Labeling of Biomolecules for Medicinal Applications—Bioorganometallic Chemistry at Its Best**

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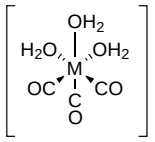
The modification of proteins with transition metal compounds has furnished valuable insights into the structure and function of biomolecules. In addition to fundamental aspects, these insights have an impact on medicinal research and applications. In the majority of investigations, transition metals in Werner-type coordination complexes were employed with "hard" donor atoms such as N, O, or S of carboxylates, alcoholates, amides, amines, and sulfides. Other properties, and consequently different reactivity, towards biomolecules is expected from transition metals in organometallic compounds. These are typically stabilized by π backbonding ligands such as CO, phosphanes, or aromatic π systems, for example η^6-arenes or η^5-cyclopentadiene (Cp). Contrary to common belief among chemists, these compounds may indeed exhibit remarkable stability in aerobic, aqueous media—given a judicious choice of metal and ligands. In addition, the special chemical or spectroscopic properties of organometallic complexes may be used advantageously for the site-selective reaction with biomolecules or the direct detection of the new bioorganometallic compound. In this rapidly growing area of interdisciplinary research, a number of groups have recently reported remarkable progress.

^{99m}Tc ($t_{1/2} = 6$ h) is a metastable isotope of the long-lived ^{99}Tc isotope ($t_{1/2} = 2.13 \times 10^5$ y). It is by far the most widely used isotope in radiopharmaceuticals.[1–5] This preeminent position is due to its very favorable physical properties: A half-life of 6 h is long enough to carry out chemical synthesis and get useful images after administration to the patient. At the same time, it is short enough to permit administration of relatively high amounts of ^{99m}Tc radioactivity without exposing the patient to dangerous radiation doses. The 140 keV photons give excellent images; the ^{99m}Tc isotope is readily available from commercial ^{99}Mo–^{99m}Tc generators. In these reactors, TcO_4^- is eluted from alumina-adsorbed [^{99}Mo]molybdate. Depending on factors such as generator age and prior elution times, the generator eluent contains between 20 and 85 % ^{99m}Tc, with the long-lived ^{99}Tc being the only by-product and the total Tc concentration in the μM range. It follows from the radiophysical bounding conditions that any ^{99m}Tc radiopharmaceutical must be prepared and used "on the spot". Preparation of the compound must be fast and ideally a "kit" formulation without the need for subsequent purification should be provided for maximum ease and safety of use by technical staff in hospitals. Recently, Roe and co-workers presented a new preparation of high specific activity Tc complexes by solid-phase methods which does not require subsequent purification by HPLC.[6] All the requirements are also met by new organometallic Tc complexes which are rapidly formed in high yield and purity when the generator eluent is directly added to a vial containing all reagents under a CO atmosphere.

Schubiger, Alberto, and co-workers reported the synthesis and applications of the astonishing complex *fac*-$[Tc(CO)_3(H_2O)_3]^+$ (**1**) .[7] This simple complex combines a



1: M = Tc
2: M = Re

"lower" organometallic half with three carbonyl ligands with an "upper" Werner-type half with three water molecules as ligands. Like its Re analogue $[Re(CO)_3(H_2O)_3]^+$ (**2**), **1** is stable for days in aqueous solution without decomposition. The interesting radioisotope ^{99m}Tc (see above) is readily available in hospitals from a ^{99}Mo reactor. From this device, a saline solution (0.9 % NaCl in H_2O) of $[^{99m}TcO_4]^-$ is obtained with typical Tc concentrations around 1 μM. In their patented method, Schubiger's group react this saline solution directly with excess $NaBH_4$ under 1 atm of CO and obtain **1** quantitatively in less than 20 min. The success of this reaction is all the more remarkable, because in the absence of CO only small amounts of TcO_2 are produced by $NaBH_4$, and CO itself is only poorly soluble in water (approx. 1 mM at 25 °C). Taking the low concentration of TcO_4^- into account, a trimolecular reaction is highly unlikely for kinetic reasons alone. Other reductants such as $SnCl_2$ and $[S_2O_4]^{2-}$ do not give **1** or **2**, and it appears very likely that an intermediate is formed from BH_4^- and CO which effects the reduction reaction and stabilizes possible metal carbonyl intermediates en route to **1** or **2**, for example the well-known $H_3B \cdot CO$ adduct.

Water and CO ligand-exchange reactions on **1** were recently reported.[8] The moderately fast exchange of ^{12}CO for ^{13}CO could be elegantly monitored by ^{99}Tc NMR spectroscopy through the subsequent appearance of a doublet, a triplet, and finally a quartet due to coupling to up to three ^{13}C nuclei, and an isotope shift $\Delta\delta = 1.05$ per carbon mass unit was determined. When **1** is left to stand under CO pressure for two weeks, all water molecules are slowly exchanged for CO, and the homoleptic $[Tc(CO)_6]^+$ carbonyl complex is formed in equilibrium with partially carbonylated

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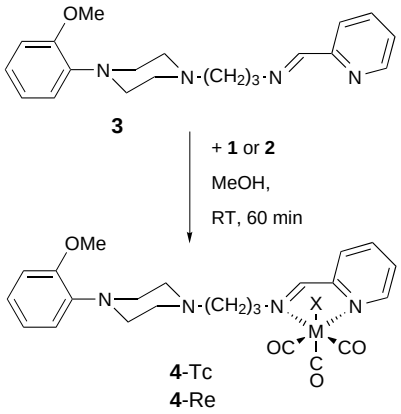
species. $[Tc(CO)_6]^+$ was prepared by Hieber and co-workers in 1965 but was characterized only by elemental analysis.[9] As a result of the high symmetry of the cation, Merbach et al. obtained beautifully resolved NMR spectra and measured values of $\delta(^{99}Tc) = -1961$, $\delta(^{13}C) = 190.2$ and $^1J(Tc,C) = 261$ Hz for $[Tc(CO)_6]^+$. In the logical next step, it was shown how **1** can actually be used for the labeling of biomolecules.[10, 11]

The challenge for a new generation of radiopharmaceuticals is the selective targeting of specific receptors. As expected, the three water ligands of **1** are readily replaced by other donors, for example amines. In particular, aromatic amines such as histidine give very stable complexes. A pharmacologically interesting complex **4**-Tc was prepared with the Schiff base derivative of arylpiperazine **3** (Scheme 1).[10] Arylpiperazines are the most thoroughly



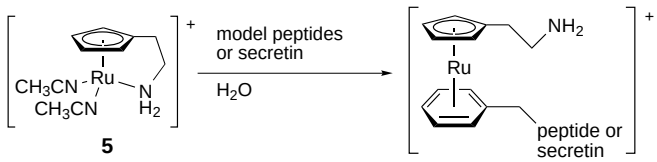
Scheme 1. Labeling of arylpiperazine **3** as a serotonergic receptor ligand with **1** or **2**.

studied class of molecules for the 5-HT_{1A} subclass of serotonergic receptors in the central nervous system (CNS). Compared to the success in radiolabeling of other peptides and proteins, there are very few examples of ^{99m}Tc labeling of CNS receptor ligands. For **4**-Tc, a labeling yield of 90–95% was achieved without subsequent purification, giving specific activities in the range of 30 GBq μmol^{-1}. This activity is about one order of magnitude better than can be achieved with more traditional Tc complexes with an 'N_2S_2' ligand set. Receptor affinity and selectivity of **4** were investigated by using the nonradioactive Re analogue **4**-Re. Conjugate **4**-Re shows high binding affinity to the 5-HT_{1A} receptor (5 ± 2 nM). Also, the selectivity of **4**-Re for its target receptor is high as shown by binding constants of >1 μM for related receptors 5-HT_{2A}, dopamine-D2, 5-HT-transporter, and D-transporter. Finally, the stability of the bioconjugate under physiological conditions is excellent. Neither UV/Vis spectroscopy nor TLC analysis of the "cold" **4**-Re showed any decomposition of the complex after 24 h incubation in serum at 37 °C.

Another very attractive target for radiolabeling is presented by single-chain antibody fragments (scFvs), which have the potential for tumor imaging. They are easily produced in pure form from combinatorial libraries and cell expression, for example in *E. coli*. Compared to whole antibodies, scFvs are less prone to inactivation and degradation in the body. In addition, they penetrate cell membranes rapidly and yield high tumor-to-background ratios. Unfortunately, there is no convenient method for ^{99m}Tc labeling of scFvs. Following expression in *E. coli*, scFvs are routinely purified by immobilized metal ion affinity chromatography (IMAC). To this end, a so-called His-tag, consisting of a number of histidine residues, is added to the N-terminus of the peptide. This His-tag provides an excellent chelating ligand for **1**, and indeed complexes of high stability were rapidly formed with a number of His-tagged scFvs.[11] In an optimized protocol, a solution of **1** was mixed with the scFv solution and activities up to 3.3 GBq mg^{-1} (corresponding to 90% labeling yield) were obtained. In a series of test experiments, the purity and identity of the $Tc(CO)_3$-labeled scFvs were established and the His-tag was confirmed to be the site of labeling by comparison with a His-tag-free scFv and monitoring the loss of radioactivity after incubation with a 100-fold excess of free histidine. In vivo stability studies were carried out with $^{99m}Tc(CO)_3$-labeled anti-mucin scFv M12 in mice. Gel filtration profiles of whole mouse serum analyzed 1 h after injection showed 75% of the activity migrating with intact scFv and most of the remainder in the albumin fraction. In accordance with its small size, the labeled scFv is secreted through the kidney and all of the activity in urine is bound to the scFv fragment. Finally, biodistribution and tumor localization of $Tc(CO)_3$-labeled scFv M12 and anti-HER2 scFv 4D5 were indistinguishable from the respective ^{125}I-labeled scFv.

The favorable properties of **1** are mainly attributed to its unusual ligand set. The σ-bound water ligands in **1** are readily displaced by other ligands, but in combination with other push–pull ligands, the carbonyl groups impart a high kinetic stability on $Tc(CO)_3^+$ derivatives like **4**. In addition, a high thermodynamic stability results from the d^6 low-spin electron configuration of the Tc^I complex. Although it was used in Tc-isocyanide compounds introduced by Davison and co-workers for heart imaging 15 years ago ("Tc-Sestamibi"),[12] the oxidation state +1 is rather unusual in biologically active Tc compounds and difficult to stabilize with Werner-type ligands (typically with an N, O, or S donor set) alone. In this respect, organometallic complexes with CO ligands expand the available range of compounds significantly.

Complexes with η-bound π ligands, for example, $[(\eta\text{-}C_6H_6)Cr(CO)_3]$ or $[(\eta\text{-}C_6H_6)RuCp]^+$, represent another typical class of organometallic compounds. Good stability of both types of compounds in aqueous solutions has been demonstrated and $[CpRu]^+$–benzene derivatives are valuable starting materials for the synthesis of aryl ethers.[13] Furthermore, the cationic $[CpRu]^+$ fragment readily coordinates to amino acid derivatives like amides and esters, and this behavior has been developed and patented by Beck and co-workers into a transition metal catalyzed solution-phase peptide synthesis scheme.[14–16] Grotjahn et al. have now found an interesting temperature effect on the coordination mode of the donor-substituted $[CpRu]^+$ derivative **5** in a series of model compounds (Scheme 2).[17, 18] Donor-substituted cyclopentadienyl complexes of early transition metals have recently received considerable attention as polymerization catalysts ("constrained geometry" catalysts). Upon addition of **5** to model peptides like Boc-Met-Phe-OMe or Boc-Phe-His-OMe



5

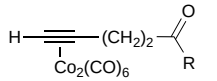
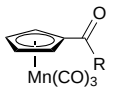
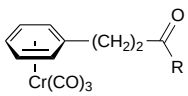
Scheme 2. Schematic representation of the reaction of **5** with aromatic rings in amino acids and peptides.

(Boc = *tert*-butoxycarbonyl, Met = methionine, Phe = phenylalanine) at room temperature, several species originating from σ bonding to the thioether or nitrogen donor groups are observed. After the sample had been heated to 60 °C for 6 h, the ^{1}H NMR spectra simplify considerably and only one species is observed with typical signals for π coordination of the $[CpRu]^+$ moiety to the phenyl ring (Scheme 2). This behavior was exploited by the authors for the selective labeling of secretin, a 27-amino-acid peptide with a molecular weight of about 3 kDa. Secretin is a gastrointestinal peptide hormone and has a unique phenylalanine residue at position 6 along with an N-terminal histidine, four arginine, one aspartate, and two glutamine residues, all of them providing good coordinating groups for transition metal fragments. When a solution of **5** is added to an aqueous solution of secretin in an NMR tube at room temperature, a relatively clean NMR spectrum is obtained after 8 h. Most significantly, the phenylalanine signals in the aromatic region are quantitatively shifted to higher field (ca. 6.2 ppm) which is diagnostic for coordination of arenes to the $[CpRu]^+$ moiety. Coordination of $[CpRu]^+$ to secretin is substantiated by electrospray ionization mass spectrometry. Finally, Edman degradation provides additional proof that the site of labeling is indeed the phenylalanine residue 6, since in the HPL chromatogram of partially degraded Ru-secretin the peak for phenylalanine is clearly missing. The site-selectivity of this reaction in the presence of over 25 concurring amide bonds, carboxylate groups, and even a histidine (remember the excellent binding of **1** to His described above!) is astonishing. Grotjahn and co-workers observed a "water-effect" in that the migration of the $[CpRu]^+$ fragment from N, S, O σ coordination to the π complex is considerably faster for secretin in water than for model peptides in water–methanol mixtures. Quite possibly, the relatively unpolar $[(benzene)RuCp]^+$ fragment is thermodynamically stabilized in water compared to $[CpRuL_3]^+$ complexes, where L are hard donor ligands from the peptide (N, S, or O). This stabilization may manifest itself already in the transition states and serve to explain the observed kinetic effect. Alternatively, intermediates with coordinated water molecules (analogous to **1**) seem very likely and this issue will surely merit further investigation. As for **1**, the organometallic moiety $[CpRu]^+$ provides unrivaled selectivity and stability which is not readily achieved with traditional coordination complexes.

Significant progress is also reported in the application of bioorganometallic compounds for biological assays. It has been recognized for some time that IR spectroscopy of organometallic carbonyl groups provides a useful handle for the detection of organometallic bioconjugates even in a complex environment like cell extracts of the serum of patients. This is attributed to the fact that the stretching vibrations of the terminal carbonyl group in organometallic complexes are observed at around 2000 cm^{-1}, a region where most organic molecules do not absorb. Moreover, these bands usually have high extinction coefficients and thus high sensitivity is obtained. The application of this technique to steroid hormones, peptides, and biogenic amines has been pioneered by Jaouen and his group and the technique has been dubbed *c*arbonyl *m*etallo*i*mmuno*a*ssay (CMIA).[19, 20] Application of this immunoassay obviates the need for radioactive markers, which constitutes the main advantage of this technique. For routine use in hospitals, the technique is in competition with other nonradioactive assays based on enzymatic methods or fluorescence, which work very reliably and in most cases easily achieve the required sensitivity and selectivity. However, none of these techniques is readily set up for simultaneous assaying of more than one analyte. On the other hand, there is considerable pressure on existing health care systems to save cost without compromising patient care. Analyzing for several species in one and the same assay seems like a promising approach to this end. Jaouen and co-workers at the Université Paris Sud have now published extensions of the CMIA technique for the simultaneous analysis of two[21] and even three compounds in a special issue of the *Journal of Organometallic Chemistry* on bioorganometallic chemistry.[22] Along with improvements in instrumental design,[23] a simultaneous quantitative analysis of several species, for example monitoring the drug levels in blood, may now be carried out on as little as 20 μL of serum.

The method is based on the diversity of metal carbonyl stretching frequencies in various carbonyl complexes.[22] Table 1 contains three examples of such complexes, **6**–**8**, together with their characteristic (analytical) stretching vibrations ν(CO). By chemical synthesis, metal complexes were covalently linked to the three anti-epileptic drugs carbamazepine (CBZ), phenobarbital (PB), and diphenylhydantoin (DPH) to yield conjugates **6**–**8**. Mono-CMIAs have been established for these drugs before. A "cocktail" of all three is often prescribed by physicians to patients suffering from epilepsy. As for many drugs, individual patient response

Table 1. Organometallic carbonyl complexes of antiepileptic drugs and their IR carbonyl stretching frequencies. The characteristic band is printed in boldface.

Metal carbonyl complex	$\tilde{\nu}$ [cm^{-1}]
	2095, **2058**, 2032
6: R = Carbamazepine	
	**2033**, 1958
7: R = Phenobarbital	
	**1973**, 1899
8: R = Diphenylhydantoin	

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varies considerably and thus, a continuous monitoring of actual serum concentrations is mandatory at least in acute care in hospitals. This way, the optimum dose for each individual patient is secured and undesirable side effects are minimized.

For each of these drugs, polyclonal antibodies were obtained by established procedures from rabbits. In preliminary mono-CMIAs, it was confirmed that none of the antibodies showed cross-reactivity with the other two drugs nor with the other two tracers. Figure 1 shows the ν(CO)

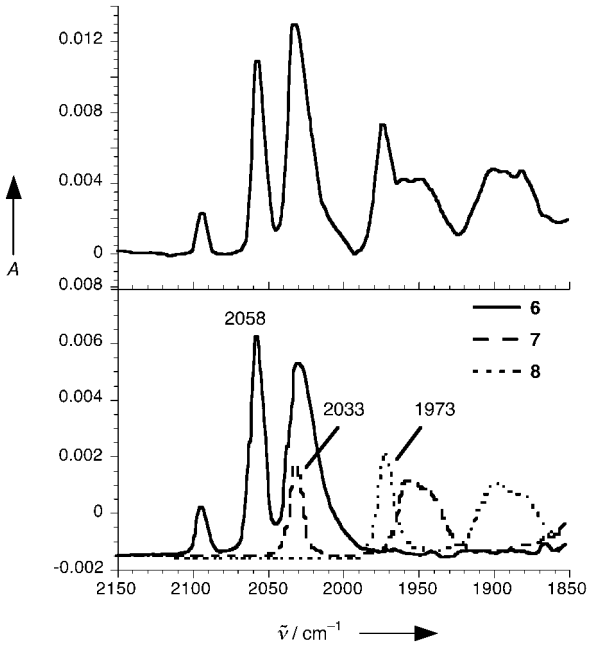

Figure 1. Carbonyl region of IR spectra of an equimolar mixture of **6**, **7**, and **8** (top) and of individual spectra of the three complexes (bottom). Characteristic bands for all three complexes are marked in the bottom spectra.

region of an IR spectrum of a mixture of **6**–**8**, along with a superimposition of IR spectra of the pure compounds at equal concentrations, which highlights the mathematical problem. The analytical band for complex **6** (at 2058 cm^{-1}) is free from interference with other bands and directly suited for quantitative analysis. In contrast, the analytical band for **7** (at 2033 cm^{-1}) is completely buried under the 2032 cm^{-1} band of **6**, and the analytical band of **8** (at 1973 cm^{-1}) partially overlaps the 1958 cm^{-1} band of **7**. Nevertheless, a quantitative deconvolution based on least-squares algorithms or a univariate method is possible and together with the known molar extinction coefficients of the three compounds yields minimum detectable quantities around 1 pmol. Validation of the multi-CMIA method was performed by comparison of standard calibration curves with those obtained from mono-CMIA for each component and yielded excellent agreement. Moreover, a very similar response of all three analytes in single and triple assays was established, further demonstrating the feasibility of the approach.

The three examples in this article exemplify an increasing interest in organometallic compounds for investigations on biological or medicinal problems. Although in different areas, they nevertheless have some features in common. The organometallic fragments react differently from coordination compounds that would be employed in more traditional bioinorganic studies. This way, a labeling of biomolecules is possible at unusual positions (as for **5**) or with unparalleled speed and selectivity (as for **1**). The metal compounds described above are very stable species due to their d^6 low-spin electronic structure with filled t_{2g} orbitals in a pseudo-octahedral environment. As such, they falsify a general wisdom among chemists which has it that organometallic compounds are sensitive towards air and moisture and can be handled safely only in glove boxes or Schlenk flasks. There are surely many more organometallic compounds that will form conjugates with unprecedented properties upon binding to biomolecules. Conceivable are, for example, conjugates of organometallic catalysts with peptides for the design of very specific, highly enantioselective catalysts. New tools for molecular biology may arise from the combination of functional organometallic fragments with oligonucleotides. Conjugates of organometallic compounds with small biologically active peptides or antibody fragments may open a new area in medical diagnosis or treatment of diseases. In all these areas, the quest has just begun.

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