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Facile solid phase peptide synthesis with a Re-lysine conjugate generated via a one-pot procedure

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Abstract

We have synthesized a Re(CO)₃-modified lysine via a one-pot Schiff base formation reaction that can be used in the solid phase peptide synthesis of peptides. To demonstrate its potential use, we have attached it to a neurotensin fragment and observed uptake into human umbilical vascular endothelial cells.

For many years, the biological chemistry of the Re(CO)₃ unit has been investigated both as a model for the chemistry of analogous Tc(CO)₃ imaging agents^{1,2} and for the design of radiological rhenium therapeutics.²⁻⁷ In general, there are two types of M(CO)₃ drug types:¹ metal-essential compounds (where the drug is comprised of the complex), and targeted compounds (where a M(CO)₃ unit is covalently bound to a fragment designed to direct the drug to specific cells or tissues). For the precise targeting of tumours, radionuclides have frequently been covalently attached to amino acids and peptides using the bifunctional chelate (BFCA) methodology.³ Moreover, various radionuclides including Re(CO)₃ were prepared and successfully coupled to the targeting peptides on via solid phase methods using a SAAC (Single Amino Acid Chelator) strategy described by Valliant and Gasser *et al.*⁸⁻¹⁰ However, among the linkers being used, either for SAAC or BFCA, one pot methods for the modification of lysine remained uninvestigated. This fact allows us to employ lysine's reactive side chain to form a Re(CO)₃ complex *in situ*.

In this report, we present the one-pot synthesis of a Re(CO)₃-modified lysine conjugate via the formation of a Schiff base chelate at the Ne position. The resultant complex can then be incorporated into a polypeptide using solid phase synthetic methods. For the present study, we incorporated the Re(CO)₃-modified lysine into a neurotensin fragment - a short peptide analogue involving in some cancer growth regulation. It has been shown that NTSR1, which is the receptor for neurotensin binding, bears the ligand binding pocket for neurotensin(8-13) and is able to bind neurotensin agonists.¹¹ Most of the known effects of neurotensin are mediated via the receptor NTSR1. The neurotensin receptor has been explored as a target for

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peptide conjugates,¹¹⁻¹² so we decided to employ this sequence as a proof of principle experiment. We were then able to observe uptake of the resultant Re-modified peptide into human umbilical vascular endothelial cells (HUVECs),^{13a-b} monitoring the process via fluorescein labelling of the peptide. As part of our investigations into the biological chemistry of $\text{Re}(\text{CO})_3$, we have synthesized numerous diimine complexes.¹⁴⁻¹⁹ One convenient methodology for the production of $\text{Re}(\text{CO})_3\text{X}(\text{diimine})$ compounds is via the one-pot reaction of pyridine-2-carboxyaldehyde, a primary amine and $\text{Re}(\text{CO})_5\text{X}$ or $\text{Re}(\text{CO})_3(\text{H}_2\text{O})^+_3$ (Scheme 1 at top). The resultant complexes are composed of a pyridine-2-carbaldehyde imine (pyca) bound to a $\text{Re}(\text{CO})_3\text{X}$ unit.¹⁹ We and others have used this reaction to generate covalent diimine adducts, often with a substituted phenyl ring attached to the imine nitrogen.^{14,20-21} However, this chemistry can also be used to produce compounds where the imine group is substituted with a biologically relevant moiety, such as a peptide or a fluorescein.^{14,17,19} We surmised that we could extend this chemistry to the Ne of a protected lysine that could subsequently be employed as a reagent for solid phase peptide synthesis (SPPS). The resultant compound would be a side chain-modified amino acid that could be incorporated anywhere into a peptide growing off the solid support matrix.

As shown in Scheme 1 (bottom), the reaction of $\text{Re}(\text{CO})_5\text{X}$ with pyridine-2-carboxyaldehyde and N α -Fmoc-lysine results in the formation of $\text{Re}(\text{CO})_3\text{X}(\text{pyca}(\text{N}\alpha\text{-Fmoc})\text{-lysine})$ (**1**: X = Cl; **2**: X = Br), where the amino acid is modified at the Ne position. The aldehyde and Fmoc-protected amino acid were refluxed in methanol for one hour, followed by addition of $\text{Re}(\text{CO})_5\text{X}$ and continued reflux for eight hours. This reaction was readily carried out with either $\text{Re}(\text{CO})_5\text{Cl}$ or $\text{Re}(\text{CO})_5\text{Br}$ as the metal source, and yields of product were ~80%. The resultant complexes **1** and **2** exhibit spectra characteristic of (diimine) $\text{Re}(\text{CO})_3(\text{diimine})$ compounds, as shown in Figure 1 for compound **2**. First, the localized C_{3v} symmetry of the $\text{Re}(\text{CO})_3$ moiety affords two IR active CO stretching vibrations corresponding to a_1 and e modes. A second key spectroscopic feature is the presence of a metal to ligand charge transfer (MLCT) band in the UV-visible spectrum, with absorption maxima at ~394 nm with extinction coefficients near $3600 \text{ M}^{-1}\text{cm}^{-1}$ (Figure 1).

We then employed compound **2** as a reagent for solid phase peptide synthesis (SPPS). The binding of Tc/Re moieties like the $\text{M}(\text{CO})_3$ unit has been an active area of investigation for more than two decades, with most strategies involving the attachment polydentate chelates (with or without the metal) to the end or a side chain of a peptide fragment.²²⁻²⁴ For the current study, we fabricated a shortened analogue of the neuropeptide neurotensin: neurotensin(8-13).²⁵ Neurotensin and other small polypeptides have been used for the fabrication of BFCA compounds incorporating the $\text{Re}(\text{CO})_3$ fragment.²⁶⁻²⁸ Previously, Metzler-Nolte *et al.* appended a rhenium complex to neurotensin(8-13) using a bis(phenanthridinylmethyl)amine-based chelate attached to the amine terminus of the fragment.²⁹ Zagermann and co-workers synthesized tungsten modified-neurotensin(8-13),³⁰ and $\text{Mn}(\text{CO})_3(\text{cymantrene})$ was attached to neurotensin using the SPPS method as presented in the work by N'Dongo *et al.*³¹ A similar strategy was recently employed to append the same moiety to a bombesin-based peptide.³² This new methodology provides several advantages versus prior work on the covalent attachment of $\text{Re}(\text{CO})_3$ to peptides. First, we are able to synthesize a protected Re-modified peptide via a one-pot method in a single step

from commercially available starting materials. Second, **2** (as well as compound **1**) can be directly incorporated into a peptide backbone via standard SPPS methods.²⁴ Finally, as compounds **1** and **2** are modified at the Ne position, the $\text{Re}(\text{CO})_3$ moiety can be incorporated as part of a growing peptide off the solid phase support, and thus could be incorporated anywhere in the peptide sequence. Scheme 2 shows the neurotensin(8-13) peptide **3** with a lysine added to the amine terminus and the rhenium pyca unit appended at Ne that was generated by use of our $\text{Re}(\text{CO})_3$ -modified amino acid as a reagent. The polypeptide was produced using standard Fmoc synthetic methods starting with Fmoc-Leu-Wang resin (Supplementary Information Scheme S1). Completeness of each coupling reaction was monitored by the Kaiser Test.³³ After synthesis, the peptide was removed from the resin using a standard TFA cleavage cocktail procedure and precipitated using cold diethyl ether. We confirmed the formation of the polypeptide using mass spectrometry (Supplementary Information Figure S1 and S3). Additionally, we observed identical UV-visible features as in the lysine complexes **1** and **2**, as shown in Figure 1, which indicates that the Schiff base chelate remains intact to relatively harsh cleavage conditions. The IR spectrum shows slight differences from that seen in the IR spectrum of **2**, however these changes can be ascribed to changes in local environment around the carbonyls, most likely due to intramolecular hydrogen bonding and solvation effects. NMR spectroscopy of peptide **3** shows peaks in the aromatic region corresponding to the terminal Fmoc group (Supplementary Information Figure S2).

The key advantage with using peptide conjugate imaging agents is that they show selective uptake into specific tissues. In order to investigate whether this occurs with these conjugates, we chose to work with HUVECs. These cells express high affinity neurotensin receptors that are coupled to intracellular calcium ion release⁸ and thus could act as a model system for uptake of this peptide conjugate. In the absence of an attached fluorophore, the $\text{Re}(\text{CO})_3\text{X}(\text{pyca})$ conjugates show little fluorescence. In order to visualize any uptake, we appended the fluorescent chromophore fluorescein to the end of the peptide, as shown in Scheme 2. As in peptide **3**, we produced fluorescein modified fluorescent peptide **4** using standard SPPS methods and purified the resultant peptide via HPLC (Supplementary Information Figure S8-S9). The resultant peptide shows absorption and emission corresponding to the presence of the fluorescein group as well as the diagnostic carbonyl stretching bands in the infrared spectrum. The MLCT band that results from the $\text{Re}(\text{CO})_3(\text{diimine})$ unit is not observable due to the high molar absorptivity of the fluorescein chromophore. The ^1H NMR spectrum of **4** exhibits peaks indicative of replacement of the Fmoc group with the fluorescein moiety (Supplementary Information Figure S4).

Fluorescence microscopy shows that cells readily take up the $\text{Re}(\text{CO})_3$ peptide conjugate. Figure 2 shows HUVECs incubated with 40 μM **4**, excited at 470 nm. The cells exhibit clear and dose-dependent uptake of the peptide and localization of the fluorescein unit (but not necessarily the peptide conjugate) within the nucleus. This is in accordance with previous work that has shown that neurotensin fragments labelled with fluorescein are taken up into cells and that this uptake can be readily monitored by fluorescence. To determine whether or not the observed fluorescence resulted from either fluorescein impurities in the peptide or from cleavage of the fluorescein from the peptide prior to uptake, we exposed the HUVECs

to similar concentrations of 5-carboxyfluorescein. The resultant cells, shown in the SI, show no uptake of the fluorescein (Supplementary Information Figure S6). However, cleavage of the peptide may be taking place in the cytoplasm; current studies are underway to determine the destination of the rhenium in these cells.

In conclusion we have synthesized a new $\text{Re}(\text{CO})_3$ -modified lysine that can be generated in one step via a one-pot reaction from commercially available reagents. This modified amino acid can be incorporated into a polypeptide using solid phase peptide synthetic methods, and is robust to standard TFA cleavage cocktail procedures. As a test case, we incorporated the $\text{Re}(\text{CO})_3$ -modified lysine into a neurotensin analogue, and monitored cellular uptake via use of a fluorescein modified variant. We are continuing our work on the functionalization of proteins and polypeptides with the $\text{Re}(\text{CO})_3$ moiety and other transition metal ions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

General scheme for one-pot syntheses (top) and preparation of **1** and **2** (bottom).

Figure 1.
The UV-visible spectra and CO stretching region of the IR spectra of compound **2** (left) and peptide **3** (right).

Scheme 2.

Neurotensin(8-13) peptide with lysine at amine terminus bound to the rhenium pyca unit; **3**: R = Fmoc, **4**: R = fluorescein.

Figure 2.
HUVECs incubated with 40 μ M **4**, excited at 470 nm, showing uptake of the poplypeptide.