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New routes to bioconjugates of rhenium using the oxobis(dithiolato)rhenate(V) core

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The development of the rhenium-188 generator has provided a convenient and economic source of radionuclide for targeted radionuclide therapy of cancer. To exploit this more widely, mild, convenient and efficient methods for stable incorporation of the radionuclide into biomolecules are required. The aim of this work is to exploit the tumour targeting therapeutic radiopharmaceutical $[ReO(dmsa)_2]^-$ (H_2dmsa = *meso*-dimercaptosuccinic acid), which is easily synthesised in radioactive form and highly stable *in vivo*, for this purpose. A new efficient synthesis of $[ReO(dmsa)_2]^-$, as a single isomer (*syn-endo*), from perrhenate using acetylhydrazine as reductant, is reported. Dehydration of the two dicarboxylic acid groups of $[ReO(dmsa)_2]^-$ with dicyclohexylcarbodiimide gives the yellow cyclic dianhydride $[ReO(dmsaa)_2]^-$ (H_2dmsaa = *meso*-dimercaptosuccinic anhydride) without disrupting the $ReOS_4$ core. As its tetraethylammonium salt, $[ReO(dmsaa)_2]^-$ was characterised crystallographically as a distorted square pyramidal complex with an axial oxo-group and a *syn-endo* orientation of the two anhydride rings with respect to this oxo-group. $[ReO(dmsaa)_2]^-$ reacts with primary and secondary amines including lysine residues of peptides, to form amide-linked conjugates, which were identified by ES-MS. The syntheses are fast, efficient and employ mild conditions, making them readily adaptable for a kit-based approach to rhenium and technetium radiopharmaceuticals.

Introduction

Rhenium radioisotopes are among the most attractive for application in targeted radionuclide therapy of cancer, in part as a result of the development of the Re-188 generator[1] as a convenient and economic source of therapeutic radionuclide. Re-188 with its relatively short half-life ($t_{½}$ = 17 h) is suitable for delivery with small molecules such as antibody fragments, peptides *etc*. while Re-186 ($t_{½}$ = 90 h) is useful for delivery with larger carriers such as whole antibodies.[2]

Despite its widening availability, the great potential clinical versatility of the Re-188 generator cannot be realised without new simple and efficient kit-based chemistry for incorporating the radionuclide into biological targeting molecules. The most significant approaches reported to date include direct labelling of antibodies and peptides using reduced cystine linkages as rhenium-chelating moieties,[3,4] and various bifunctional chelator approaches,[2] none of which yet meet fully the criteria for ideal robust kit-based synthesis: rapid, clean, high radiochemical yield reaction under mild conditions with minimal need for manual handling and purification, and high *in vivo* stability. Among bifunctional chelator methods, the HYNIC system is currently popular for Tc[5] but has been unsuccessful with Re.[6] Moreover, the details of the interaction between the metal complex and the biomolecule are obscure with this method and labile ancillary ligands leave the conjugate vulnerable to cross-linking with endogenous chelators *in vivo*. Mild methods for synthesising the $Tc(CO)_3^+$ synthon and incorporating it into biomolecules have been developed[5,7] and are gaining popularity but there are still unresolved difficulties in applying this to rhenium.[8] S_2N_2 and N_3S tetradentate chelating systems for both Re and Tc were among the earliest developed,[2] but labelling procedures remain lengthy and low yielding. Direct methods of antibody and peptide labelling with Tc[9] and Re[3,4,10] are convenient but structures of the products are ambiguous[11] and their stability is poor.[3]

During the development of the tumour-targeting complex $[ReO(dmsa)_2]^-$ ("pentavalent rhenium dmsa"; H_2dmsa = *meso*-2,3-dimercaptosuccinic acid)[12] we made a number of observations that prompted us to evaluate this complex as a starting point for bioconjugate synthesis with rhenium. It has a number of attractive properties for this purpose: it is readily synthesised in radiolabelled form in quantitative radiochemical yield;[12–14] it has a discrete, well understood mononuclear structure;[15] it is highly stable *in vivo*;[12] it does not accumulate unduly in any specific normal tissues;[12] and it has pairs of carboxylate groups offering a means of covalent linkage to biomolecules *via* possible anhydride or reactive ester intermediates. Anhydride functionalised chelators have been used previously for synthesis of protein–metal conjugates: an anhydride derivative of a diphosphine was reported for synthesis of bioconjugates of copper radionuclides,[16] and the dianhydride of diethylenetriaminepentaacetic acid has been used routinely for many years for labelling proteins and peptides with indium-111.[17] Here we report the synthesis and structure of the anhydride derivative of $[ReO(dmsa)_2]^-$ and its reactivity with amines and the lysine side chain of an analogue of the 32-amino acid peptide salmon calcitonin (sCTa). We also report an improved mild synthesis of $[ReO(dmsa)_2]^-$ from perrhenate using acetylhydrazine as reducing agent in place of stannous chloride.

Results

Synthesis of $[ReO(dmsa)_2]^-$

Acetyl hydrazine reduces ammonium perrhenate to a rhenium(V) intermediate in acetonitrile at room temperature. Addition of H_2dmsa to this solution gives $[NH_4]^+[ReO(dmsa)_2]^-$ **1a** as an orange crystalline precipitate in high yield. This can be converted to the tetraethylammonium salt $[Et_4N]^+[ReO(dmsa)_2]^-$ **1b** by precipitating from water with tetraethylammonium bromide. The analogous tetrabutylammonium salt **1c** was prepared similarly. The reaction also proceeds efficiently in aqueous solution to give a clear orange solution of **1a**. The reactions are summarised in Scheme 1.

Negative ion ES-MS of complexes **1a**–**c** showed a molecular ion envelope at *m*/*z* 563 (^{187}Re isotope). Fragmentation of the ligand by C–S bond cleavage in the mass spectrometer was

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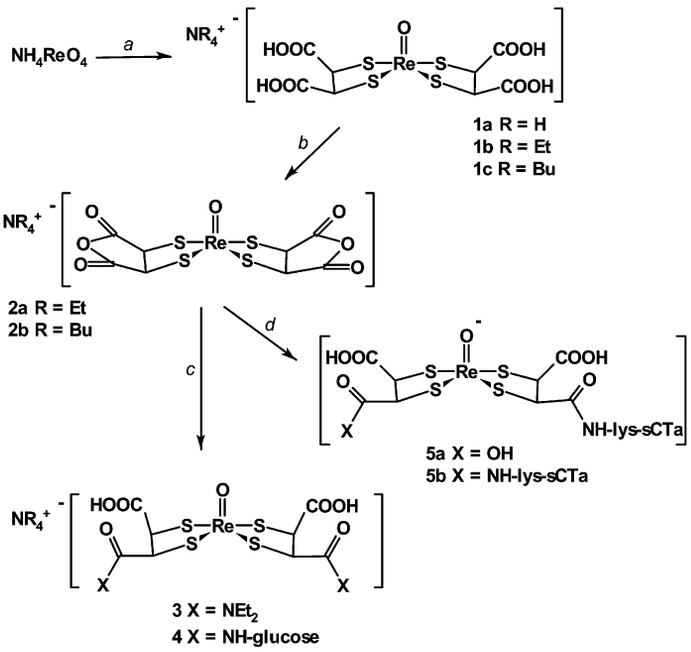
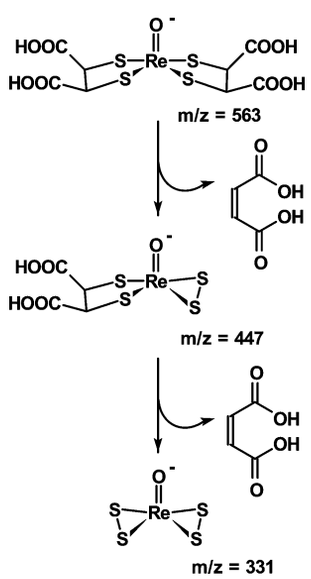


Scheme 1 Synthesis of complexes and peptide conjugates: *a* 1. acetyl hydrazine, HCl, dimercaptosuccinic acid in MeCN or H_2O, 2. NR_4^+ salt; *b* dicyclohexylcarbodiimide in acetonitrile or acetic anhydride; *c* amine in acetonitrile; *d* salmon calcitonin analogue in bicarbonate buffer.

indicated by ions at *m*/*z* 447 $([ReO(dmsa)S_2])^-$ and 331 $([ReOS_4]^-)$. These probably correspond to sequential loss of the alkene *cis*-fumaric acid (Scheme 2), in a fragmentation



Scheme 2 Probable sequential loss of fumaric acid HOOC–CH=CH=COOH by C–S bond cleavage accounting for fragment ions observed in the ES-MS.

analogous to the sequential losses of ethene previously observed in the ES-MS of 1,3,7-trithiacyclononane complexes.[18]

IR spectroscopy shows bands characteristic of the carboxylic acid groups (3600–2500 cm^{-1}, 1715–1690 cm^{-1}), and of the Re=O stretch at 955 cm^{-1} for **1a**, 961 for **1b**, and 971 for **1c** (*cf*. 974 previously reported for **1c**[13]). These values are comparable with the reported values for $[ReO(SCH_2CH_2S)]_2^-$ of 952[19] and 954.[20]

^{1}H NMR of **1a** and **1b** in dmso-d^6 showed only a single sharp resonance at 3.88 ppm corresponding to the C–H groups of the ligands, indicating that in contrast to the previous synthesis of this complex in hot aqueous solution,[13] only one of the three possible isomers (Fig. 1), is present. This conclusion was supported by HPLC, which showed a single peak eluting at 18.9

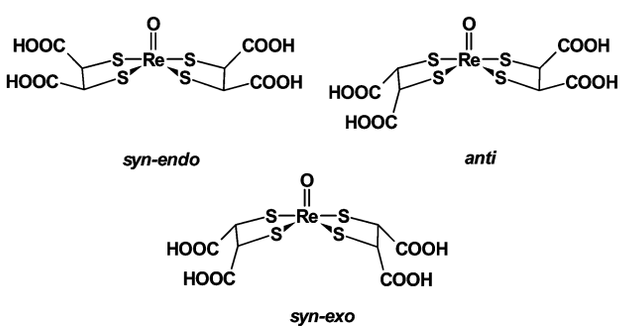


Fig. 1 Possible isomers of $[ReO(dmsa)_2]^-$ complexes (**1**).

min, whereas the mixed isomers, formed on standing in aqueous solution, gave two additional peaks eluting at 5.1 min (*anti*) and 22.6 min (*syn-exo*). The single isomer was identified as *syn-endo* by NMR and HPLC comparison with a crystallographic sample retained from earlier work.[15] The ^{1}H NMR of **1c** suggests that the other two isomers are present as a minor component (*ca*. 1% initially), perhaps because the lower solubility of this salt in water prevented preferential crystallisation of the *syn-endo* isomer (it is assumed that the highest field SCH protons of the *anti*-isomer overlap with the SCH protons of the *syn-endo* isomer, giving three peaks instead of the expected four). ^{1}H NMR showed that even the crude reaction mixture contained at least 91% *syn-endo* isomer.

Synthesis of anhydride $[ReO(dmsaa)_2]^-$

Complex **1b** reacts smoothly with 1,3-dicyclohexylcarbodiimide in acetic anhydride or acetonitrile solution at room temperature to give yellow $[NEt_4]^+[ReO(dmsaa)_2]^-$ **2a** (H_2dmsaa = *meso*-2,3-dimercaptosuccinic anhydride). The tetrabutylammonium salt **2b** was prepared similarly from **1c**. The identity of complex **2a** was confirmed by negative ion ES-MS, which showed the expected molecular ion at *m*/*z* 527, and as before, fragmentation peaks corresponding to sequential loss of fumaric anhydride at 429 and 331 $([ReO(dmsa)S_2]^-$ and $[ReOS_4]^-$ respectively.

^{1}H NMR of **2a** in dmso-d^6 showed a single sharp resonance due to ligand (C–H) protons consistent with the presence of a single isomer (*syn-endo*). These protons appeared at 4.77 ppm, shifted about 0.9 ppm downfield from the corresponding

Table 1 Selected bond lengths (Å) and angles (°) for $[NEt_4]$-$[ReO(dmsaa)_2]$ **2a** and comparison with $[NEt_4][ReO(dmsa)_2]$ **1b**[15]

	1b	**2a**
Re–O(1)	1.699(8)	1.673(3)
Re–S(1′)	2.322(4)	2.3105(9)
Re–S(2′)	2.301(4)	2.2961(10)
Re–S(1″)	2.329(4)	2.3201(9)
Re–S(2″)	2.286(3)	2.3067(9)
Mean Re–S	2.3095	2.30835
O(1)–Re–S(1′)	106.7(4)	109.17(11)
O(1)–Re–S(2′)	111.2(3)	110.28(11)
O(1)–Re–S(1″)	105.3(4)	107.97(11)
O(1)–Re–S(2″)	113.2(3)	109.64(11)
S(1′)–Re–S(2′)	85.4(1)	85.79(3)
S(2′)–Re–S(1″)	81.9(1)	81.69(3)
S(1″)–Re–S(2″)	85.7(1)	85.91(3)
S(2″)–Re–S(1′)	83.0(1)	81.62(3)
S(1′)–Re–S(1″)	147.9(1)	142.86(4)
S(2′)–Re–S(2″)	135.5(1)	140.08(4)

protons in **1a**–**c** consistent with the electron-deficient character of the anhydride group. IR spectroscopy showed vibrations characteristic of five-ring anhydrides between 1300–1050 cm^{-1} (C–O) and between 1870–1845 and 1800–1775 cm^{-1} (C=O), and Re=O at 964 cm^{-1}. There were no bands assignable to carboxylic acid groups between 1715–1680 cm^{-1}. In the range 280–700 nm the electronic spectrum shows a principal band at 328 nm with a shoulder at 352 nm and further weak shoulders to lower energy. In this respect it does not differ substantially from that of the parent **1b** or $[ReO(SCH_2CH_2S)]_2^-$, which have principal peaks at 325 and 328 nm, with shoulders at 365 and 363 nm respectively, and similar weak shoulders to lower energy.[13,19] Importantly in view of the intended application of this chemistry, the combined spectroscopic data indicate that the ReO(dithiolate)$_2^-$ core remained intact under the conditions required to dehydrate the ligands.

X-Ray structure of $[NEt_4][ReO(dmsaa)_2]$, 2a

Complex **2a** was further characterised by X-ray crystallography. The structure is shown in Fig. 2, with selected bond distances

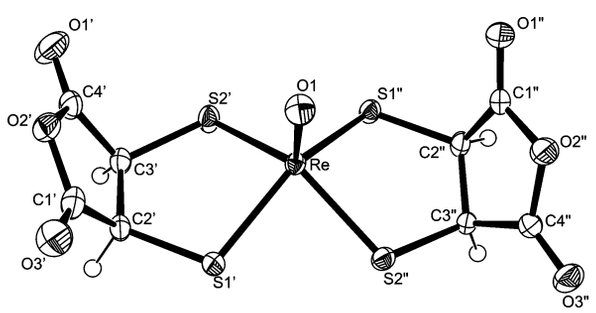


Fig. 2 ORTEP[32] view of the complex anion of **2a**, $[ReO(dmsaa)_2]^-$.

and angles shown in Table 1. This confirmed the expected distorted square-pyramidal structure with an apical oxo-group and four basal thiolate ligands, with the anhydride rings disposed to form the *syn-endo* isomer. The gross geometry is similar to that of related complexes $[ReO(dmsa)_2]^-$ [15] and $[ReO(SCH_2CH_2S)_2]^-$ [19,20] but detailed dimensions differ significantly. The Re–O distance of 1.673(3) Å in **2a** is significantly shorter than that found in the analogous complexes, which have less electron-withdrawing dithiolate ligands {1.742(16) Å for $[ReO(SCH_2CH_2S)_2]^-$,[20] and 1.699(8) Å for $[ReO(DMSA)_2]^-$ [15]}. This is consistent with the Re–O bond distance being controlled largely by the strength of π-donation from O to Re, which is enhanced in **2a** by the greater electron withdrawing nature of the dithiolate ligands. The average Re–S bond length in **2a** does not differ significantly from that in $[ReO(dmsa)_2]^-$. The overall geometry of **2a** is very close to idealised square pyramidal, and is less distorted towards trigonal bipyramidal than that of $[ReO(dmsa)_2]^-$ [for example S(1′)–Re–S(1″) differs from (S2′)–Re–S(2″) by only 2.78° in **2a**, but by 12.4° in $[ReO(dmsa)_2]^-$].

Reaction of 2a with amines

The anhydride groups in **2a** underwent rapid and quantitative aminolysis with primary and secondary amino-groups. As illustrative examples we examined the reactions with diethylamine, glucosamine and an analogue of the 32-amino-acid therapeutic peptide salmon calcitonin. In dry acetonitrile, diethylamine (in excess) reacted with yellow **2a** to give the expected orange condensation product **3** (Scheme 1), again without disruption of the ReO-dithiolate core. The crude product was identified by ES-MS, ^{1}H NMR, IR and UV-VIS spectra. ES-MS showed the expected molecular ion $[ReO\{SCH(CO_2H)C(S)HCON(C_2H_5)_2\}_2]^-$ at *m*/*z* 673, as well as a trace of $[ReO(dmsaa)\{SCH(CO_2H)C(S)HCON(C_2H_5)_2\}]^-$ (*m*/*z* 600) and $[ReOS_4]^-$ at 331. The ^{1}H NMR of **3**, in addition to the expected ethyl groups, showed two AB systems, one centred at 4.15 ppm (J = 129.4 and 4.15 Hz) and one at 3.87 ppm (J = 71.7 and 4.9 Hz). This suggests the presence of two isomers (Fig. 3),

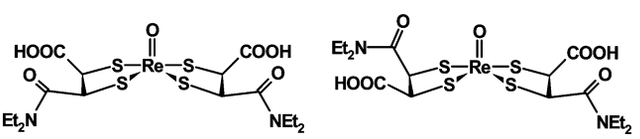


Fig. 3 Isomers formed upon reaction with **2a** with diethylamine. The isomer on the right is chiral and is assumed to be racemic under the synthesis conditions used.

one with a plane of symmetry, the other with a two-fold rotation (assumed to be a racemic mixture of enantiomers), in the ratio of approximately 2 : 1. This is consistent with the absence of *syn*/*anti* isomerism, as inferred from the ^{1}H NMR of **1a** and **2a**. The IR spectrum of **3** showed the expected amide (1627 cm^{-1}) and carboxylate (1708 cm^{-1}) peaks and ν(Re=O) at 962 cm^{-1}. The electronic spectrum of **3** was similar to that of **1b** and **2a** (331 nm, with a shoulder at 361 nm and addition weak shoulders to lower energy).

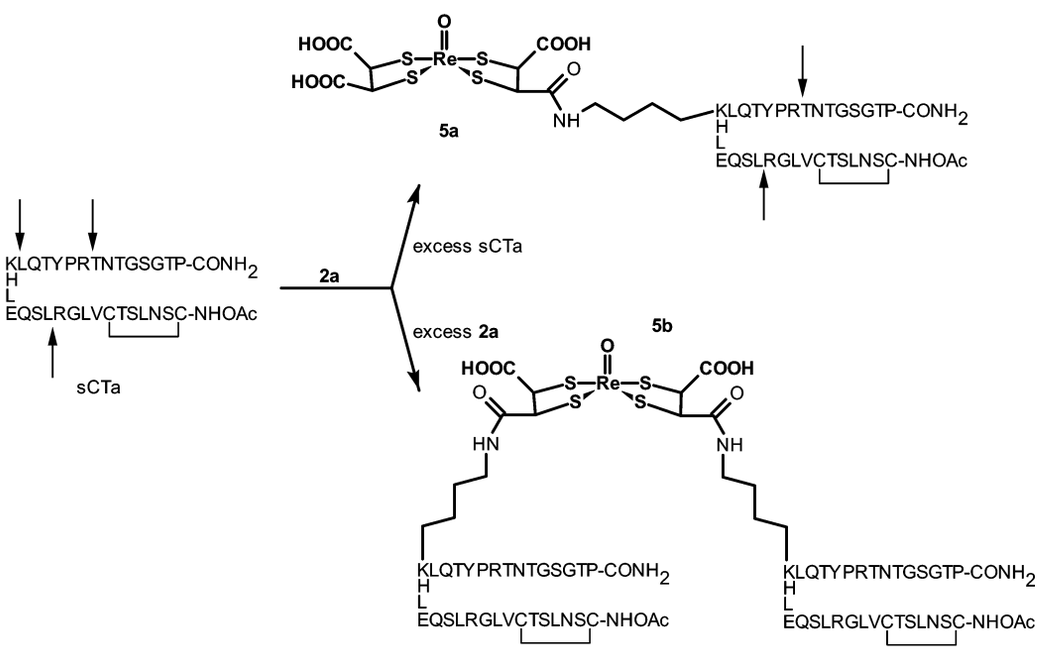
Reaction of **2a** with glucosamine hydrochloride and triethylamine in hot acetonitrile also gave a product with ES-MS consistent with the expected bis-amide conjugate **4** (Scheme 1) (*m*/*z* 885, 100%), as well as a trace of the intermediate mono-glucosamine conjugate (*m*/*z* 706, 10%) and the fragment ion $ReOS_4^-$ (*m*/*z* 331, 35%).

Conjugation of 2a with salmon calcitonin analogue peptide

Salmon calcitonin analogue (sCTa) is a 32-amino-acid peptide (Scheme 3) in which one of the two native lysine residues has been replaced by arginine and the N-terminus has been acetylated. It was synthesised in our laboratory to provide a calcitonin receptor targeting agent, in which conjugation to chelators by aminolysis at a single specific site (lysine-18) was assured. The peptide was incubated with **2a** in 0.1 M sodium hydrogen carbonate buffer, pH 8.2 with **2a** : peptide stoichiometric ratios of 1 : 1, 38 : 1 and 0.3 : 1 for 1 h. The main peptide conjugate peaks and ions observed in the positive ion ES-MS are listed in Table 2 together with their assignments. With **2a** : peptide ratio of 1 : 1, a new HPLC peak was observed, eluting at 24.7 min, in addition to those of the unmodified peptide (23.5 min), **2a** (30.3 min) and its hydrolysis product **1b** (isomers at 17.9 and 20.4 min) and the partially hydrolysed product $[ReO(dmsa)(dmsaa)]^-$ (25.5 min). The new peak was identified by in line ES-MS as the 1 : 1 conjugate **5a** shown in Scheme 3. With a **2a** : peptide ratio of 38 : 1 no significant new peptide-

Table 2 HPLC-ES-MS of sCTa peptide conjugates with **2a**

m/z assignment	Elution time/min	Observed *m/z*	*m/z* shift from (unmodified sCTA + 2H)$^{2+}$	
			Observed	Calculated
Re : sCTa 1 : 1				
[Unmodified sCTa + 2H]$^{2+}$	23.5	1752.1	0	0
[sCTa–dmsa–ReO–dmsa + 3H]$^{2+}$ (**5a**)	24.7	2025.0	272.9	273.0
Re : sCTa 38 : 1				
[sCTa–dmsa–ReO–dmsa + 3H]$^{2+}$ (**5a**)	24.7	2025.1	273.0	273.0
Re : sCTa 1 : 3				
[Unmodified sCTa + 2H]$^{2+}$	23.5	1752.3	0	0
[sCTa–dmsa–ReO–dmsa–sCTa + 6H]$^{5+}$ (**5b**)	25.0	1506.8	−245.5	−244.7
[sCTa–dmsa–ReO–dmsa–sCTa + 5H]$^{4+}$ (**5b**)	25.0	1884.0	131.7	132.0
[sCTa–dmsa–ReO–dmsa–sCTa + 4H]$^{3+}$ (**5b**)	25.0	2509.8	757.5	759.8
[sCTa–dmsa–ReO–dmsaa + 3H]$^{2+}$	25.0	2015.6	264.0	263.3



Scheme 3 Salmon calcitonin analogue sequence and conjugation products. Arrows indicate expected trypsin cleavage points.

containing peaks were observed, only a greatly enhanced **2a** peak indicating that the peptide is not easily conjugated at residues other than lysine and that the great majority of the non-conjugated rhenium complex is in the form of **2a** and not its hydrolysis products. With a **2a** : peptide ratio of 0.3 : 1 a significant new peptide-containing HPLC peak was observed at 25.0 min, identified by ES-MS (Table 2) as the divalent peptide **5b** (Scheme 3) in which two peptides are linked to one rhenium centre.

The site of conjugation in **5a** was unambiguously identified as the lysine-18 residue by means of a trypsin digest[21] analysed by ES-MS. Unmodified sCTa is expected to be cleaved by trypsin at three sites (two arginines and one lysine) giving four peptide fragments A, B, C and D, all of which were observed by ES-MS (Table 3). The conjugation reaction mixture gave, in addition, the expected ion E corresponding to the combination of fragments B and C with the Re complex. This is consistent with the lysine cleavage site being blocked by the modification of the lysine side chain. Additional peaks were observed (F, G, H, I, J, see Table 3) all of which were consistent with the proposed conjugation site. No other significant assignable fragments were observed. This confirms that **2a** links covalently to sCTa exclusively at the lysine residue, that there is no internal crosslinking through **2a**, and that there is no transchelation of the rhenium from the dimercaptosuccinate to the cysteines.

Discussion

In principle, the ideal approach to efficient and convenient labelling of biomolecules with short half-life radioisotopes is to introduce the radiolabel as the last step of the synthesis ("post-conjugation labelling"). Methods that combine this advantage with ease of synthesis under mild conditions to give products with very high *in vivo* stability are rare especially for rhenium labelling. *Meso*-dimercaptosuccinic anhydride was used in such an approach previously by conjugating its dithiane-protected form with antibodies, and transchelating metallic radionuclides to this conjugate. The structure of the metal complex part of the conjugate was not identified; probably only one dimercaptosuccinate ligand bound to the metal, with the remainder of the coordination sphere completed by donor atoms from within the protein.[22] The chemistry we describe here is not a post-labelling approach, so that some radiochemistry with the radioisotope has to be performed prior to the actual labelling. However, it has the advantage that the two-step synthesis of the reactive rhenium anhydride complex **2a** is a very rapid, mild method which proceeds in quantitative yield with minimal handling and is amenable to a "kit" formulation. Since the structure and stability of $[TcO(dmsa)_2]^-$ is the same as that of $[ReO(dmsa)_2]^-$,[23,24] the method should be equally applicable to technetium labelling, although we have not as yet demonstrated

Table 3 HPLC-ES-MS analysis of products from trypsin digest of **5a**

Assigned fragment[a]	Elution time/min[a]	Observed *m*/*z*	Calculated *m*/*z*
A Cys^{1}–Arg^{11} + H^+	19.4–20.5	1192.9	1193.4
B Leu^{12}–Lys^{18} + H^+	19.4–20.5	856.0	855.0
C Leu^{19}–Arg^{24} + H^+	19.4–20.5	777.6	777.9
D Thr^{25}–Pro^{32} + Na^+	5.3	756.1	755.8
E Leu^{12}–Arg^{24}·$ReO(DMSA)_2$	19.4–20.5	2158.9	2158.9
F Leu^{12}–Arg^{24} + H^+ [b]	19.4–20.5	1613.3	1613.9
G Leu^{19}–Pro^{32} + H^+ [b]	5.3	756.1	756.9
H Leu^{12}–Pro^{32} + $2H^{2+}$ [b]	19.4–20.5	1436.5	1438.4
I Leu^{12}–Arg^{24}·fumaric acid + H^+ [c]	19.4–20.5	1711.3	1711.9
J Leu^{12}–Arg^{24}·fumaric acid + $2H^{2+}$ [c]	19.4–20.5	856.0	857.5

[a] Assignments of expected ions A–E are discussed in the text. [b] Fragments F, G and H are due to incomplete cleavage of unmodified peptide between Lys^{18} and Leu^{19}. [c] Fragments I and J are due to C–S bond cleavage between peptide and rhenium complex in fragment E occurring in the mass spectrrometer as observed also for the unconjugated complexes **1**–**4**.

this. Successful synthesis of both 1 : 1 and 2 : 1 (peptide : rhenium) conjugates was demonstrated in reasonable yield, despite the fact that no steps have yet been taken to optimise the conjugation conditions (pH, buffer, solvent, temperature). The conjugate should be highly invulnerable to transfer of Tc or Re to *in vivo* chelators because of the observed high *in vivo* kinetic stability of the rhenium/technetium oxobis(dithiolate) core.[12,25] The metal complex also has the advantage of being rendered hydrophilic by the carboxylate groups, unlike some of the recently developed conjugates with labels such as rhenium/technetium tricarbonyl,[26] cyclopentadienyl[27] and organophosphine[28] complexes, in which lipophilicity and water insolubility may adversely affect biodistribution.

The synthesis incorporates a significant improvement on the previous synthesis of $[ReO(dmsa)_2]^-$,[13] in that it is complete in a few minutes and the work-up is straightforward. An additional unexpected benefit is that it gives the product in the form of a single isomer rather than the equilibrium mixture of three produced by the literature method.[12] This is significant because $[ReO(dmsa)_2]^-$ itself is a tumour targeting agent for medullary thyroid carcinoma[29] and for bone metastases.[12,23] Since the *anti*, and then the *syn-exo* isomers are formed to a greater extent on standing in aqueous solution, as observed in this and earlier[15] work, it is clear that there is only a marginal thermodynamic preference for the *syn-endo* isomer, and that under the present conditions of synthesis the overwhelming preference for this isomer is kinetic in origin. The reasons for this remain unclear with the available data.

The successful use of acetylhydrazine as reducing agent for perrhenate in this reaction suggests that it may have wider utility as a general purpose, clean, low-toxicity reducing agent in radiopharmaceutical synthesis since it produces only acetic acid and dinitrogen as by-products and is effective at room temperature in water and non-aqueous solvents.

We are now evaluating the use of the new reactive complex $[ReO(dmsaa)_2]^-$ and its Tc analogue, using generator-produced Re-188 and Tc-99m, at the radiochemical level for synthesis of protein- and peptide-based radiopharmaceuticals.

Experimental

General

All reactions were undertaken at room temperature without precautions to exclude air and moisture unless otherwise indicated. Elemental analyses (C, H, N) was performed by the Chemical Laboratory, University of Kent. NMR (^1H) measurements were made in dmso-d^6 unless otherwise indicated, using a JEOL 270 MHz spectrometer. Chemical shifts were quoted relative to internal $SiMe_4$. ES-MS were recorded in negative ion mode for small complexes and in positive ion mode for peptides, using acetonitrile or aqueous acetonitrile solutions, in some cases in-line with reverse-phase HPLC (Vydac C18 column, 250 × 21.2 mm), at the EPSRC Mass Spectrometry Centre, Swansea, UK using a QUATTRO II "triple" quadrupole instrument, or at the Wellcome Protein Science Laboratory, University of Kent, using a Finnegan MAT instrument. Infrared spectra were recorded in the range 700–3300 cm^{-1} as Nujol mulls using a Perkin-Elmer ATI Mattson Genesis series FTIR spectrophotometer. All chemicals were from Aldrich, UK. Electronic spectra were recorded using acetonitrile solutions (0.1 mg ml^{-1}) on a Helios α Unichem spectrophotometer.

Crystallography

Crystals of **2a** suitable for an X-ray crystal structure determination were grown from anhydrous acetonitrile–diethyl ether. The data were collected on a Bruker AXS SMART 1K CCD diffractometer[30] at 150(2) K using synchrotron radiation (λ = 0.6900 Å; SRS station 9.8 CLRC Daresbury Laboratory). The structure was solved by direct methods and refined by full matrix least-squares on F^2 using SHELXTL,[31] with anisotropic displacement parameters for all non-hydrogen atoms. The hydrogen atoms were placed in calculated positions and allowed to ride on the carbon atom to which they were attached. Crystal data for $[NEt_4][ReO(dmsaa)_2]$, $C_{16}H_{24}NO_7ReS_4$, M = 656.80, crystal dimensions 0.10 × 0.08 × 0.02 mm, orthorhombic, space group $Pna2_1$, a = 12.4936(4), b = 19.2134(6), c = 9.2046(3) Å, Z = 4, U = 2209.51(12) $Å^3$, D_c = 1.974 g cm^{-3}; 15074 reflections were collected to θ = 29.50°; 5995 unique reflections (R_{int} = 0.0300); which were corrected for Lorentz and polarisation effects and for absorption, μ = 5.915 mm^{-1}. $R1$ = 0.0235, $wR2$ = 0.0573 for 5813 reflections with [$I > 2\sigma(I)$] and $R1$ = 0.0243, $wR2$ = 0.0576 for all data.

CCDC reference number 195018.

See http://www.rsc.org/suppdata/dt/b2/b209820b/ for crystallographic data in CIF or other electronic format.

Synthesis of *syn-endo*-$[NH_4][ReO(dmsa)_2]$, 1a

Ammonium perrhenate (0.536 g, 2.0 mmol) and acetylhydrazine (0.148 g, 2.0 mmol) were stirred in acetonitrile (50 cm^3) for 10 min at room temperature. Addition of 0.23 ml conc. HCl followed by stirring for 25 min yielded a yellow solution with a white or pale yellow precipitate. *Meso*-2,3-dimercaptosuccinic acid (0.728 g, 4.0 mmol) was then added and left to stir for 3 h to give a microcrystalline orange solid, which was isolated by filtration, washed with acetonitrile and dried under vacuum. Yield 0.87 g (75%). $C_8H_{12}NReO_9S_4$: calc. C 16.5, H 2.1, N 2.4; found C 17.2, H 2.8, N 3.2%. IR ν_{max}/cm^{-1} 3100br (NH_4^+, OH), 1685s (C=O), 955 (Re=O); ^1H NMR δ_H (270 MHz; dmso-d^6) 11.9 (br s, COOH), 7.19 (t, J_{NH} = 51 Hz, NH_4^+), 3.88 (s, CH); ES-MS *m*/*z* 562.9 (M^-, 90%), 446.9 (M^- − fumaric acid, 15%), 330.9 ($ReOS_4^-$, 100%). To check the isomeric composition of the crude reaction mixture prior to filtration of the crystalline product, the solution was evaporated to dryness and the residue

dissolved in dmso-d^6 or 0.1 M Na_2CO_3 in D_2O. ^{1}H NMR spectra of these solutions were as follows. In dmso-d^6, the SCH protons appeared as a singlet with $\delta = 3.88$ (*syn-endo* isomer). In 0.1 M Na_2CO_3/D_2O they appeared as a singlet with $\delta = 4.12$ (*syn-endo* isomer, 91% of total ReSCH signal), two small singets with $\delta = 4.16$ and 4.18 (*anti* isomer, 8% of total ReSCH signal, increasing on standing), and a further small singlet with $\delta = 4.20$ (*syn-exo* isomer, 1% of total ReSCH signal, increasing on standing). The isomeric composition of a freshly prepared aqueous solution was also checked by HPLC using a Hamilton PRP-1 reversed-phase column and a solvent gradient (flow rate 0.3 ml min^{-1}) comprising 0.1% trifluoroacetic acid in water (solvent A) and 0.085% trifluoroacetic acid in 70 : 30 acetonitrile–water (solvent B) defined as follows (min, %B): 0, 5; 0, 5; 30, 64; 31, 5; 40, 5.

Synthesis of *syn-endo*-[NEt$_4$][ReO(dmsa)$_2$], 1b

1a (0.876 g, 1.5 mmol) was dissolved in water and an excess of Et_4NBr was added. The orange precipitate was filtered off. Yield 0.73 g, 1.06 mmol (70%). $C_{16}H_{28}NReO_9S_4 \cdot 2H_2O$: calc. C 26.4, H 4.4, N 1.9; found C 26.6, H 4.3, N 2.0%; IR ν_{max}/cm^{-1} 3440br, (OH), 1686s (C=O), 961m (Re=O); ^{1}H NMR δ_H (270 MHz; dmso-d^6) 12.01 (4H, br s, COOH), 3.88 (4H, s, SCH), 3.20 (q, $J_{HH} = 7.4$ Hz, NCH_2); 1.16 (tt, $J_{HH} = 7.4$ Hz, $J_{NH} = 1.9$ Hz, CH_3); ^{1}H NMR (D_2O) 4.10 (*syn-endo* isomer, >98% of total ReSC*H* signal), two small singlets with $\delta = 4.16$ and 4.18 (*anti* isomer, <2% of total ReSC*H* signal); ES-MS as for **1a**.

Synthesis of [NBu$_4$][ReO(dmsa)$_2$], 1c

This salt was prepared analogously to **1b** in 70% yield using tetrabutylammonium bromide as the source of cations. $C_{24}H_{44}NReO_9S_4$: calc. C 35.8, H 5.5, N 1.7; found C 35.8, H 5.5, N 2.2%. IR ν_{max}/cm^{-1} 3400 br (OH), 1688 (C=O), 971 (Re=O); ^{1}H NMR δ_H (270 MHz; dmso-d^6) 11.99 (4H, br s, COOH), 3.95, 3.93, 3.87 (all s, total 4H, SCH), 3.16 (8H, br m, NCH_2), 1.57 (8H, m, NCH_2CH_2), 1.31 (hextet, $J = 7.2$ Hz, CH_2CH_3), 0.93 (12H, t, $J = 7.2$ Hz, CH_3); ES-MS as for **1a**.

Synthesis of [NEt$_4$][ReO(dmsaa)$_2$], 2a

To compound **1b** (0.347 g, 0.5 mmol) dissolved in acetic anhydride or MeCN (30 cm^3), was added 1,3-dicyclohexylcarbodiimide (0.206 g, 1.0 mmol) in the same solvent (20 cm^3). The solution was stirred for 3 h during which the orange solution rapidly changed to yellow with formation of white solid, dicyclohexyl urea. This was removed by filtration under dry dinitrogen and the yellow solution was then evaporated to dryness under vacuum at 40 °C. The solid was washed repeatedly with dichloromethane to remove residual dicyclohexyl urea. Pure samples were obtained by slow recrystallisation from acetonitrile–diethyl ether. $C_{16}H_{24}NReO_7S_4$: calc. C 29.3, H 3.7, N 2.1; found C 28.7, H 4.1, N 2.4%; IR ν_{max}/cm^{-1} 1855, 1785 (CO), 964 (Re=O); ^{1}H NMR δ_H (270 MHz; dmso-d^6) 4.77 (4H, s, SCH), 3.20 (8H, q, $J = 7.4$ Hz, CH_2), 1.16 (tt, $J_{CH} = 7.4$ Hz, $J_{NH} = 1.9$ Hz, CH_3); UV-VIS: λ_{max}/nm (MeCN) 328 (ε/M^{-1} cm^{-1} 5040), 352 (sh), further weak shoulders to lower energy; ES-MS *m*/*z* 526.9 (M$^-$, 80%), 428.9 (M^- − fumaric anhydride, 10%), 331.0 ($ReOS_4^-$, 100%).

Synthesis of [NBu$_4$][ReO(dmsaa)$_2$], 2b

Complex **2b** was prepared from **1c** by a method analogous to that for **2a**. The pale yellow solid obtained by evaporating the filtered reaction solution to dryness was dried under vacuum. Yield 0.331 g, 0.43 mmol (86%). $C_{24}H_{40}NReO_7S_4$: calc. C 37.5, H 5.2, N 1.8; found C 36.2, H 5.7, N 1.7%; IR ν_{max}/cm^{-1} 1856, 1786 (CO), 964 (Re=O); ^{1}H NMR δ_H (270 MHz; dmso-d^6) 4.77 (4H, s, SCH), 3.16 (8H, m, NCH_2), 1.57 (8H, m, NCH_2CH_2), 1.31 (8H, hextet, $J = 7.4$ Hz, CH_2CH_3), 0.94 (12H, t, $J = 7.4$ Hz, CH_3); ES-MS as for **2a**.

Reactions of [ReO(dmsaa)$_2$]$^-$ with amines

Complex **2a** (0.034 g, 0.05 mmol) was dissolved in dried distilled acetonitrile (2.0 cm^3) and diethylamine (0.05 ml) was added whilst stirring to give immediately an orange solution. This was stirred 2 h at room temperature to give an orange suspension. This was evaporated under vacuum and the residue **3** analysed by spectroscopic methods. IR ν_{max}/cm^{-1} 3400br (OH), 1708, 1630 (carboxylic acid and secondary amide C=O), 961 (Re=O); UV-VIS: λ_{max}/nm (MeCN) 331 (ε/M^{-1} cm^{-1} 4140), 361 (sh) and additional weak shoulders at lower energy; ^{1}H NMR δ_H (270 MHz; dmso-d^6) 4.15 (AB q, $J = 129.4$ and 4.15 Hz) and 3.87 (AB q, $J = 71.7$ and 4.9 Hz, total 4H); 3.2 (8H, q, $J = 7.4$ Hz, $[N(CH_2CH_3)_4]^+$), 2.62 (8H, q, $J = 7.2$, amide NCH_2), 1.16 (12H, tt, $J_{CH} = 7.4$, $J_{NH} = 1.9$ Hz, $[N(CH_2CH_3)_4]^+$), 1.04 (12H, t, $J = 7.2$, amide NCH_2CH_3); ES-MS *m*/*z* 673.2 (M^-, 50%), 331.0 ($ReOS_4^-$, 100%).

The reaction with glucosamine hydrochloride was performed similarly after addition of triethylamine, to give crude **4**. ES-MS: *m*/*z* 885.1 (100%, M$^-$), 330.9 (30%, $ReOS_4^-$).

Reaction of 2a with salmon calcitonin analogue (sCTa)

100 µl sCTa solution (1 mg ml^{-1} in 100 mM sodium bicarbonate buffer, pH 8.2), was treated with **2a** dissolved in anhydrous dmso at a concentration of 10 mg ml^{-1} (2 µl for a **2a** : sCTa ratio of 1 : 1 and 75 µl for 38 : 1) and incubated in darkness at room temperature for 1 h. The conjugation reaction was then analysed with reverse phase HPLC-ES-MS. The elution gradient consisted of aqueous trifluoroacetic acid (0.05%) as solvent A and acetonitrile (70%) as solvent B, flow rate 0.2 ml min^{-1}, gradient as follows (minutes, %B): 0, 0; 5, 0; 30, 100; 35, 100; 40, 0. Peaks eluting in the first 40 min were analysed directly in the ES-MS in positive ion mode. To study the effect of incubating **2a** with excess peptide, 50 µl of the above sCTa solution (1.43×10^{-8} mol) was evaporated to dryness and the peptide redissolved in 10 µl bicarbonate buffer. To this solution was added 3 µl of a solution of **2a** in dmso (1 mg ml^{-1}) to give a **2a** : sCTa ratio of 0.3 : 1. The mixture was incubated as above before adding 50 µl water prior to HPLC and ES-MS analysis.

Trypsin digest of sCTa conjugate

sCTa was incubated overnight with a four-fold excess of **2a** using the reagents and conditions described above. The product was purified by RP HPLC using the method described above, collecting 24.7 min (conjugate) and 23.5 min (unmodified control) peaks (these peaks were incompletely resolved). The partially purified labelled peptide conjugate (24.7 min fraction, now in ~30% acteonitrile–0.05% trifluoroacetic acid pH 2.5 aqueous solution), was added to an equal volume of 100 mM sodium bicarbonate at pH 8.2 to maintain the pH at 8.2. Modified Trypsin Sequencing Grade (Promega, UK) (2 mg ml^{-1}, 20 µl) was added and left at room temperature for 120 min. The sample was then dried in a vacuum centrifuge and reconstituted with 100 µl of 10 mM acetic acid (pH 3.5). Analysis was performed by RP HPLC ES-MS. Major peaks were eluted at 5.3 and 19.4–20.5 min. The latter was an incompletely resolved group of peaks which were analysed together in the ES-MS. This was compared to a control digestion of unmodified sCTa.

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