A NEW STRATEGY FOR THE PREPARATION OF Re AND Tc METALLOCARBORANES

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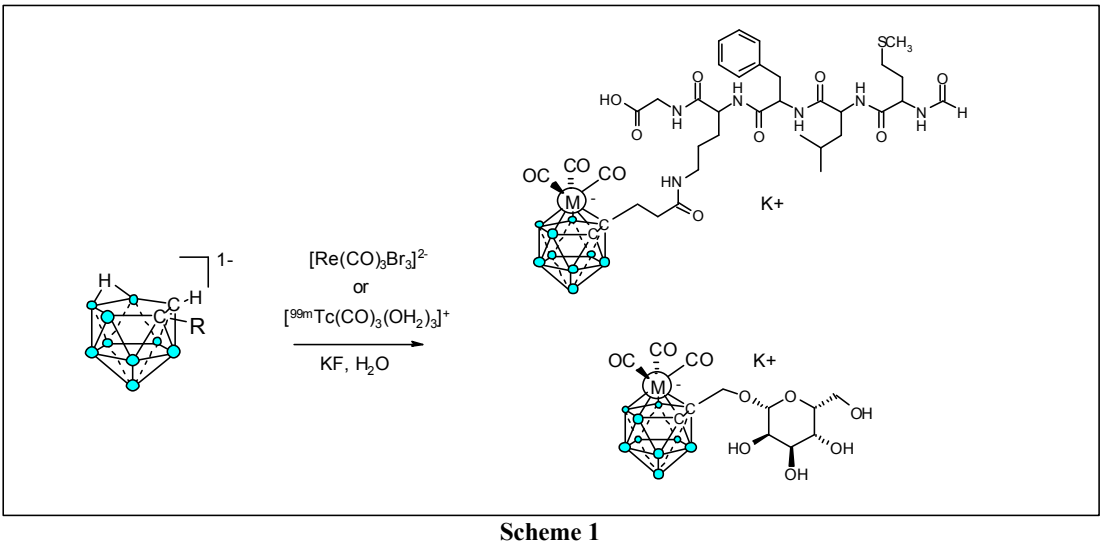
Keywords: Carboranes, Tc/Re(I), Bioconjugates, Organometallic

Having access to $([M(CO)_3(OH_2)_3]^+$ (M = Tc, Re) has created the opportunity to create a wide range of novel radiopharmaceuticals including those derived from organometallic ligands. Alberto and coworkers, for example, demonstrated that cyclopentadienide (Cp^-), the quintessential organometallic ligand, can be used to prepare complexes of the type $RCpM(CO)_3$ in water so long as R contains a carbonyl group alpha to the Cp ring [1]. The presence of the acyl group is essential in order to avoid side reactions associated with the highly reactive Cp ligand.

Our approach to the preparation of organometallic radiopharmaceuticals is to use carboranes in place of Cp^- [2]. The dicarbollide dianion $[nido\text{-}C_2B_9H_{11}]^{2-}$ is isolobal to cyclopentadienide and can be prepared by the degradation of commercially available *ortho*-carborane. $[nido\text{-}C_2B_9H_{11}]^{2-}$, which is stable in water, can be easily functionalized with a range of functional groups, including carboxylic acids, amines, alcohols and hydrazines, as sites for attaching targeting agents.

The aim of the presentation is to describe our recent work on the synthesis and radiolabeling of unique carborane ligands and their biomolecule conjugates. The focal point of the presentation will be a new method for preparing Re(I) and Tc(I) carborane complexes that is superior, and milder, than the strategy we reported previously. This new methodology minimizes the formation of Re and Tc byproducts when performing reactions on a macroscopic scale and enhances radiochemical yields at the tracer level. Our investigation of the factors affecting radiochemical yields and a study on the stability of the carborane complexes will also be presented.



Scheme 1

1. Wald J, Alberto R, Ortner, K, Candreia, L. *Angew Chem, Int Ed Engl* 2001; 40: 3062-3066.
2. Valliant J F, Morel P, Schaffer P, Kaldis, J H. *Inorg Chem* 2002; 41: 628-629.

SYNTHESIS AND SUBSTITUTION BEHAVIOR OF ^{99M}TECHNETIUM AND RHENIUM CARBONYL-NITROSYL COMPLEXES FOR POTENTIAL USE IN RADIOPHARMACY

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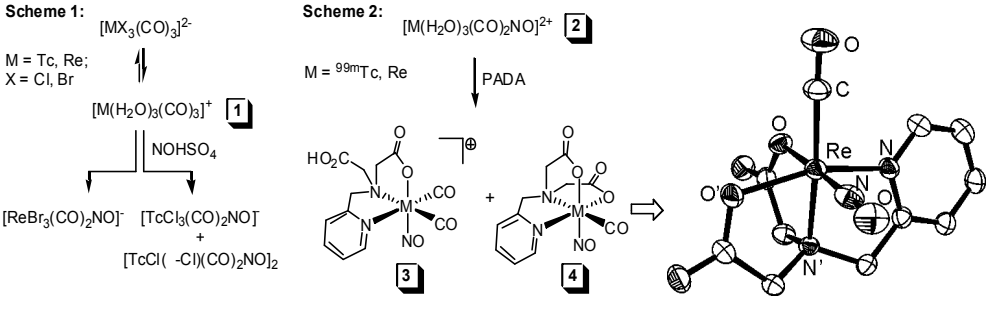
Keywords: Technetium(I), rhenium(I), organometallic, nitrosyl, carbonyl

Continuing the development of organometallic technetium and rhenium precursors for potential biomedical applications, we present herein the first aqueous based synthesis of mixed carbonyl-nitrosyl complexes of Tc(I)/Re(I) on the carried added and n.c.a. level. In respect of radiopharmaceutical use, the access to such precursors could lead to compounds with different and improved pharmacokinetic properties than those labeled with the $[M(CO)_3]$-fragment or simplify the functionalization of biomolecules for radiolabeling with Tc-99m or Re-188.

Water soluble $NOHSO_4$ were used as the NO^+-source for the synthetic approach. $[M(H_2O)_3(CO)_3]^+$, **1** (M = ^{99}Tc, Re), was the starting material. Time dependent analyses of the reactions by means of IR- and ^{99}Tc-NMR spectroscopy in solution revealed the almost quantitative formation of complexes with a '*fac*-$[MNO(CO)_2]^{2+}$' entity. In case of technetium the formation of the monomeric $[TcCl_3(CO)_2NO]^-$ and the dimeric species $[TcCl(\mu\text{-}Cl)(CO)_2NO]_2$ was observed, whereas in case of rhenium the $[ReBr_3(CO)_2NO]^-$ was solely produced (Scheme 1). The halo-nitrosyl-carbonyl complexes form readily the aqua complexes $[MX(H_2O)_2(CO)_2NO]^+$ and $[M(H_2O)_3(CO)_2NO]^{2+}$, **2**, in diluted solutions as evident from HPLC and IR spectroscopy. On the n.c.a. level the desired complex $[^{99m}Tc(H_2O)_3(CO)_2NO]^{2+}$ was almost quantitative formation after 10 min at 100°C in saline in presence of excess $NOHSO_4$.

Ligand exchange reactions on the carrier-added and n.c.a. level with multi-dentate chelating systems have been performed. Other than in case of the tricarbonyl precursor **1**, the nitrosyl pendant **2** reveals a much more diverse and complex substitution pattern. E.g. reaction with the ligand system picolyamine diacetic acid (PADA) produced two different complexes as evident from IR and NMR investigations. Beside the anticipated complex $[Re(PADA)(CO)_2NO]^+$ **3** (tridentate N,N',O-coordinated), the tetradentate coordinated (N,N'O,O'), mono carbonyl-mono nitrosyl complex [Re(PADA)(CO)NO] **4** was predominantly formed. The x-ray structure of complex **4** is shown in Scheme 2. In case of complex **4**, one CO ligand of **2** has been displaced enabling a tetradentate, "umbrella-like" coordination of the PADA ligand. On the n.c.a. level with ^{99m}Tc the desired tridentate complex $[^{99m}Tc(PADA)(CO)_2NO]^+$ was predominantly formed as evident from HPLC analyses. In vitro analyses performed in buffered media and human serum revealed a similar stability as the corresponding ^{99m}Tc-tricarbonyl complex. Direct labeling experiments of large proteins with precursor **2** revealed a slightly faster reaction kinetic compare to **1** but at the same time a higher plasma aggregation. Further in vitro and in vivo experiments will be presented.



NOVEL SITE-SPECIFIC ENZYMATIC RADIOLABELING OF PROTEINS AND PEPTIDES WITH THE 99M-TC-TRICARBONYL COMPLEX USING TRANSGLUTAMINASE

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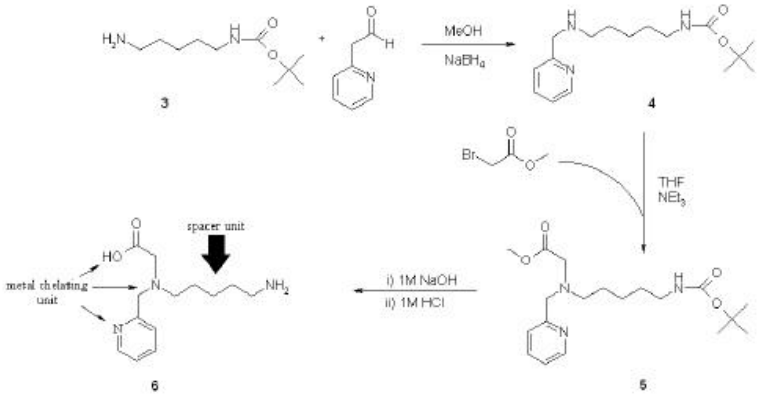
Keywords: Site-specific, radiolabeling, technetium-tricarbonyl, enzymatic

Transglutaminases irreversibly catalyze covalent cross-linking of proteins by forming isopeptide bonds between peptide-bound glutamine and lysine residues. In this present work, the first enzyme mediated $^{99m}Tc(CO)_3$ radiolabeling of peptide and proteins with the bifunctional chelating agent [(5-amino-pentyl)-pyridine-2-yl-methyl-amino]-acetic acid (APPA) is presented.

We synthesized the APPA-chelater to mach the following criteria: first, the ligand system should coordinate the *fac*-$[M(CO)_3]^+$ core ($M=^{99m}Tc$, ^{188}Re) in a tridentate way, thus not offering a residual coordination site for unspecific coordination. Therefore the chelating system should contain one or more amine (preferentially aromatic N-heterocycles) functionalities in combination with a carboxylic acid function. Second, the ligand system should have a five-carbonatom alkylamine side chain which mimics a lysine residue and thus should be recognized by transglutaminase (Tgase) as a substrate for site specific radiolabeling of peptides and proteins.

Synthesis of APPA



The peptide RPLPQQF and bovine b-casein were used as model substrates for radiolabeling with $^{99m}Tc(APPA)(CO)_3$ and free APPA, using Ca^{2+} dependent guinea pig liver TGase and Ca^{2+} independent microbial TGase. A time dependent incorporation of the ^{99m}Tc-complex was observed, which was completed after 4 h. Compared to the control, enzyme mediated incorporation of the ^{99m}Tc-complex was one to two orders of magnitude higher.

In conclusion we present a new strategy to radiolabel peptides and proteins site-specific and under physiological conditions, using solely the enzymatic activity of TGases.

SELECTION OF RADIOLABELING SITES BY SITE-DIRECTED MUTAGENESIS

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Keywords: Peptides, Tc-99m, Bifunctional Chelators, HYNIC, Thrombus Imaging

Bitistatin is an 83-amino acid polypeptide which binds to the alpha IIb beta 3 receptor on the surface of platelets and shows promise as a radiopharmaceutical for imaging thrombi and emboli. Recombinant bitistatin (rBitistatin) is now produced by recombinant DNA expression in E.Coli. Bitistatin has been labeled by direct electrophilic radioiodination at Tyr71 or by attachment of hydrazino nicotinamide (HYNIC) bifunctional chelators to one or more of the five Lys residues (positions 35, 52, 54, 55, and 75). Although all Lys residues are on the opposite side of the bitistatin molecule from the putative RGD binding domain, attachment of 1.0-2.5 HYNIC per molecule resulted in lower binding to platelets in vitro compared with iodination. Molecular modeling suggested that modification of certain Lys sidechains with Tc-Tricine-HYNIC might affect the orientation of the binding domain. The purpose of this study was to selectively eliminate points of attachment of HYNIC to maintain higher binding to platelets.

Two mutants were created by altering the DNA sequence of the gene for bitistatin within its expression vector using a commercially available kit for site-directed mutagenesis. Lysines at position 35 or 75 were mutated to Arg (R) to prevent their reaction with the N-succinimidyl ester of HYNIC. The mutated vectors were inserted into E.Coli and used to express the desired polypeptides. After refolding and purification to a single peak, each polypeptide was modified with HYNIC and labeled with Tc-99m using Tricine as coligand. The peptides were tested for binding to platelets in whole blood in vitro, for binding to ADP-activated platelets in a plasma-free environment (Stim GFP), and for the rate of labeled polypeptide dissociation from Stim GFP after adding a physiologic concentration of fibrinogen (offrate).

The results are summarized in Table 1. Tc-HYNIC-[R35]rBitistatin had better binding to platelets in whole blood compared to Tc-HYNIC-rBitistatin(wild-type) and was similar to I-rBitistatin. Tc-HYNIC-rBitistatin(wild-type) has a slower offrate than I-rBitistatin from Stim GFP and this was preserved in the [R35] mutant. In contrast, the [R75] mutant had the same level of platelet binding as Tc-HYNIC-rBitistatin(wild-type) and had a slightly faster offrate. Nonspecific binding was 5% for all radiotracers.

Table 1. Platelet binding properties of labeled rBitistatin and mutants

Peptide	% bound to platelets in blood	P(vs I-B)	P(vs Tc-B)	Offrate T50*	P(vs I-B)
I-rBitistatin (wild-type)	40.5 ± 1.1			53 min	
Tc-HYNIC-rBitistatin(wild-type)	31.2 ± 0.7	<0.001		67 min	<0.005
Tc-HYNIC-[R35]rBitistatin	42.3 ± 1.0	NS	<0.001	64 min	<0.002
Tc-HYNIC-[R75]rBitistatin	35.4 ± 0.5	<0.001	NS	54 min	NS

I-B = I-rBitistatin(wild-type); Tc-B= Tc-HYNIC-rBitistatin(wild-type);
NS = not significant (P<0.05)
* Time to 50% dissociation from plasma-free platelets after challenge with 8.8 μM fibrinogen

In conclusion, attachment of HYNIC at Lys35 appears to adversely affect the binding of labeled bitistatin to platelets. Selective replacement of Lys35 with Arg to prevent conjugation yielded a Tc-labeled molecule which retained the platelet binding of iodinated bitistatin. Tc-HYNIC-rBitistatin(wild-type) remains bound to platelets longer than I-rBitistatin, which may explain its better targeting in vivo despite lower platelet binding. When tested in vivo, Tc-HYNIC-[R35]rBitistatin may provide a radiopharmaceutical with improved thrombus uptake and retention.

Supported by NIH grant R01 HL 54578.

TRICARBONYL COMPLEXES OF Re AND Tc WITH A BIFUNCTIONAL TRIDENTATE NNO LIGAND CARRYING THE 2-PHENYL-BENZOTHIAZOLE MOIETY

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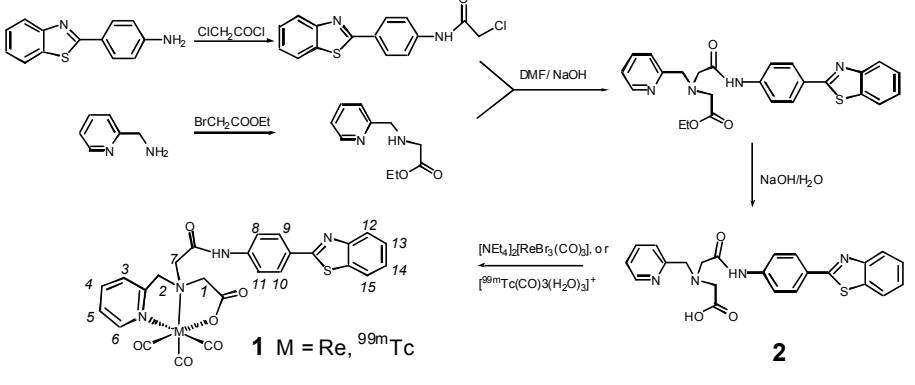
Keywords: rhenium(I), technetium(I), radiopharmaceuticals, tricarbonyl complexes, 2-phenylbenzothiazole

2-Substituted derivatives of benzothiazole comprise a novel class of potent and selective antitumor agents. In addition, 2-substituted benzothiazole derivatives have been shown to bind to Alzheimer's amyloid aggregates with high affinity by *in vitro* and *in vivo* techniques. In view of the interesting properties of benzothiazole derivatives we proceeded in the design of rhenium (Re) and technetium (Tc) complexes of this class of compounds for potential radiopharmaceutical applications. ^{186}Re and ^{188}Re as β-emitters are two of the most promising candidates for tumor targeting radiotherapy while the ^{99m}Tc is the radionuclide of choice for diagnostic imaging.

Herein we report the synthesis of tricarbonyl Re- and ^{99m}Tc- complexes (**1**) with the bifunctional tridentate NNO ligand N-(pyridin-2-yl-methyl)-N-(4-(benzothiaz-2-yl)-phenylaminocarboxymethyl)aminoacetic acid (**2,** synthetic scheme outlined below) which carries the 2-(4-aminophenyl)benzothiazole moiety attached through an amide bond.

The Re(I)-complex **1** was prepared in good yield by refluxing equimolar amounts of **2**, $[NEt_4]_2[ReBr_3(CO)_3]$ and NaOH in acetonitrile for 2 hours. Its identification was based on mass spectral analysis and NMR spectroscopy (DMSO-d_6, ppm: 4.71 and 4.52 (H-1), 3.80 and 4.16 (H-2), 7.86 (H-3), 8.16 (H-4), 7.58 (? -5), 8.77 (H-6), 5.27 and 4.78 (H-7), 8.09 (H-8/H-11), 7.82 (H-9/H-10), 8.12 (? -12), 7.44 (? -13), 7.53 (? -14), 8.02 (? -15), numbering of the protons is shown in the scheme below). Synthesis of the ^{99m}Tc complex **1** was carried out by incubating the *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor for 20 min at 70 ^oC with 0.4 mg of **2** in saturated solution of sodium chloride at pH 1. The pH of the solution was subsequently adjusted to 8 and, after standing for 30 min at room temperature, the reaction mixture was analyzed by HPLC. The HPLC analysis demonstrated that the reaction results in the formation of a single complex (yield > 90 %) which is stable for more than 6 h. The identity of the ^{99m}Tc complex **1** was established by comparative HPLC studies using a sample of the well characterized Re(I) complex as reference. Biodistribution studies of the ^{99m}Tc complex **1** in mice are being currently carried out.



^{99m}TECHNETIUM LABELING OF PEGYLATED PN_2S LIGAND IN PRESENCE OR ABSENCE OF TIN(II)

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Keywords: PN_2S, BFCA, Technetium, PEG

The PN_2S ligands N-(N-(3-diphenylphosphinopropionyl)glycyl)-S-trytilcysteine [PN_2S(Trt)-OH, **1**] was previously labeled via ^{99m}Tc-gluconate. The reaction at pH 7 led to a single species identified as the anti isomer of pentacoordinated complex ^{99m}TcO[PN_2S-OH]. The labeled compound exhibited high stability in vivo being the PN_2S set promising for labeling biomolecules.

In these studies ligand 1 was conjugated to the methoxy-PEG-amine (MW 5000) to evaluate the increase of water-solubility of ^{99m}TcO[PN_2S-OH] (to reduce hepathobiliary elimination), the use of bifunctional PEGs as a linker for labeling biomolecules and the possible use of PN_2S chelating system for PEG-dendrimers labeling. The PN_2S(Trt)-PEG derivative **2** was easily obtained in high yields (>95%) by DCCI/NHS ligand activation and detrytilated with TFA/TES before labeling. The PN_2S-PEG ligand **3** was isolated (yields >90%) and both compounds were characterized with MALDI-TOF spectrometry and ^{1}H-NMR, ^{31}P-NMR and UV-Vis spectroscopies.

The labeling of compound 3 with in situ produced ^{99m}Tc-gluconate was quantitative in 5 min (pH 7, 37°) and HPLC analysis showed one major peak followed by some minor peaks. The minor peaks were ascribed to an unspecific labeling of PEG by Tc hydrolysed species. The reaction performed after Sep-Pak purification of ^{99m}Tc-gluconate led to a single homogeneous peak coincident with the major one previously described. No labeling of unconjugated PEG with purified ^{99m}Tc-gluconate was observed, indicating that the major peak was due to the transchelation reaction gluconate-PN_2S chelating system leading to the ^{99m}TcO[PN_2S-PEG] complex identified by comparison with the analogous ReO[PN_2S-PEG] (same HPLC retention time).

Taking into consideration the reductive properties of phosphine phosphorous and the catalytic properties of PEG the labeling reaction was also performed just mixing the PN_2S-PEG solution with TcO_4^- at different pH (4, 7, 9), in aqueous medium or mixed solvents (H_2O/EtOH) and at RT or 37°C. All the different conditions led to the same labeled compound. The absence of Tin(II) suggested that the phosphine phosphorous of the ligand acts as reducing agent of TcO_4^-. Then reduced TcO(V) can be coordinated by the PN_2S set or alternatively by the new (P)ON_2S set. In order to confirm the coordination properties of the oxidized PN_2S-PEG ligand, the PON_2S-PEG derivative **4** was prepared (complete oxidation of phosphorous ensured by ^{31}P-NMR). The reaction of compound 4 with purified ^{99m}Tc-gluconate led effectively to a stable labeled species with a retention time in the same range of that obtained from reduction of TcO_4^- by the PN_2S-PEG ligand. This implies a possible reduction-coordination mechanism which could be affected by micelles formation due to amphiphilic characters of 3 (under evaluation). Moreover the reaction performed by mixing equivalent amount of PN_2S-OMe ligand and free methoxy-PEG with TcO_4^- did not showed an increase on the reductive ability of the ligand by itself, indicating that the PEG has a catalytic effect just when it is covalently bound to the PN_2S moiety.

The labeled species obtained from the reduction of TcO_4^- by the PN_2S-PEG ligand was evaluated in vivo. These studies showed a rapid and efficient clearance from the bloodstream mainly by kidneys (>50% ID) induced by PEG conjugation, and demonstrated the high stability of the compound recovered unmodified from the urines. The low activity accumulation in the stomach (<1.5% ID) indicated that $^{99m}TcO_4^-$ was not produced in relevant amount.

These results indicate that the conjugation of the PN_2S ligand with bi- or polifunctional-PEGs can be useful for the labeling of targeting biomolecules, for the diagnosis of tumor exploiting the EPR effect and for the design of labeled drug delivery systems.