a colorless oil (162 mg, 90 %). $R_f = 0.2$ ($CH_2Cl_2$/MeOH 40:1); $^1H$ NMR (500 MHz, $CD_3CN$, 25 °C): $\delta = 7.37$–7.28 (m, 9H; 2 × 4H-$CH_{ph}$, $CH_{im}$), 7.16 (d, $J = 8.23$ Hz, 2H; 2 × $CH_{ph}$), 6.77 (brd, $J = 7.65$ Hz, 1H; NH), 6.69–6.66 (m, 2H; $CH_{im}$, NH), 5.05 (s, 2H; $CH_2$-Bn), 4.61 (dt, $J = 7.86$ Hz, 1H; CH-Phe), 4.52 (s, 2H; $CH_2N_{im}$), 4.43–4.41 (m, 1H; CHCO), 4.11 (q, $J = 7.15$ Hz, 2H; $CH_2CH_3$), 3.62 (s, 3H; $OCH_3$), 3.10 (dd, $J = 8.19$ Hz, 1H; $CH_2$-Phe), 2.99–2.93 (m, 3H; $CH_2$-Phe, $CH_2CH$), 2.57 (s, N-$CH_3$), 1.18 (t, $J = 7.13$ Hz, 3H; $CH_3$); $^{13}C$ NMR (500 MHz, $CD_3CN$, 25 °C): $\delta = 173.3$, 172.0, 167.8, 157.0, 139.1, 138.3, 138.2, 137.8, 130.4, 129.5, 129.4, 129.0, 128.9, 127.9, 118.9, 67.2, 62.2, 55.2, 54.8, 52.8, 49.9, 38.0, 30.3, 14.5; MS (ESI): m/z (%): 537.53 (100) [$M^+$+H]; elemental analysis calcd (%) for $C_{28}H_{32}N_4O_7$ + 0.5$[N(CH_3)_2]_3P{=}O$ + 0.5$H_2O$ (634.5): C 58.63, H 6.62, N 12.14; found: C 58.98, H 6.89, N 12.48.

**Methyl 1-{2-[(1-ethoxycarbonyl-2-phenylethyl)amino]-2-oxoethyl}-*N*-(9*H*-fluoren-9-ylmethoxycarbonyl) histidinate (8b)**: The title compound was prepared under the same condition as described for **8a**. A solution of **7b** (13 mg, 0.03 mmol), prepared from compound **6b** in TFA/$CH_2Cl_2$, BOP (13 mg, 0.03 mmol), and $Et_3N$ (8 µL, 0.06 mmol) in $CH_2Cl_2$ was stirred for 1.5 d at room temperature. Flash column chromatography yielded **8b** (13 mg, 74 %). $R_f = 0.15$ ($CH_2Cl_2$/MeOH 60:1); $^1H$ NMR (300 MHz, $CD_3CN$, 20 °C): $\delta = 7.83$ (d, $J = 7.61$ Hz, 2H; 2 × $CH_{fluoren}$), 7.63 (d, $J = 7.44$ Hz, 2H; 2 × $CH_{fluoren}$), 7.43–7.23 (m, 9H; 4 × $CH_{fluoren}$, 4 × $CH_{ph}$, $CH_{im}$), 7.14 (d, $J = 6.25$ Hz, 1H; $CH_{ph}$), 6.71–6.69 (m, 2H; $CH_{im}$, NH), 6.60 (brd, $J = 7.83$ Hz, 1H; NH), 4.61 (dt, $J = 7.82$ Hz, 1H; CH-Phe), 4.51 (s, 2H; $CH_2N_{im}$), 4.44–4.33 (m, 3H; $CH_2$-fluoren, CH-fluoren), 4.22 (t, $J = 6.41$ Hz, 1H; CHCO), 4.09 (q, $J = 7.15$ Hz, 2H; $CH_2CH_3$), 3.61 (s, 3H; $OCH_3$), 3.09 (dd, $J = 5.64$ Hz, 1H; $CH_2$-Phe), 2.96 (dd, $J = 6.91$ Hz, 3H; $CH_2$-Phe, $CH_2CH$), 1.17 (t, $J = 7.12$ Hz, 3H; $CH_3$); $^{13}C$ NMR (300 MHz, $CD_3CN$, 20 °C): $\delta = 173.5$, 172.2, 168.0, 145.4, 142.4, 139.2, 137.9, 130.5, 129.4, 128.9, 128.4, 128.1, 126.3, 121.2, 119.1, 67.3, 62.3, 55.3, 54.8, 52.8, 50.0, 48.1, 38.1, 30.9, 30.4, 14.4; MS (ESI): *m/z* (%): 625.68 (100) [$M^+$+H]; elemental analysis calcd (%) for $C_{35}H_{36}N_4O_7$+$H_2O$ (642): C 65.42, H 5.92, N 8.72; found: C 65.38, H 5.99, N 8.49.

**Re complex 15**

**From compound 8a**: To remove the Cbz group, **8a** (100 mg, 0.19 mmol) was dissolved in a solution of MeOH/$H_2O$/AcOH (7:2:1 *v*/*v*) (20 mL) which was added dropwise by syringe into the reaction flask containing Pd/C (100 mg). $H_2$ gas was purged through the reaction solution for 2.5 h at room temperature. The reaction mixture was filtered through Celite, rinsed with the same solution (5 mL), concentrated in vacuo, and purified by column chromatography to provide the methyl ester of **9** (59 mg, 79 %). $R_f = 0.15$ ($CH_2Cl_2$/MeOH/25 % $NH_4OH$ 10:1:0.1); $^1H$ NMR (300 MHz, $CD_3CN$, 20 °C): $\delta = 7.33$–7.14 (m, 6H; 5 × $CH_{ph}$, $CH_{im}$), 6.78 (brd, $J = 7.53$ Hz, 1H; NH), 6.69 (s, 1H; $CH_{im}$), 4.61 (dt, $J = 7.75$ Hz, 1H; CH-Phe), 4.53 (s, 2H; $CH_2N_{im}$), 4.10 (q, $J = 7.14$ Hz, 2H; $CH_2CH_3$), 3.70–3.63 (m, 4H; CHCO, $OCH_3$), 3.13–3.07 (m, 3H; $NH_2$, $CH_2$-Phe), 2.97 (dd, $J = 7.69$ Hz, 1H; $CH_2$-Phe), 2.86 (dd, $J = 5.02$ Hz, 1H; $CH_2CH$), 2.73 (dd, $J = 7.06$ Hz, 1H; $CH_2CH$), 1.18 (t, $J = 7.13$ Hz, 3H; $CH_3$); $^{13}C$ NMR (300 MHz, $CD_3CN$, 20 °C): $\delta = 176.3$, 172.0, 167.9, 139.1, 138.8, 137.7, 130.4, 129.5, 127.9, 120.3, 62.3, 55.5, 54.8, 52.4, 49.9, 38.1, 33.8, 14.5; IR (THF): $\tilde{\nu} = 1744$, 1696, 1196 $cm^{-1}$; MS (ESI): *m/z* (%): 403.13 (100) [$M^+$+H].

To remove the two ester groups, Cbz-deprotected phenylalanine-histidine (16 mg, 0.04 mmol) was dissolved in MeOH (2 mL) and 0.5M LiOH aqueous solution (1 mL) was added. After 17 h, the reaction solution was neutralized with 1N HCl solution. The reaction mixture was concentrated in vacuo and the crude product **9** was used in the next step without any further purification.

For the complexation, the crude all-deprotected compound **9** was dissolved in $H_2O$ (3 mL) and the pH of the solution was adjusted until 7–8 by adding

1 N HCl or 0.5 M LiOH solution. $[Et_4N]_2[ReBr_3(CO)_3]$ (30 mg, 0.04 mmol) was added immediately in one portion. A white solid precipitated at RT which was filtered after 19 h and rinsed with cold water (1 mL). The filtrate was concentrated in vacuo and purified by preparative HPLC to afford **15** (10 mg, 40%). HPLC gradient (%): 0–3 min = 0.1% TFA aqueous solution (100), 3.1–20 min = 0.1% TFA/MeOH (75:25–0:100), 21–30 min = MeOH (100). $^1$H NMR (500 MHz, $CD_3OD$, 25 °C): $\delta$ = 7.93 (s, 1H; $CH_{im}$), 7.26–7.17 (m, 5H; $CH_{ph}$), 6.84 (s, 1H; $CH_{im}$), 4.67 (s, 2H; $COCH_2$), 4.55–4.52 (m, 1H; CH-Phe), 3.98 (q, $J$ = 4.54 Hz, 1H; CHCO), 3.34–3.26 (m, 2H; $CH_2$-Phe), 3.24–3.10 (m, 2H; $CH_2CH$); $^{13}$C NMR (500 MHz, $CD_3OD$, 25 °C): $\delta$ = 198.6, 197.5, 197.3, 185.3, 167.4, 143.6, 139.6, 135.6, 130.5, 129.4, 127.6, 121.0, 57.4, 53.4, 50.8, 39.4, 30.9, 28.8; IR (KBr): $\tilde{\nu}$ = 3436, 2024, 1904, 1677, 1636, 1434, 1381, 1203, 1138 cm$^{-1}$; MS (ESI): $m/z$ (%): 631.07 (100) $[M^+ + H]$.

**From compound 13**: BOP (7.4 mg, 0.02 mmol) and $Et_3N$ (2 µL, 0.02 mmol) were added at room temperature to the solution of the complex **13** (8 mg, 0.02 mmol) in a solution of $CH_2Cl_2$/DMF (3:0.2 mL). After 30 min, a solution of phenylalanine ethyl ester (4 mg, 0.02 mmol) and $Et_3N$ (2 µL, 0.02 mmol) in $CH_2Cl_2$ (2 mL) was added dropwise to the solution by syringe. The reaction mixture was stirred overnight. The reaction solution was concentrated in vacuo. The residue was treated with diethyl ether (2 × 5 mL). The white solid was dissolved in THF (10 mL) and insoluble solid was filtered off. The filtrate was concentrated in vacuo to provide the ethyl ester of complex **15** (75%). $^1$H NMR (500 MHz, $CD_3CN$, 25 °C): $\delta$ = 7.8 (s, 1H; $CH_{im}$), 7.34–7.21 (m, 5H; $CH_{ph}$), 6.8 (s, 1H; $CH_{im}$), 4.63–4.59 (m, 1H; CHCO), 4.53 (d, 2H; $CH_2N_{im}$), 4.08 (q, 2H; $CH_2CH_3$), 3.92–3.88 (m, 1H; CH-His), 3.18–3.10 (2 × dd, 2H; $CH_2$-His, $CH_2$-Phe), 3.05–2.96 (2 × dd, 2H; $CH_2$-His, $CH_2$-Phe), 1.17 (t, 3H; $CH_3$); $^{13}$C NMR (500 MHz, $CD_3CN$, 25 °C): $\delta$ = 198.1, 196.6, 196.5, 180.4, 170.8, 165.5, 142.0, 136.7, 134.3, 129.4, 128.4, 126.8, 124.9, 120.3, 119.6, 61.2, 54.1, 51.4, 49.2, 37.2, 27.7, 13.4; IR (KBr): $\tilde{\nu}$ = 2020, 1886, 1733, 1636 cm$^{-1}$; MS (ESI): $m/z$ (%): 659 (100) $[M^+ + H]$; elemental analysis calcd (%) for $C_{22}H_{24}N_4O_8Re$ (658.6): C 40.12, H 3.67, N 8.51; found: C 39.31, H 3.83, N 8.25.

The ethyl ester group of the complex was hydrolyzed by stirring the complex overnight in solution of 0.5 M LiOH and MeOH (1:2) at room temperature, as mentioned above, to afford complex **15** quantitatively.

**Re complex 11**: The compound **7b** was treated with aniline, BOP, $Et_3N$ in a solution of $CH_2Cl_2$/DMF under the same condition as described for **8a**, followed by deprotection of functional groups with piperidine to prepare the compound **10**. The compound **10** was coordinated with $[Et_4N]_2[ReBr_3(CO)_3]$ to afford the complex **11** in the same manner as described for **8a** in overall yield 75%. IR (KBr): $\tilde{\nu}$ = 3478, 2020, 1889, 1689, 1635 cm$^{-1}$; MS (ESI): $m/z$ (%): 558.2 (100) $[M^+ + H]$; elemental analysis calcd (%) for $C_{17}H_{18}N_4O_6Re$ (557.35): C 36.6, H 2.71, N 10.05; found: C 36.98, H 3.03, N 9.95. Crystal structure is shown in Figure 3. Crystals suitable for X-ray structure analysis were obtained from EtOH/toluene (1:1).

**1-(Carboxymethyl)histidine (5)**: The crude compound **4** (300 mg) was dissolved in 6 N HCl aqueous solution (10 mL), heated under reflux for 2 d and concentrated under reduced pressure. The sticky oily residue was solidified in diethyl ether (20 mL) and the white solid was washed with diethyl ether (2 × 10 mL) and THF (5 mL) dried in vacuo and used in the next step without further purification. $^1$H NMR (300 MHz, $D_2O$, 20 °C): $\delta$ = 8.69 (s, 1H; $CH_{im}$), 7.38 (s, 1H; $CH_{im}$), 4.96 (s, 2H; $CH_2N_{im}$), 4.18 (t, $J$ = 6.3 Hz, 1H; CHCO), 3.31 (t, $J$ = 5.8 Hz, 2H; $CH_2CH$); MS (ESI): $m/z$ (%): 213.82 (87) $[M^+ + H]$, 167.88 (100) $[M^+ - COOH]$.

**Re complex 14a**: Ethyl bromoacetate (29.5 mg, 0.176 mmol) in acetonitrile (5 mL) was added to a solution of complex **12**, (25 mg, 0.059 mmol) and $Cs_2CO_3$ (20.4 mg, 0.065 mmol) in acetonitrile (25 mL). The reaction mixture was heated at 35 °C for 1.5 h. Glacial acidic acid was added to the mixture to neutralized the pH. After standard work-up, the crude substance was purified by a silica gel chromatography to provide complex **14a** (30 mg, 90%). $R_f$ = 0.15 (EtOAc/EtOH 5:1); $^1$H NMR (300 MHz, $CD_3CN$, 20 °C): $\delta$ = 7.95 (s, 1H; $CH_{im}$), 6.92 (s, 1H; $CH_{im}$), 4.78 (s, 2H; $CH_2N_{im}$), 4.23–4.16 (q, $J$ = 7.1 Hz, 2H; $CH_2CH_3$), 3.91–3.87 (m, 1H; CHCO), 3.21–2.98 (q, 2H; $CH_2CH$), 1.28–1.23 (t, $J$ = 7.5 Hz, 3H; $CH_3$); $^{13}$C NMR (300 MHz, $CD_3CN$, 20 °C): $\delta$ = 199.5, 197.8, 197.8, 181.8, 168.8, 143.4, 135.7, 120.9, 63.0, 52.6, 49.4, 28.7, 14.4; IR (KBr): $\tilde{\nu}$ = 2020, 1883, 1741, 1636 cm$^{-1}$; MS (ESI): $m/z$ (%): 511.8 (100) $[M^+ + H]$, 1020.7 (55) $[2M^+]$; elemental analysis calcd (%) for $C_{15}H_{18}N_3O_7Re$ (510.5): C 30.59, H 2.76, N 8.23; found: C 30.84, H 3.0, N 8.06.

**Re complex 14b**: The preparation is similar to compound **14a**. To compound **12**, (25 mg, 0.059 mmol) and $Cs_2CO_3$ (20.4 mg, 0.065 mmol) in acetonitrile (25 mL) was added *tert*-butyl bromoacetate (34.5 mg, 0.176 mmol). The reaction mixture was stirred at 35 °C for 1.5 h. The reaction mixture was filtered, dried under vacuum and purified by silica gel chromatography (EtOAc/EtOH 5:1) to yield complex **14b** (29 mg, 90%). $^1$H NMR (300 MHz, $CD_3CN$, 20 °C): $\delta$ = 7.93 (s, 1H; $CH_{im}$), 6.90 (s, $CH_{im}$), 4.65(s, 2H; $CH_2N_{im}$), 3.94–3.25 (m, 1H; CHCO), 3.27–3.23 (q, 2H; $CH_2CH$), 1.45 (s, 9H; *t*Bu); $^{13}$C NMR ($CD_3CN$): $\delta$ = 181.3, 167.3, 143.1, 135.2, 120.6, 83.8, 52.3, 49.7, 28.5, 28.0, 27.8; IR (KBr): $\tilde{\nu}$ = 2021, 1892, 1738, 1635, 1155 cm$^{-1}$; MS (ESI): m/z (%): 539.9 (100) $[M^+]$, 1076.8 (50); elemental analysis calcd (%) for $C_{15}H_{18}N_3O_7Re$ (538.5): C 33.43, H 3.34, N 7.80; found: C 33.30, H 3.85, N 7.68.

**Re complex 13**

**From compound 5**: The crude **5** (300 mg) was dissolved in $H_2O$ (15 mL). The pH of the solution was adjusted until 7–8 by adding 1 N NaOH aqueous solution. $[Et_4N]_2[ReBr_3(CO)_3]$ (531 mg, 0.69 mmol) was added. After stirring for 21 h at room temperature, the solution was concentrated in vacuo. The residue was treated with $CH_2Cl_2$ (3 × 10 mL) and diethyl ether (2 × 10 mL) and dried in vacuo. The sticky crude product was purified by flash column chromatography to afford **13** as a white solid (240 mg, 68% from **3** in three steps). $R_f$ = 0.1 (EtOH/$CH_2Cl_2$/AcOH 10:1:0.1); $^1$H NMR (500 MHz, $CD_3CN$/$CD_3OD$, 25 °C): $\delta$ = 7.99 (s, 1H; $CH_{im}$), 6.95 (s, 1H; $CH_{im}$), 4.78 (d, $J$ = 7.08 Hz, 2H; $CH_2N_{im}$), 3.99–3.96 (m, 1H; CHCO), 3.24–3.20 (m, 1H; $CH_2CH$), 3.10–3.05 (m, 1H; $CH_2CH$); $^{13}$C NMR (500 MHz, $CD_3CN$/$CD_3OD$, 25 °C): $\delta$ = 199.2, 197.7, 197.5, 183.8, 169.8, 143.7, 135.5, 121.2, 53.1, 28.8; IR (KBr): $\tilde{\nu}$ = 3428, 2023, 1889, 1731, 1623, 1521, 1437, 1181 cm$^{-1}$; MS (ESI): $m/z$ (%): 505.8 (23) $[M^+ + Na]$, 483.67 (100) $[M^+ + H]$.

**From complex 14a and 14b**: To hydrolyze the ester groups, compound **14a** (30 mg, 0.057 mmol) was stirred in a solution of methanol (5 mL) and LiOH (0.5 M, 2 mL) overnight at room temperature and compound **14b** (15 mg, 0.028 mmol) was stirred in a solution of methylene chloride (2 mL) and trifluoroacetic acid (2 mL) for 2 h at room temperature. Two crude substances were purified by column chromatography (EtOH/THF/AcOH 10:1:0.1) to yield complex **15** (95% and 90%, respectively).

**General procedure for the oxidation of rhenium in the complexes 15 and 13**: A solution of compound **15** or **13** (5 mM in $H_2O$, 500 µL) and acid (HCl, TFA, or acetic) solution (1.0, 0.1, or 0.01 M in $H_2O$, 70 µL) were added to a vial, which was sealed and then degassed with nitrogen (10 min). $H_2O_2$ (0.43, 0.86, or 1.29 M in $H_2O$, 60 µL) was added to the degassed vial, followed by heating of the sample at 50 °C. Monitoring of the reaction mixture was conducted by HPLC at 250 nm, where the reaction mixture (10 µL) was injected on the HPLC at 4, 8, 24, and 48 h or until the rhenium complex was not visible in the spectrum. The effectiveness of the reaction condition was calculated by determining the peak area ratio of the rhenium complex over the formation of perrhenate. When the rhenium complex was no longer observed, the reaction mixture was treated with manganese dioxide to remove residual $H_2O_2$ from the reaction mixture then filtered with a Wattman 0.2 µm filter to yield the uncoordinated ligand in solution to be used in $^{99m}$Tc labeling.

**General procedure for labeling 5 and 9 with $[^{99m}Tc(H_2O)_3(CO)_3]^+$**: A solution of ligand ($10^{-3}$ or $10^{-4}$ M in $H_2O$, 100 µL) obtained from either organic synthesis or through rhenium oxidation pathway was added to a vial, which was then sealed and degassed with a stream of nitrogen gas for 10 min. A solution of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ (900 µL) was added to the vial through a syringe and the vial was heated to 70–90 °C for 30 min to yield the corresponding $[^{99m}Tc(CO)_3]^+$ complexes, $[(\mathbf{5})^{99m}Tc(CO)_3]$ **16** and $[(\mathbf{9})^{99m}Tc(CO)_3]$ **17** in high yield which was quantified by HPLC with radioactive detection. All the results are described in Table 1.

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CCDC-193892 (**3**), -193893 (**6a**) and -193894 (**11**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; (fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk).
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