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Facile rhenium-peptide conjugate synthesis using a one-pot derived $\text{Re}(\text{CO})_3$ reagent

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Abstract

We have synthesized two $\text{Re}(\text{CO})_3$ -modified lysine complexes (**1** and **2**), where the metal is attached to the amino acid at the Ne position, via a one-pot Schiff base formation reaction. These compounds can be used in the solid phase synthesis of peptides, and to date we have produced four conjugate systems incorporating neurotensin, bombesin, leutenizing hormone releasing hormone, and nuclear localization sequence. We observed uptake into human umbilical vascular endothelial cells as well as differential uptake depending on peptide sequence identity, as characterized by fluorescence and rhenium elemental analysis.

Graphical Abstract

Introduction

The functionalization of biological molecules with organometallic groups continues apace.¹ Investigations into this chemistry have been driven by both older applications and new developments in bioinorganic chemistry.^{2–7} Organometallic compounds have been used as phasing agents for protein structure elucidation, and compounds containing the $\text{Tc}(\text{CO})_3$ moiety are used for SPECT imaging applications.^{8–10} More recently, metal carbonyl compounds have been employed as electron transfer reagents as well as probes for protein dynamics via vibrational relaxation studies. Organometallic compounds have also been used

as drugs that can interact with proteins, as seen in their use as kinase inhibitors^{11,12} or as carbon monoxide releasing molecules (CORMs).^{1,13–15}

Another active area of investigation is the covalent attachment of organometallic units to biomolecules via conjugate formation.^{16–21} The bifunctional chelate method (BFCA)^{22,23} has frequently been used, which involves the construction of an organic linker that both chelates the metal unit and forms a covalent bond to the biological molecule.^{24–26} Accordingly, this strategy has been applied to biomolecules such as polypeptides. This has been successfully implemented to attach units such as the $\text{Re}(\text{CO})_3$ moiety to peptides and can be applied to solid phase peptide synthesis (SPPS) methods.^{27–30} However, the construction of such linkages requires several steps,^{31–33} that may limit their usefulness in radiopharmaceutical applications, especially with isotopes of limited lifetimes.

For several years we have been interested in the fundamental chemistry of the $\text{Re}(\text{CO})_3$ unit, and have used this chemistry to functionalize biological molecules with this group. Originally used to model the chemistry of $\text{Tc}(\text{CO})_3$ compounds, $\text{Re}(\text{CO})_3$ is now also relevant to many alternate uses of organometallic compounds in biological systems.^{1,13–15} One such method to append biological molecules to $\text{Re}(\text{CO})_3$ is to use metal-mediated Schiff base formation, which can be readily accomplished using pyridine-2-carboxaldehyde (pyca) as a reagent. We and others have used $\text{Re}(\text{CO})_5\text{X}$ or $\text{Re}(\text{CO})_3(\text{H}_2\text{O})_3^+$ with pyca and primary amines to produce $\text{Re}(\text{CO})_3$ diimine conjugate complexes (Scheme 1).^{10,34–42} This method is broadly applicable to a wide variety of primary amines and can be carried out using one-pot methods. We have used this reaction to synthesize amino acid $\text{Re}(\text{CO})_3$ conjugates and surmised that we would be able to use the same process to generate a sidechain-modified amino acid that could be incorporated into SPPS methods.

In this report we present the one pot synthesis of a sidechain-modified lysine using pyca and $\text{Re}(\text{CO})_5\text{X}$ to afford an N_ϵ substituted $\text{Re}(\text{CO})_3$ complex. A portion of this work was presented recently in a communication.⁴³ The resultant modified lysine is quite stable and can be used in SPPS methods, and we have incorporated it into four biologically active peptides: neurotensin (NEU: RRPYIL), bombesin (BBN: MLHGVAWQ), luteinizing hormone releasing hormone (LHRH: EHWSYGLRPG), and a nuclear localization sequence (NLS: KRRRC). Additionally, we exposed these rhenium-modified peptides to human umbilical vascular endothelial cells (HUVECs), and demonstrated uptake using a fluorescent label as well as rhenium elemental analysis. We observed that the identity of the peptide affects the degree of uptake in HUVECs, which supports the hypothesis that this uptake is at least in part mediated by receptors on the surface of the cells.

Experimental

Materials and methods

All single amino acid loaded resins, Fmoc-amino acids, HOBt (hydroxybenzotriazole), HBTU (*O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate) and *N*-methylmorpholine, and other chemicals were purchased and used without further purification. NMR spectra were recorded on a Varian Mercury 300 MHz and Varian NMRS 500 MHz (2 channel with automation) spectrometers. Chemical shifts were reported with

respect to residual solvent peaks as internal standard (^1H : DMSO- d_6 , δ = 2.50 ppm; ^{13}C : DMSO- d_6 , δ = 39.7 ppm). MALDI-TOF mass spectra were carried out on a Bruker Ultraflex-III TOF/TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA) equipped with a Nd:YAG laser (355 nm). All spectra were measured in positive reflection mode. UV-visible spectroscopy was carried out on a Hitachi U-3010 spectrometer.

Synthesis of 1 and 2

Pyridine-2-carboxaldehyde (0.012 mL, 0.125 mmol) and Fmoc-Lys-OH (46 mg, 0.125 mmol) were heated in methanol (15 mL) for 0.5 hour. Upon reaction the solution's colour changed from very pale yellow to light brown, $\text{Re}(\text{CO})_5\text{Cl}$ (45 mg, 0.125 mmol) was then added into reaction mixture. The solution was further refluxed for 8 hours. The resulting orange solution was allowed to cool down to room temperature. The solvent was removed and the orange solid was dried under vacuum for a few days. The orange solid was repeatedly washed with ether and air-dried. Compound **1** was obtained in 76% yield (66 mg, 0.088 mmol). Compound **2** can be synthesized by the same method using $\text{Re}(\text{CO})_5\text{Br}$ and was obtained as orange solid in 79% yield (80 mg, 0.099 mmol). **1**: ^1H -NMR (500 MHz in DMSO- d_6): 9.23 (s, 1H, $\text{HC}=\text{N}$), 8.98 (m, 1H, H on py), 8.19–8.28 (m, 2H, H on py), 7.88 (m, 2H, H on py and NH), 7.60–7.76 (m, 4H, H on Fmoc), 7.28–7.43 (m, 4H, H on Fmoc), 4.25 (m, 2H, $\text{O}-\text{CH}_2$), 4.20 (m, 1H, $\text{HC}-\text{NH}$), 4.03 (m, 2H, $\text{CH}_2-\text{CH}-\text{NH}$), 3.95 (m, 1H, H on Fmoc), 1.90–1.95 (m, 2H, $\text{CH}_2-\text{CH}_2-\text{CH}-\text{NH}$), 1.69–1.75 (m, 2H, $=\text{N}-\text{CH}_2$), 1.42 (m, 2H, $=\text{N}-\text{CH}_2-\text{CH}_2$). ^{13}C -NMR (125 MHz in DMSO- d_6): 198.2, 197.9, 188.2, 174.3, 170.0, 156.6, 155.2, 153.4, 144.3, 141.2, 129.7, 128.1, 127.5, 125.7, 120.5, 66.1, 64.3, 54.2, 47.1, 30.7, 29.2, 23.1 ppm **2**: ^1H -NMR (500 MHz in DMSO- d_6): 9.21 (s, 1H, $\text{HC}=\text{N}$), 9.02 (m, 1H, H on py), 8.23–8.28 (m, 2H, H on py), 7.89 (m, 1H, H on py), 7.87 (s, 1H, NH), 7.31–7.76 (m, 8H, H on Fmoc), 4.20 (m, 1H, H on Fmoc), 4.26 (m, 2H, $\text{O}-\text{CH}_2$), 3.95 (m, 1H, $\text{HC}-\text{NH}$), 4.05 (m, 2H, $\text{CH}_2-\text{CH}-\text{NH}$), 1.87–2.01 (m, 2H, $\text{CH}_2-\text{CH}_2-\text{CH}-\text{NH}$), 1.30–1.45 (m, 2H, $=\text{N}-\text{CH}_2$), 1.66–1.81 (m, 2H, $=\text{N}-\text{CH}_2-\text{CH}_2$). ^{13}C -NMR (125 MHz in DMSO- d_6): 197.7, 197.5, 187.5, 174.3, 169.9, 156.6, 155.3, 153.6, 144.3, 141.2, 129.6, 128.1, 127.5, 125.7, 120.6, 66.1, 64.4, 54.3, 47.1, 30.7, 29.3, 23.1 ppm.

Peptide synthesis

Rhenium-peptide conjugates were synthesized using standard Fmoc solid phase peptide synthesis methods. The completeness of each coupling reaction was monitored by the Kaiser Test.⁴³ Fmoc removal between each cycle was performed by 30-minute treatment with 20% piperidine in dimethyl formamide (DMF).

Each synthesis was initiated by using 100 milligrams of Fmoc-Xxx-Wang resin. The resin was soaked in DMF for 1–2 h to swell the resin beads. Prior to coupling, Fmoc groups were removed using 20% piperidine in DMF for 30 minutes followed by washing with DMF, methanol and dichloromethane several times. Five equivalents of Fmoc-Xxx-OH along with 4.5 equivalents of HBTU and HOBt and 10 equivalents of 4-methylmorpholine were used in this synthesis. The coupling process was performed for 2 hours followed by washing and Kaiser test was carried out prior to the next cycle. All reactions were performed on a mechanical shaker. In cases where free NH_2 termini remained present on the resin, the coupling process was repeated. After completion of all cycles with Fmoc-Xxx(s), 5

equivalents of the rhenium complex **2** were coupled overnight using HBTU/HOBt 4-methylmorpholine in the amounts described above. The coupling reaction using 5(6)-carboxyfluorescein was performed in a similar manner. The coupled resin turned yellow/orange after completion of the last cycle and was washed with DMF. In each case the resin was moved to a clean round bottom flask and air-dried. Methylene chloride was added into the flask and the resin was left until swollen with solvent (1 hour). Methylene chloride was removed under vacuum and the TFA (trifluoroacetic acid) cleavage method was started immediately.

The TFA/water/triisopropylsilane (95:2.5:2.5% v/v/v) cocktail solution (10 mL: 1 mL per 10 mg resin) was added to dry resins. The suspension was gently stirred for three hours. The mixture was filtered and resin was washed twice with a small amount of fresh TFA cocktail. The filtrate was kept and the volume was reduced to be around ¼ of original volume. An excess amount of cold ether (> 10 times the initial TFA cocktail volume) was added causing the crude yellow peptide to precipitate. The suspension was cooled and centrifuged for 5 minutes at 5000 rpm and the supernatant was discarded. The peptide was solubilized in acetonitrile/water/TFA (50:50:0.1% v/v/v) and was lyophilized to give yellow-orange powder.

Conjugates **3–6** were purified by HPLC. The purification process was performed on a GE Äkta Purifier equipped with a UV900 detector using a semipreparative Vydac 218TP C₁₈ reversed-phase column. The solvent system used was 99.9% water/0.1% TFA (v/v) and 95% acetonitrile/5% water/0.1% TFA v/v/v. The detector was set to detect absorption at 254 and 280 nm. Mass spectra of the purified sample fractions showed the expected m/z values for all conjugates.

Cell studies

Human umbilical vascular endothelial cells (HUVEC) were maintained in EBM2 medium with SingleQuots™ and 10% fetal bovine serum (FBS) in a humidified 5% CO₂ environment at 37°C. For staining experiments, cells were seeded on 24-well 0.1% gelatine coated plates (75,000 cells/ well) and incubated for 24 h prior to incubation with the rhenium-peptide conjugates at different concentrations.

Incubation of compounds **3–6** was carried out on adherent HUVECs. Concentrated DMSO stock solutions of each compound were diluted using cell culture medium to achieve desired final concentrations of 80, 40, 20, 10, and 5 µM. The amount of DMSO used in each dilution was lower than 1% to prevent cytotoxicity from DMSO itself.⁴⁴ After incubation, the cells were washed three times with phosphate buffered saline (PBS) (pH 7.4), fixed in 2% paraformaldehyde (PFA), for 15 minutes at room temperature and washed again three times with PBS to remove residual PFA. Nuclei were stained using 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI).

Rhenium uptake studies

The uptake of rhenium by HUVECs was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). The cells were seeded on 6-well plates and were

incubated with each compound as described above. 24 hours later, cells were washed thoroughly with PBS several times, lysed with lysis buffer (RIPA, 100 μ L per well), at 4°C. The adherent cells were scraped off the dish using a plastic cell scraper and cell lysates were stored at -20°C for ICP-OES analysis.

The Agilent 700 series ICP-OES with Expert II software was used to confirm the cellular uptake of rhenium. Rhenium standard solutions were prepared using commercially available BDH® ARISTAR® single element standards Re (1,000 μ g/mL in 3% HNO₃). All measurements were conducted in triplicate and the results were found to be reproducible (with <6% RSD).

Results and Discussion

For many years, researchers have looked to append the Re(CO)₃ unit to peptides. Historically, these studies aimed to produce new methods for generating radionuclide conjugates that could be directed to specific tissues or cell types. Frequently, organic metal-bridging ligands have been employed to couple the Re(CO)₃ unit to a polypeptide, however recently several groups have focused on modifying the N_ε of lysine to convert it into a metal binding unit. In 2004, Stephenson *et al.*^{45,46} reported a new strategy for the synthesis of a Re(CO)₃ complex with a lysine-derived bis(pyridyl) amine ligand. This strategy was referred to as the single amino acid chelate (SAAC) method. *N*- α -Fmoc-L-lysine was reacted with either pyridine or quinoline aldehyde to give the bifunctional ligand that was subsequently reacted with [NEt₄]₂[Re(CO)₃Br₃], and formed a Re(I)/Tc(I)-binding amino acid mimic as a result. This inert SAAC complex with a M(CO)₃³⁺ core (M = Re, Tc) can be incorporated into peptides in a similar way as a natural amino acid. The SAAC technology was used to make a variety of Tc/Re(CO)₃ biomolecules.^{32,33} Cyclic peptides for the urokinase plasminogen activator receptor were prepared by using a series of *N*- α -Fmoc-lysine derivatives with two heterocyclic donor groups at the ϵ -amine.³² Moreover, a SAAC-Re complex was integrated within fMLF, a targeting sequence guiding radionuclides to the formyl peptide receptor (FPR). The peptide conjugate internalized into human leukocytes and can be fluorescently monitored.⁴⁵ Alberto *et al.* reported work in which Fmoc-L-Lys(Dap(Boc)) (Dap=2,3-diamino propionic acid) was used to conveniently post-label Tc(CO)₃ and Re(CO)₃ units to a BBN peptide.⁴⁷

For several years, we have been reporting on Re(CO)₃ mediated Schiff base formation using pyca and primary amines. This chemistry is broadly applicable and can be used to synthesize Re(CO)₃ pyridine-2-carboxaldimines with a variety of groups off of the imine nitrogen. Additionally, this chemistry can either be carried out sequentially after initial formation of a pyridine-2-carboxaldehyde Re(CO)₃ complex, or via one pot conditions. We decided to use the latter method to produce a sidechain-modified lysine, as shown in Scheme 1. By reacting Re(CO)₅X with the N-terminal protected lysine using one pot conditions, we were able to isolate the N_ε functionalized pyridine-2-carboxalimine complex. This reaction can be out with either Re(CO)₅Cl or Re(CO)₅Br as the source of the Re(CO)₃ group, and yields of these reactions were approximately 80%. It is important to note that the metal centre is chiral and that both enantiomers are produced in this reaction. Compounds **1** and **2** exhibit spectra that are typical for (diimine)Re-(CO)₃X(diimine) compounds. As can be seen in

Figure 1 for compound **2**, two key spectroscopic features can be observed. First, in the vibrational spectra, the localized C_{3v} symmetry of the $\text{Re}(\text{CO})_3$ moiety affords two IR active CO stretching vibrations corresponding to the a_1 and e modes. Another key spectroscopic feature is the presence of metal to ligand charge transfer (MLCT) bands in the UV-visible spectra of **1** and **2**, with absorption maxima at ~ 394 nm with extinction coefficients near $3600 \text{ M}^{-1} \text{ cm}^{-1}$ (Fig. 3).

We hypothesized that the kinetically inert nature of the d^6 Re(I) center in $\text{Re}(\text{CO})_3$ diimine compounds would make them useful as SSPS reagents. Incorporating $\text{Re}(\text{CO})_3/\text{Tc}(\text{CO})_3$ groups into polypeptides has been actively pursued since the 1990s. For the $\text{Re}(\text{CO})_3$ unit, a good portion of this work has focused on integrating the moiety into a peptide via a tridentate-ligand linker that could either be incorporated into the end of the polypeptide or in a modified lysine or alternate sidechain.

As a proof of principle for our approach, we used compound **2** to synthesize several polypeptides via standard SPPS methods. The peptides constructed for this study are shown in Figure 1. Previously, we reported in a communication the synthesis of a neurotensin $\text{Re}(\text{CO})_3$ derivative,⁴² and we have extended this to three additional peptides, including bombesin, LHRH, and NLS. All four sequences have been used to transfer metal ions into cells, but each does so via a different mechanism. After synthesis of each of the peptides, we were able to confirm the identity of the peptide conjugates via mass spectrometric methods and the purity via HPLC (Figure 2).

Additionally, we were able to observe two distinct spectroscopic features in these rhenium-peptide conjugates that establish the presence of a $\text{Re}(\text{CO})_3$ diimine unit. First, all four systems show IR bands that result from the facial coordination mode of the three carbonyl ligands (Figure 3). The pattern of the carbonyl stretching frequency results from the localized C_{3v} symmetry present in the $\text{Re}(\text{CO})_3$ unit, producing bands between 2040 and 1880 cm^{-1} that correlate to the a_1 and e modes. The second key feature is a metal-to-ligand charge transfer (MLCT) band that is diagnostic for $\text{Re}(\text{CO})_3$ diimine compounds. These bands appear at $370\text{--}380$ nm and exhibit extinction coefficients ranging from 2000 to $3900 \text{ M}^{-1} \text{ cm}^{-1}$ (Table 1, Figure 3). Due to the charge transfer nature of these transitions, they are solvent dependent, exhibiting shifts in the absorption maxima with changes in solvent polarity. In the peptide conjugates **4–6**, we also observe similar charge transfer bands in their UV-visible spectra and the presence of fluorescein absorbance bands that appear as shoulders on the MLCT band. Additionally, all of the three new peptide conjugates exhibit the expected carbonyl stretching modes in their IR spectra.

Previously, we showed that our rhenium pyridine-2-carboxaldimine compounds were taken up by human vascular endothelial cells (HUVECs).⁴³ It is known that HUVECs express NTSR1, which is the receptor for neurotensin binding and was chosen in our prior work since conjugates that contain neurotensin(8–13) are able to bind to this receptor.⁴⁸ We wanted to use the same cell line for the current study, and these cells also have receptors that can bind bombesin and LHRH based peptides. Bombesin, a homologue of mammalian gastrin-releasing peptide (GRP), possesses specific binding ability to the G-coupled protein receptors expressed on breast, lung, prostate and pancreatic cancers.^{49,50} It was observed

that a bombesin-like peptide called neuromedin B can enter and stimulate the growth of HUVECs.⁵¹ The presence of luteinizing hormone/human chorionadotropin receptors (LH/hCG) expressing in HUVECs has also confirmed and hCG mediated angiogenic effects can take place through interaction with this receptor.⁵²

We extended this to the three new peptides presented in the current study. As in the original study with peptide **3**, we appended a fluorescein unit to the end of the polypeptide chain **4**, **5** and **6** to more easily monitor uptake. Figures 4 and S9 (supplementary information) show fluorescent micrographs of HUVECs treated with these three peptides. The left column shows whole cell fluorescence, the middle shows DAPI staining, and the right shows merged images. All the three sets of micrographs show the presence of fluorescein in the cells, which we can attribute to uptake of the rhenium modified peptides. Uptake is seen at both the higher concentration (Figure 4, 40 μ M) and at an eight fold smaller concentration (Figure S9, 5 μ M). Fluorescence is detected both in the cytoplasm as well as the nuclei of these cells. In the more concentrated sample, minimal difference between the three peptide sequences was observed. This might arise either from similar degrees of uptake, or from lysis of the fluorescein unit within the cells. At low concentrations, we observed differential uptake, with **5** showing the least uptake.

We can quantify the amount of peptide conjugate uptake in the HUVECs by use of rhenium elemental analysis via ICP OES (Figure S12). One of the advantages of this method is that there is no naturally abundant rhenium in cells, and therefore it can provide an absolute measure of peptide uptake. Table 2 shows the results of our analysis. All three peptides investigated do show uptake, however we observe significantly higher uptake for the peptide **4**, which contains the nuclear localization sequence. Peptide conjugate **4** shows more than four times greater uptake as measured by rhenium analysis, clearly showing that the identity of the peptide sequence affects this process. This is not too surprising since K/R rich peptides are often also used as cell penetrating peptides for efficiently shuttling various cargos into cells.⁴⁷

Conclusions

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. Although other methods have been used to append the $\text{Re}(\text{CO})_3$ unit to the sidechain of lysine, the pyca-Schiff base forming reaction affords a one-pot/one step method for carrying out this chemistry. We have shown that 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. To demonstrate the flexibility of this new modified lysine reagent, we incorporated it into four different peptide sequences, 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.

Acknowledgments

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Fig. 1.

Structures of $\text{Re}(\text{CO})_3$ conjugates with a neurotensin fragment (**3**), a nuclear localization sequence (RRRC, NLS, **4**), a bombesin fragment (**5**), and a luteinizing hormone releasing hormone fragment (LHRH, **6**).

Fig.2.

The chromatograms of **4** (100% A $\rightarrow$ 100% B in 25 min): 19 min, **5** (100% A $\rightarrow$ 100% B in 30 min): 25 min, and **6** (100% A $\rightarrow$ 100% B in 32 min): 25 min with the MALDI-TOF spectra. (Chromatograms: blue trace: 280 nm, red trace : 254 nm detection)

Fig. 3.
FT-IR and UV-visible spectra of compounds 1, 2 and 4–6

Fig. 4. Fluorescent micrographs of HUVECs incubated with 40 μ M of compound 4 (first row), 5 (second row), and 6 (third row) for 24 hours. Images were achieved in GFP (a), DAPI (b), and merged (c) modes.

Scheme 1.

Top: General scheme for a one pot pyca Schiff base Re(CO)₃X reaction. Bottom: Preparation of **1** and **2**.

Scheme 2.

Synthesis of peptide conjugates **3–6** where amides were formed using the HOBt/HBTU method and cleaved from the resin using TFA: i. Fmoc-deprotection, ii. Washing, iii. Coupling, iv. Repeat washing, v. TFA Cleavage.

Table 1

Absorption characteristics data of 2–6 in DMSO

Compound	λ_{max}	ϵ ($\times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$)
2	394	3.6
3	380	2.0
4	379	2.1
5	364	3.9
6	372	2.9

Table 2

Cellular uptake and accumulation percent values for compounds 4–6 in HUVECs.

Peptide	Loaded peptide (ppm)	Cellular uptake (ppm)	Accumulation (%)	%RSD
4	2.68	0.237	8.85	5.6
5	2.98	0.049	1.66	3.0
6	3.38	0.054	1.61	1.1