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Re(I) and ^{99m}Tc(I) tricarbonyl complexes with ether-containing pyrazolyl-based chelators: Chemistry, biodistribution and metabolism

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We dedicate this paper to Professor Maria José Calhorda on the occasion of her 65th birthday.

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ABSTRACT

Tris(pyrazolyl)methane chelators, **L1**–**L3**, containing one or two ether groups at different positions of the azole rings, were synthesized and fully characterized. These chelators enabled the synthesis of *fac*-[^{99m}Tc(CO)$_3${HC[4-(ROCH$_2$)pz]$_3$}]$^+$ (R = Me (**Tc1**), Et (**Tc2**)) and *fac*-[^{99m}Tc(CO)$_3${HC[3,5-(EtOCH$_2$)$_2$pz]$_3$}]$^+$ (**Tc3**) which were identified by HPLC in comparison with the rhenium counterparts. The evaluation of **Tc1**–**Tc3** in CD-1 mice has shown that the number and/or nature of the ether groups greatly influence the biodistribution profile, pharmacokinetics and metabolic stability of these complexes. **Tc1** and **Tc2**, bearing a unique ether substituent at the 4-position of the pyrazolyl ring, undergo metabolic transformation *in vivo* while **Tc3** is not metabolized. The metabolization of **Tc1** and **Tc2** enhanced their rate of excretion but, most probably, also justify their negligible heart uptake in contrast with the high heart uptake of congener non-metabolizable complexes (^{99m}Tc-DMEOP and ^{99m}Tc-TMEOP), which have recently emerged as potential myocardial imaging agents.

The attempts made to identify the metabolites of **Tc1** and **Tc2** have shown that the metabolization of these compounds must involve the ether functions with probable formation of carboxylic acid derivatives. A comparative study with the congener *fac*-[^{99m}Tc(CO)$_3${[4-(MeOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Tc6**) led us to confirm the formation of such type of metabolites. In fact, **Tc6** is also metabolized in mice with formation of *fac*-[^{99m}Tc(CO)$_3${[4-(HOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Tc7**) and *fac*-[^{99m}Tc(CO)$_3${[4-(HOOC)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Tc8**), which were chemically identified by HPLC in comparison with the Re congeners (**Re7** and **Re8**).

1. Introduction

Tris(pyrazolyl)methanes are tridentate and tripodal ligands that have been used to stabilize coordination and organometallic complexes with a large variety of d- and f-transition metals, aiming mainly at exploring their reactivity in stoichiometric and/or catalytic transformation of organic substrates [1]. The research studies on tris(pyrazolyl)methane complexes for biomedical applications are more scarce and are restricted to a few examples, such as CO releasing molecules (CORM) based on Mn(I) tricarbonyl complexes [2]. Searching for new metal-based compounds with relevance for nuclear medicine applications, our group has investigated in the past few years tris(pyrazolyl)methane ^{99m}Tc(I) organometallic complexes as radioprobes for myocardial imaging by Single Photon Emission Computerized Tomography (SPECT) [3–6].

We have introduced the complexes *fac*-[^{99m}Tc(CO)$_3${HC[3,5-(MeOCH$_2$)$_2$pz]$_3$}]$^+$ (**^{99m}Tc-DMEOP**) and *fac*-[^{99m}Tc(CO)$_3${HC[3,4,5-(CH$_3$OCH$_2$)$_3$pz]$_3$}]$^+$ (**^{99m}Tc-TMEOP**) (Fig. 1), anchored by tris(pyrazolyl)methane chelators that contain two and three methoxymethyl substituents at each azolyl ring, respectively. The biological evaluation of **^{99m}Tc-DMEOP** and **^{99m}Tc-TMEOP** indicated that these complexes hold potential as myocardial imaging agents, particularly **^{99m}Tc-TMEOP** [4–6]. Biodistribution studies and SPECT images have demonstrated that **^{99m}Tc-TMEOP** exhibits a cardiac uptake comparable to ^{99m}Tc-Sestamibi and ^{99m}Tc-Tetrofosmin (Fig. 1), which are the ^{99m}Tc-radiopharmaceuticals in clinical use for perfusion cardiac imaging. Most importantly, **^{99m}Tc-TMEOP** showed a significantly faster liver clearance than these radiopharmaceuticals, pointing out that our ^{99m}Tc(I) tricarbonyl complex might improve the diagnostic accuracy of coronary artery disease (CAD). In fact, **^{99m}Tc-TMEOP** is among the best performing cationic and lipophilic ^{99m}Tc complexes that have been evaluated in recent years as potential myocardial imaging agents, which include mixed-ligand ^{99m}Tc(V) nitrido complexes and a ^{99m}Tc(I) tricarbonyl

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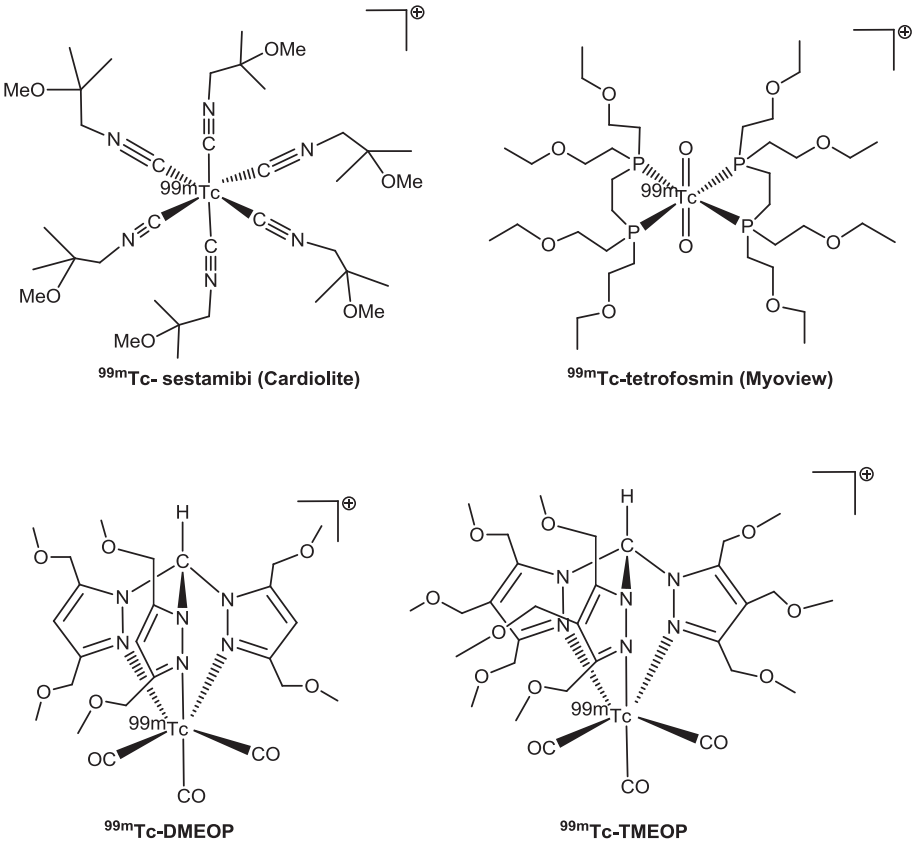


Fig. 1. Molecular structures of ^{99m}Tc-Sestamibi, ^{99m}Tc-Tetrofosmin, ^{99m}Tc-DMEOP and ^{99m}Tc-TMEOP.

complex stabilized by a tridentate PNP ligand bearing a pendant crown ether [7–16].

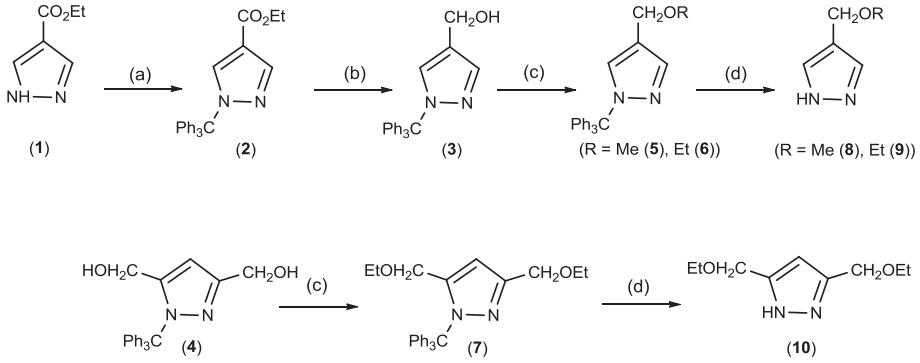
The encouraging pre-clinical results that we have obtained with **^{99m}Tc-TMEOP** prompted us to study related tris(pyrazolyl)methane Re(I) and ^{99m}Tc(I) tricarbonyl complexes having different ether functions at different positions of the azole rings, expecting to understand the influence of the ether substitution pattern on the physico-chemical (i.e. lipophilicity) and biological properties (i.e. heart uptake, heart/liver and heart/lung ratios, metabolic stability) of this family of complexes. Herein, we describe the new tris(pyrazolyl)methane chelators HC[4-(ROCH$_2$)pz]$_3$ (R = Me (**L1**), Et (**L2**)) and HC[3,5-(EtOCH$_2$)$_2$pz]$_3$ (**L3**) and report on the synthesis and characterization of the respective tricarbonyl complexes *fac*-[M(CO)$_3${HC[4-(ROCH$_2$)pz]$_3$}]$^+$ (M = Re, R = Me (**Re1**), Et (**Re2**); M = ^{99m}Tc, R = Me (**Re1**), Et (**Re2**)) and *fac*-[M(CO)$_3${HC[3,5-(EtOCH$_2$)$_2$pz]$_3$}]$^+$ (M = Re (**Re3**), ^{99m}Tc (**Tc3**)). The biological evaluation of **Tc1**–**Tc3**, comprising biodistribution and metabolism studies in mice, is also presented. Looking for a better understanding on the biological fate of ^{99m}Tc(I) tricarbonyl complexes with ether-containing pyrazolyl-based chelators, we have also studied the *in vivo* biological behaviour of *fac*-[^{99m}Tc(CO)$_3${[4-(MeOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Tc6**), containing a N$_3$-donor ligand with a single ether-containing azolyl ring.

2. Results and discussion

2.1. Synthesis of ether-containing tris(pyrazolyl)methane chelators

To obtain pyrazoles functionalized with ether groups at different positions of the azolyl ring we have started from alcohol derivatives, 1-trityl-4-hydroxymethylpyrazole (**3**) and 1-trityl-3,5-bis(hydroxymethyl)pyrazole (**4**), having the N–H function protected with a trityl group. Compound **3** was obtained using the same approach that we have previously described for the synthesis of **4**, by reaction of ethyl pyrazole-4-carboxylate (**1**) with trityl chloride [4]. The resulting tritylated pyrazole, compound **2**, was reduced almost quantitatively with $LiAlH_4$ to the corresponding alcohol **3** (Scheme 1). Compounds **3** and **4** were used to prepare the ether-containing N-tritylated pyrazoles **5**–**7** by a Williamson reaction [17]. This comprised the *in situ* preparation of the respective alcoholates followed by reaction with excess of the adequate alkyl halide. Removal of the trityl protecting group under acidic conditions gave the final ether-containing pyrazoles **8**–**10** (Scheme 1). Compounds **8**–**10** are colourless to pale yellow oils that have been characterized by ^{1}H and ^{13}C NMR spectroscopy.

As depicted in Scheme 2, the syntheses of the ether-containing tris(pyrazolyl)methane chelators, HC[4-(ROCH$_2$)pz]$_3$ (R = Me (**L1**), Et (**L2**)) and HC[3,5-(EtOCH$_2$)$_2$pz]$_3$ (**L3**) have been performed by $CHCl_3/H_2O$ phase transfer reaction with the pyrazoles **8**–**10** under alkaline conditions, which is the most common synthetic approach to obtain this type of chelators [18]. **L1**–**L3** are colourless or pale yellow compounds that have been isolated with yields ranging from 16 to 73%, after adequate purification. **L2** was purified by extraction with ether followed by washing with distilled water. The purification of **L1** and **L3** was achieved, respectively, by HPLC or silica gel column chromatography. **L1**–**L3** were characterized by ^{1}H and ^{13}C NMR spectroscopy and by high resolution FT/ICR mass spectrometry. The analytical data collected for **L1**–**L3** were consistent with their formulation as chelators of the tris(pyrazolyl) methane type. In particular, the FT/ICR mass spectra obtained in the



Scheme 1. Synthesis of ether-containing pyrazoles. (a) (i) NaH, DMF, 30 min, rt, (ii) triphenylmethylchloride, overnight (o.n.), rt; (b) $LiAlH_4$, THF, o.n., rt; (c) (i) NaH, THF, 4 h, rt, (ii) CH_3I (**5**) or CH_3CH_2I (**6**, **7**), 2 h, rt. (d) CF_3COOH, CH_2Cl_2/MeOH (1:1), 75–80 °C, 24 h (**8**) or HCl, EtOH/acetone, 80 °C, 2 h (**9** and **10**).

positive mode showed the presence of prominent peaks (M^+ or MH^+) which were assigned to the expected molecular-ions.

2.2. Synthesis and characterization of the ether-containing organometallic Re(I) and ^{99m}Tc(I) complexes

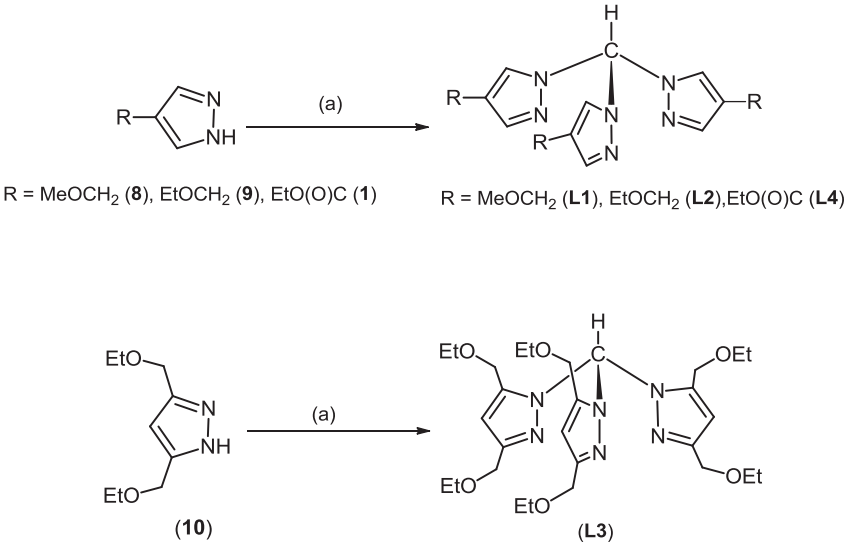
The synthesis of the compounds *fac*-[Re(CO)$_3${HC[4-(ROCH$_2$)pz]$_3$}]Br (R = Me (**Re1**), Et (**Re2**)) and *fac*-[Re(CO)$_3${HC[3,5-(EtOCH$_2$)$_2$pz]$_3$}]Br (**Re3**) was achieved in moderate to high yields (46–92%), by treatment of a methanolic solution of *fac*-[Re(CO)$_3$(H$_2$O)$_3$]Br with a stoichiometric amount of the respective tris(pyrazolyl)methane ligand (Scheme 3).

Complexes **Re1**–**Re3** are air- and water-stable microcrystalline solids which were fully characterized by the common analytical techniques, and were used to identify the chemical nature of the ^{99m}Tc congeners by HPLC, as described below. Due to the similarities between the physico-chemical properties of Re and Tc compounds, this is a common and well accepted practice in radiopharmaceutical chemistry that avoids the manipulation of ^{99}Tc, the long lived and soft β^- emitter used to isolate technetium compounds at the macroscopic level, i.e. at the millimolar range.

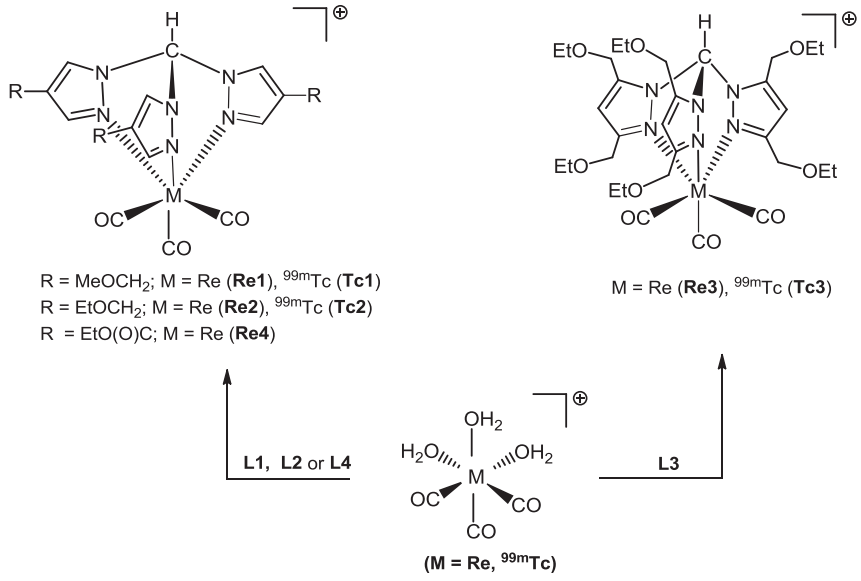
The IR spectra of **Re1**–**Re3** displayed two intense bands in the 1923–2038 cm^{-1} range with the typical pattern of tricarbonyl complexes with C_{3v} symmetry [19]. The frequencies found for each of these two bands, associated respectively with the asymmetrical E mode and symmetrical A_1 mode, are very similar for all the complexes. The resonances present on the ^{1}H and ^{13}C NMR spectra of **Re1**–**Re3** display a splitting pattern consistent with the magnetic equivalence of the three pyrazolyl rings. Some of these resonances are downfield shifted compared with those in the respective free tris(pyrazolyl)methane ligands, which corroborates the coordination of the azolyl rings to the *fac*-[Re(CO)$_3$]$^+$ unit. Mass spectrometry analysis of **Re1**–**Re3** led to the expected molecular-ions (M^+) in the respective positive ESI mass spectra. In summary, the spectroscopic data obtained for **Re1**–**Re3** support their formulation as cationic tricarbonyl complexes anchored by tridentate and facially coordinated tris(pyrazolyl)methanes, a coordination mode that has been confirmed in several instances by X-ray diffraction analysis for other complexes of the type *fac*-[Re(CO)$_3$(RCpz$_3$*)]$^+$ [3,20–23].

At the no-carrier added (n.c.a) level, the syntheses of *fac*-[^{99m}Tc(CO)$_3${HC[4-(ROCH$_2$)pz]$_3$}]$^+$ (R = Me (**Tc1**), Et (**Tc2**)) and *fac*-[^{99m}Tc(CO)$_3${HC[3,5-(EtOCH$_2$)$_2$pz]$_3$}]$^+$ (**Tc3**) were done in aqueous medium by reaction of the organometallic precursor *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ with **L1**–**L3** (Scheme 3), using the experimental conditions summarized in Table 1.

The ^{99m}Tc complexes, **Tc1**–**Tc3**, were obtained with high radiochemical yield (>95%), using ligand (**L1**–**L3**) concentrations in the 10^{-3}–1.5×10^{-3} M range, and running the reactions at 100 °C



Scheme 2. Synthesis of tris(pyrazolyl)methane chelators. (a) [NBu$_4$]Br, Na_2CO_3, H_2O/$CHCl_3$, reflux, 3 days.



Scheme 3. Synthesis of ^{99m}Tc and Re complexes with tris(pyrazolyl)methane chelators.

for 30–60 min. The chemical identity of **Tc1**–**Tc3** was established by comparing their HPLC radiochromatograms with the UV/vis trace of the Re congeners, **Re1**–**Re3** (Fig. 2).

The *in vitro* evaluation of **Tc1**–**Tc3** comprised the study of their *in vitro* stability in PBS (pH 7.4, 37 °C) and the determination of the respective lipophilicity by measurement of the log $P_{o/w}$ values (*n*-octanol/0.1 M PBS, pH 7.4). HPLC analysis of **Tc1**–**Tc3** has shown that the compounds did not undergo any transformation, including reoxidation to pertechnetate, even after 24 h of incubation under physiologic conditions. The log *P* values measured for **Tc1**–**Tc3** spanned between 0.45 and 2.64. The log *P* value of 0.45 found for **Tc1** is rather similar to the values that we have previously found for *fac*-[^{99m}Tc(CO)$_3${HC[3,5-(MeOCH$_2$)$_2$pz]$_3$}]$^+$ (**^{99m}Tc-DMEOP**) (log *P* = 0.58) and *fac*-[^{99m}Tc(CO)$_3${HC[3,4,5-(CH$_3$OCH$_2$)$_3$pz]$_3$}]$^+$ (**^{99m}Tc-TMEOP**) (log *P* = 0.64) [5]. This result suggests that the number of methoxymethyl substituents at the azolyl rings has little influence on the lipophilicity of tris(pyrazolyl)methane ^{99m}Tc(I) tricarbonyl complexes. By contrast, the replacement of methoxy by an ethoxy group leads to an important increase of lipophilicity, independently of the number of substituents per ring, as can be seen by comparing the log *P* values of **Tc1** and **Tc2** (0.45 *vs* 1.80) and ^{99m}Tc-DMEOP and **Tc3** (0.58 *vs* 2.64).

The lipophilicity is a physico-chemical parameter having a quite determinant influence on the biological performance of ^{99m}Tc compounds for myocardial imaging. It is generally considered that cationic ^{99m}Tc radiotracers must have a log *P* value of 0.5–1.2 in order to obtain high heart uptake and fast liver clearance, although there is no clear-cut "optimal" log *P* value [7]. The introduction of a slightly longer ether, like ethoxymethyl groups, into the framework of tris(pyrazolyl)methane chelators increases the lipophilicity of the ^{99m}Tc(I) complexes to log *P* values higher than 1.5, which are expected to induce a slow liver clearance.

Table 1
Experimental conditions for the synthesis of complexes **Tc1**–**Tc3** and **Tc6** and their radio-HPLC retention times and log *P* values.

Complex	Yield (%)	[L]/M	Time (min)	*T*/°C	pH	t_R (min)[a]	Log $P_{o/w}$
Tc1	≥98	1.5×10^{-3}	30	100	4.0	17.9 (17.5)[b]	0.45 ± 0.022
Tc2	≥98	1.5×10^{-3}	30	100	4.0	18.5 (18.2)[b]	1.80 ± 0.019
Tc3	≥96	10^{-3}	60	100	4.0	21.9 (21.4)[b]	2.64 ± 0.002
Tc6	≥97	10^{-4}	30	100	7.4	15.9 (15.5)[c]	n. d.

[a] The values in parentheses are for the corresponding Re complexes.
[b] Using a gradient of acetonitrile and aqueous 0.1% CF_3COOH as the solvent.
[c] Using a gradient of acetonitrile and 0.01 M sodium acetate buffer (pH = 5).
(n. d. = not determined).

2.3. Biodistribution and metabolism studies in mice

The evaluation of the biodistribution and pharmacokinetics of **Tc1**–**Tc3** has been performed in female CD-1 mice. The organ distribution (% ID/g) of **Tc1**–**Tc3** in mice as a function of time is summarized in Table 2.

All the evaluated complexes presented a relatively fast blood clearance in mice displaying blood activities between 0.36 ± 0.03% ID/g for **Tc2** and 1.2 ± 0.2% ID/g for **Tc3** at 1 h p.i. The dominant excretory pathway is hepatobiliary as expected for lipophilic complexes but the overall rate of excretion depends very much on the degree of substitution and/or on the nature of the ether substituents. Compared with **Tc3** that presents an overall excretion of 4.9 ± 0.8% ID at 2 h p.i., the compounds containing

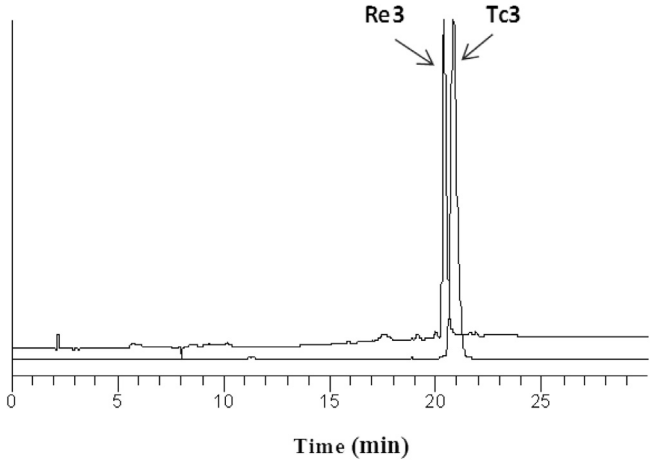


Fig. 2. HPLC chromatograms of co-injected rhenium complex (**Re3**) (UV detection) and ^{99m}Tc complex (**Tc3**) (γ detection).

Table 2
Biodistribution results of complexes **Tc1**–**Tc3** in CD-1 mice at 1 and 2 h after i.v. administration, expressed in % ID/g tissue ± SD (n = 3–5).

Organ	**Tc1**		**Tc2**		**Tc3**	
	1 h	2 h	1 h	2 h	1 h	2 h
Blood	0.4 ± 0.2	0.08 ± 0.02	0.36 ± 0.03	0.6 ± 0.4	1.2 ± 0.2	0.75 ± 0.01
Liver	4.9 ± 1.5	1.4 ± 0.9	6.0 ± 1.4	4.9 ± 2.0	16.3 ± 2.6	8.9 ± 1.7
Intestines	17.9 ± 3.5	18.9 ± 2.9	14.1 ± 0.8	17.6 ± 3.9	11.9 ± 3.3	18.9 ± 6.4
Spleen	0.4 ± 0.2	0.16 ± 0.06	1.1 ± 0.3	1.7 ± 0.4	3.8 ± 0.6	2.8 ± 0.5
Heart	0.3 ± 0.1	0.07 ± 0.01	1.0 ± 0.2	0.5 ± 0.1	3.7 ± 0.6	2.5 ± 0.7
Lung	0.27 ± 0.06	0.31 ± 0.05	0.69 ± 0.06	0.49 ± 0.07	3.2 ± 0.6	1.9 ± 0.6
Kidney	11.8 ± 2.7	4.4 ± 1.0	9.2 ± 2.9	6.2 ± 0.2	29.1 ± 5.8	27.1 ± 9.1
Muscle	0.21 ± 0.04	0.2 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
Bone	0.27 ± 0.06	0.12 ± 0.05	0.45 ± 0.03	0.30 ± 0.06	1.7 ± 0.1	1.3 ± 0.1
Stomach	2.4 ± 0.9	2.9 ± 1.6	0.8 ± 0.2	1.1 ± 0.5	7.3 ± 2.2	10.0 ± 2.9
Excretion (% ID)	29.1 ± 7.9	39.4 ± 5.5	25.8 ± 1.1	28.6 ± 2.8	3.7 ± 1.1	4.9 ± 0.8

ether groups uniquely at the 4-position of the pyrazolyl rings, **Tc1** and **Tc2**, display a fastest rate of excretion, respectively 39.4 ± 5.5 and 28.6 ± 2.8% ID at 2 h p.i.

Tc1 and **Tc2** have a negligible or very low heart uptake in mice (<1% ID/g). On the contrary, complex **Tc4** has higher heart uptake (3.7 ± 0.03% ID/g, 1 h p.i.) but display a quite unfavourable heart/liver ratio of 0.3 ± 0.6% at 2 h p.i., which certainly reflects its slow rate of excretion.

The metabolic stability of **Tc1**–**Tc3** was assessed by HPLC analysis of the urine and blood serum of mice injected with these complexes. Likewise the previously reported ^{99m}Tc-DMEOP and ^{99m}Tc-TMEOP with two or three ether substituents in the respective azolyl rings, **Tc3** does not undergo any metabolic transformation in mice [4,5]. Contrastingly, **Tc1** and **Tc2** are metabolized *in vivo*. A considerable fraction of the urine activity at 1 h p.i. still corresponds to intact **Tc1** (57%) and **Tc2** (35%) but the presence of three prominent metabolites was also detected (Fig. 3). The same metabolites were also detected in the serum, although in a smaller proportion than in urine. The three metabolites have lower retention times than the original compounds, being eluted with retention times between 13 and 18 min. Co-injection of the urine of mice injected independently with **Tc1** and **Tc2** indicated that the metabolite with the lowest retention time (t_R = 13.0 min) corresponds to the same compound, as revealed by the coincident retention time in both analysis. At 2 h p.i., this metabolite is already the major radiochemical species in the urine, corresponding to 46% and 51% of the excreted activity for **Tc1** and **Tc2,** respectively (Fig. 3).

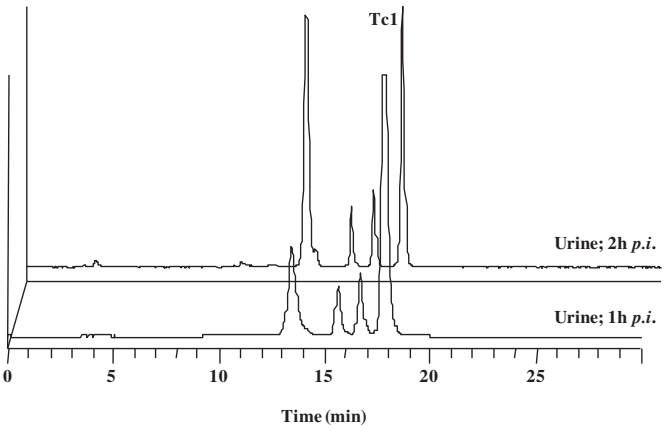
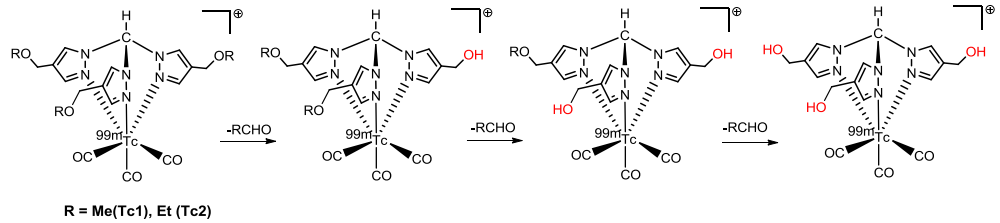


Fig. 3. HPLC analysis (radiometric detection) of urine from mice administered with **Tc1** at 1 h and 2 h p.i.

2.4. Chemical identification of metabolites

A common feature of all ^{99m}Tc complexes with adequate biological profile for cardiac imaging is the presence of several ether groups on their structure. The role of the ether groups is not fully understood, but it is generally accepted that they allow a fine-tuning of the lipophilicity of the complexes, without changing their overall charge and enhancing the liver washout kinetics. It has been also invoked that the metabolic transformation of the ether functions is another possibility to enhance the liver washout kinetics of myocardial imaging radiotracers. Unlike **Tc1** and **Tc2**, almost all of the ether-containing ^{99m}Tc complexes are excreted as non-metabolized species. The mechanisms involved in the metabolic fate of complexes **Tc1** and **Tc2** have not been investigated in detail, but we have considered that the formation of the three metabolites could result from successive O-dealkylation reactions of the ether groups, occurring most probably in the rodent liver microsomes and involving catalysis by an isoform of cytochrome P-450 [24]. O-Dealkylation reactions of methyl or ethyl ethers of the type R_1–O–R_2 (R1 = Me, Et) are usually accompanied by the release of these alkyl groups as formaldehyde or acetaldehyde and by the formation of R_2–OH alcohol derivatives. As proposed in Scheme 4, in the case of **Tc1** and **Tc2** this process should end-up with the formation of a tri-alcohol derivative of these organometallic complexes, corresponding to the common metabolite appearing at t_R = 13.0 min. Searching to confirm this possibility, we have synthesized the congener tri-alcohol Re complex (**Re4**), aiming at its use in the chemical identification of this metabolite by means of HPLC analysis.

As depicted in Scheme 5, complex **Re4** has been obtained by demethylation of the ether functions of **Re1** with BBr_3 [25]. **Re4** has been isolated as a white solid, after HPLC purification, and has been fully characterized by mass spectrometry, spectroscopic techniques (IR and ^{1}H/^{13}C NMR) and HPLC. The characterization of **Re4** confirmed the formation of a tri-alcohol derivative as a consequence of the full demethylation of the three ether functions. By HPLC analysis **Re4** has shown a t_R = 19.8 min, which is considerably longer than the one (t_R = 13.0 min) exhibited by the common metabolite, under the same analytical conditions. This result indicated that the metabolites detected for **Tc1** and **Tc2** do not correspond to alcohol derivatives, as hypothesized initially. If formed, such alcohol derivatives undergo themselves a fast metabolic transformation, eventually oxidation to aldehydes and subsequently to carboxylic acids [26]. To check for this possibility, we have studied the synthesis of *fac*-[Re(CO)$_3${HC[4-(HOOC)pz]$_3$}]$^+$. However, we could not synthesize this complex as we were unable to obtain the HC[4-(HOOC)pz]$_3$ chelator. Alternatively, we have prepared the Re complex of HC[4-(EtOOC)pz]$_3$ (**L4**), *fac*-[Re(CO)$_3${HC



Scheme 4. Possible *O*-dealkylation reactions of complexes **Tc1** and **Tc2**.

[4-(EtOOC)pz]$_3$}]$^+$ (**Re5**), expecting that the hydrolysis of its ester functions could afford "*fac*-[Re(CO)$_3${HC[4-(HOOC)pz]$_3$}]$^+$". Complex **Re5** was obtained as described above for the congeners **Re1**–**Re3**, and its characterization by ^{1}H/^{13}C NMR and ESI-MS confirmed the proposed formulation. The hydrolysis of **Re5** was also followed by decomposition processes, and we were unable to isolate the desired "*fac*-[Re(CO)$_3${HC[4-(HOOC)pz]$_3$}]$^+$".

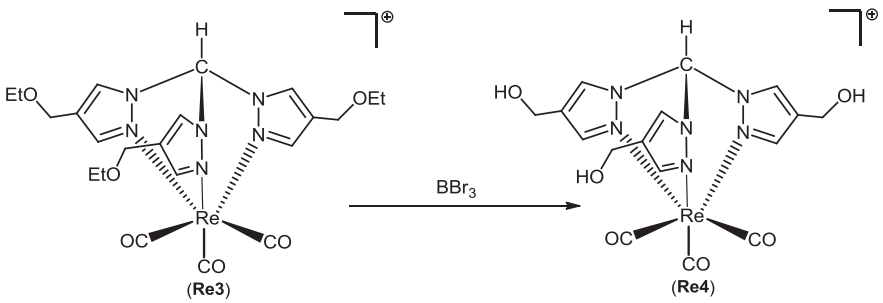
Finally, we decided to synthesize and evaluate a pyrazolyl-diamine ^{99m}Tc(I) tricarbonyl complex containing a single ether-containing pyrazolyl ring: *fac*-[^{99m}Tc(CO)$_3${[4-(MeOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Tc6**). By studying this compound, we have considered that it would also undergo metabolization in mice and we have anticipated a more straightforward identification of the involved transformations, i.e. conversion of the ether function into an alcohol or an acid. **Tc6** was synthesized in high yield by reaction of *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ with the respective pyrazole-diamine ligand (**L5**), which has been obtained using the synthetic approach that we have previously reported for other pyrazolyl-diamine derivatives (Scheme 6) [27]. The chemical identification of **Tc6** has been achieved by HPLC comparison with the congener *fac*-[Re(CO)$_3${[4-(MeOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Re6**) (Scheme 6), isolated at macroscopic level and fully characterized by the common analytical techniques. **Tc6** has been administered to female CD-1 mice, and the urine and serum of the injected mice was analyzed by HPLC. The HPLC analysis of urine revealed the formation of two metabolites with retention times of 9.8 and 11.8 min, respectively, as well as the presence of **Tc6** (Fig. 4). These findings confirmed that **Tc6** also underwent metabolic transformations, like **Tc1** and **Tc2** bearing the same ether-containing pyrazolyl ring. Moreover, **Tc6** has shown a biodistribution profile (Table S1) similar to the one of **Tc1** and **Tc2**, although exhibiting a faster clearance from main organs and an increased overall excretion.

We have synthesized the alcohol and acid derivatives *fac*-[Re(CO)$_3${[4-(HOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]Br (**Re7**) and *fac*-[Re(CO)$_3${[4-(HOOC)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]Br (**Re8**), aiming the identification of the **Tc6** metabolites by HPLC. Complex **Re8** has been reported previously by our research group [28]. The alcohol derivative, **Re7**, is a new compound that has been synthesized by reacting *fac*-[Re(CO)$_3$(H$_2$O)$_3$]Br with 4-(HOCH$_2$)pz(CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$ (**L6**). In its turn, **L6** was obtained by treatment of **L5** with BBr$_3$ (Scheme 6). Complex **Re7** has been characterized by ESI-MS, by the common spectroscopic techniques and X-ray diffraction analysis. The solid state structural analysis confirmed the tridentate coordination of **L6** and the presence of the alcohol function at the 4-position of the pyrazolyl ring, as can be seen in the molecular structure of the cation of **Re7** presented in Fig. 5. The rhenium atom is six-coordinate with a distorted octahedral coordination geometry The angles and bond distances found for **Re7** are comparable to the values that we have previously reported for *fac*-[Re(CO)$_3${(3,5-Me$_2$pz)(CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]Br [27].

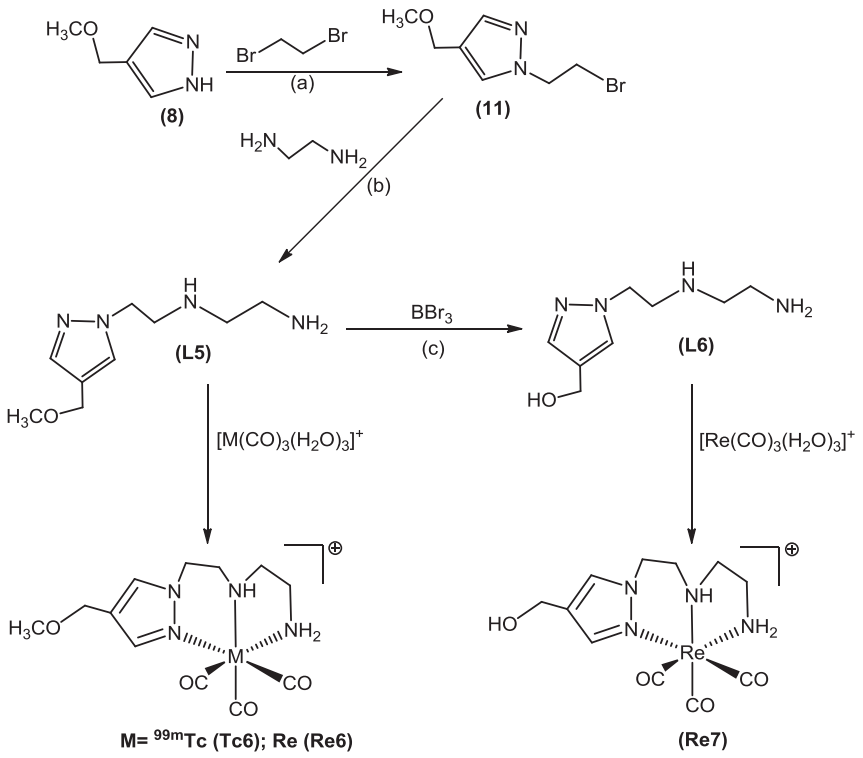
The co-injection of **Re7** and **Re8** with the urine from a mouse injected with **Tc6** showed that the **Tc6** metabolites, with retention times of 9.8 and 10.8 min, correspond to the acid and alcohol derivatives, respectively. These findings point out that in the case of the tris(pyrazolyl)methane Tc(I) complexes, **Tc1** and **Tc2**, their metabolization also takes place at the methoxymethyl substituent of the azolyl ring, and proceeds most probably with its oxidation to a carboxylic acid function.

3. Conclusions

The functionalization of tris(pyrazolyl)methane chelators with methoxymethyl or ethoxymethyl groups at different position of the azole rings has a marked influence on the biodistribution profile, pharmacokinetics and metabolic stability of the respective ^{99m}Tc(I) tricarbonyl complexes. The complexes containing ether groups uniquely at the 4-position of the azolyl ring, *fac*-[^{99m}Tc(CO)$_3${HC[4-(ROCH$_2$)pz]$_3$}]$^+$ (R = Me (**Tc1**), Et (**Tc2**)), undergo metabolic transformation to more polar compounds. Interestingly, the presence of additional ether groups at the 3- and 5-positions prevents the metabolization of the ethers at the 4-position, as the previously reported *fac*-[^{99m}Tc(CO)$_3${HC[3,4,5-(CH$_3$OCH$_2$)$_3$pz]$_3$}]$^+$ (**^{99m}Tc-TMEOP**) is not metabolized in mice. Despite being cationic and lipophilic, **Tc1** and **Tc2** did not accumulate in the mice heart, on contrary to **^{99m}Tc-TMEOP** that holds potential as a SPECT radioprobe for cardiac imaging. The ether metabolization observed for **Tc1**



Scheme 5. Synthesis of complex **Re4**.



Scheme 6. Synthesis of pyrazolyl-diamine chelators and respective ^{99m}Tc and Re complexes. (a) [NBu_4]Br, NaOH, reflux, 1.5 h; (b) EtOH, reflux, 5 h; (c) CH_2Cl_2, −20 °C, 3 h.

and **Tc2** enhanced their rate of excretion and certainly justifies their negligible heart uptake.

The metabolic transformation of **Tc1** and **Tc2** is most probably due to O-dealkylation processes. However, the final metabolites are not of the alcohol type, as confirmed by HPLC in comparison with the fully characterized *fac*-[Re(CO)$_3${HC[3,5-(HOCH$_2$)$_2$pz]$_3$}]Br (**Re4**). Most probably, such metabolites are carboxylic acid derivatives which, despite our efforts, could not be synthesized. Nevertheless, we have proved that *fac*-[^{99m}Tc(CO)$_3${[4-(MeOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Tc6**), containing a single ether-containing pyrazolyl ring, is metabolized to the corresponding alcohol and acid derivatives. These findings corroborate the possible oxidation of the ether functions of **Tc1** and **Tc2** to carboxylic acid groups.

In summary, we have proved for the first time that ether-containing ^{99m}Tc complexes can be metabolized *in vivo* with formation of alcohol and carboxylic acid derivatives. Such possibility was invoked previously by other authors but was not demonstrated before [7]. The metabolization observed for the ether-

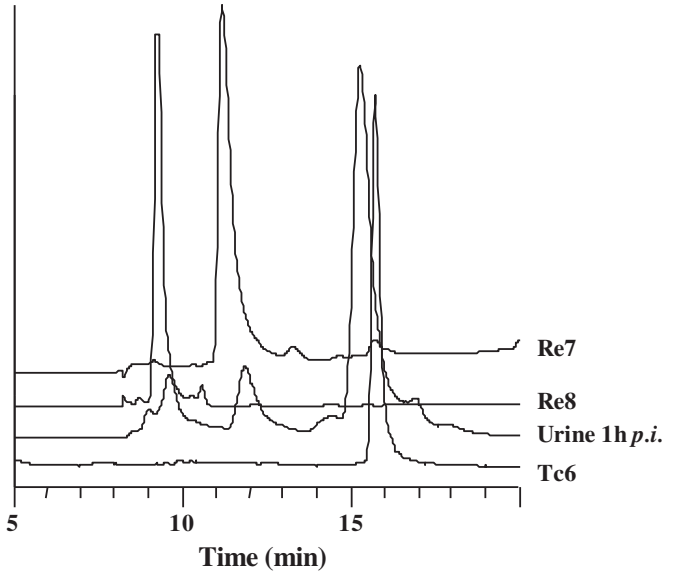


Fig. 4. HPLC analysis (radiometric detection) of urine from mice administered with **Tc6** at 1 h p.i. and comparison with the chromatograms of complexes **Re7** and **Re8**.

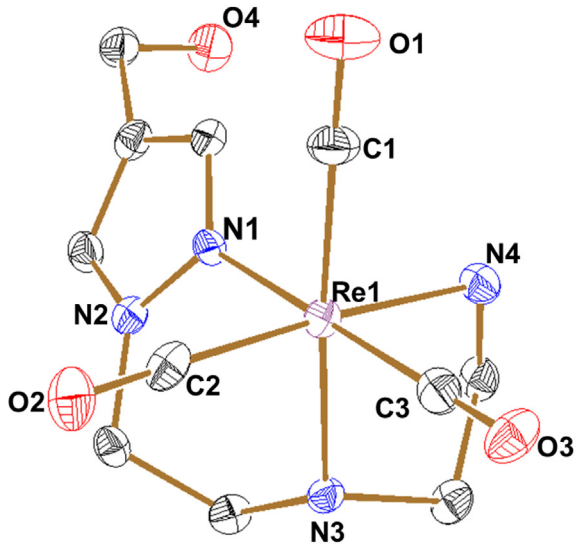


Fig. 5. ORTEP view of complex **Re7** with ellipsoids drawn at the 40% probability level. Selected bond distances (Å): Re(1)–C(1) 1.919(6), Re(1)–C(2) 1.923(6), Re(1)–C(3) 1.923(7), Re(1)–N(1) 2.169(5), Re(1)–N(3) 2.227(4), Re(1)–N(4) 2.217(5).

containing tris(pyrazolyl)methane (**Tc1** and **Tc2**) and pyrazolyl-diamine (**Tc6**) ^{99m}Tc(I) tricarbonyl complexes involves the ether function, did not affect their coordination sphere and enhanced their overall excretion. These features indicate that the introduction of ether functions at the 4-position of the azolyl ring of tris(pyrazolyl)methane and pyrazolyl-diamine ligands might allow the tuning of the pharmacokinetics of the corresponding ^{99m}Tc(I) tricarbonyl complexes, which is a key issue in radiopharmaceutical applications.

4. Experimental section

4.1. General procedures

All chemicals were of reagent grade and were used without purification. ^{1}H and ^{11}C NMR spectra were recorded on a Varian Unity 300 MHz spectrometer; ^{1}H and ^{11}C chemical shifts were referenced with the residual solvent resonances relative to tetramethylsilane. IR spectra were recorded as KBr pellets on a Bruker 27 Tensor spectrometer. C, H and N analyses were performed on an EA 110 CE Instruments automatic analyser. The compounds ethyl 1*H*-pyrazole-4-carboxylate (**1**) [29], 1-trityl-3,5-bis(hydroxymethyl) pyrazole (**4**) [4], $[Re(H_2O)_3(CO)_3]Br$ [30] and *fac*-$[Re(CO)_3\{[4\text{-}(HOOC)pz](CH_2)_2NH(CH_2)_2NH_2\}]Br$ (**Re8**) [28] were prepared according to published methods. The description of the synthesis of compounds **2**, **3**, **5** and **6**–**10** is presented as supplementary data. The radioactive precursor *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ was prepared using a IsoLink® kit (Covidien) following described procedures [31]. $Na[^{99m}TcO_4]$ was eluted from an Elumatic ^{99}Mo/^{99m}Tc generator (CisBio) with 0.9% saline. HPLC analysis of the Re and ^{99m}Tc complexes was performed on a Perkin–Elmer LC pump 200 coupled to a LC 290 tunable UV/Vis detector and to a Berthold LB-507A radiometric detector. Separations were achieved on a Nucleosil column (10 μm, 250 mm × 4 mm), using a flow rate of 1 mL/min; UV detection, 254 nm; eluents, A – aqueous 0.1% CF_3COOH solution, B – acetonitrile; method, t = 0–3 min, 0% B; 3–3.1 min, 0–25% B; 3.1–9 min, 25% B; 9–9.1 min, 25–34% B; 9.1–20 min, 34–100% B; 20–22 min, 100% B; 22–22.1 min, 100-0% B; 22.1–30 min, 0% B. The HPLC analysis of the **Tc6** metabolites and complexes **Re7** and **Re8** was performed using the following eluent: The HPLC analysis of the metabolites of **Tc6** and complexes **Re7** and **Re8** was performed under the same experimental conditions but using the following eluent: A – 0.01 M sodium acetate buffer (pH = 5), B – acetonitrile.

4.2. Synthesis of the ligands

4.2.1. $HC[4\text{-}(MeOCH_2)pz]_3$ (*L1*)

To a suspension of 4-methoxymethylpyrazole (**8**) (60 mg, 0.54 mmol) and $[NBu_4]Br$ (9 mg) in distilled water (2 mL) was slowly added Na_2CO_3 (500 mg). Then, 0.5 mL of $CHCl_3$ was added and the mixture was gently refluxed for 3 days. The organic phase was separated, washed with water and dried over $MgSO_4$. Evaporation of the solvent under vacuum gave a brown oily residue which was purified by gradient HPLC (100% aqueous 0.1% CF_3COOH solution → 100%·CH_3CN) using a Nucleosil column (10 μm, 250 mm × 4 mm) and a flow rate of 1 mL/min. Compound **L1** was recovered as a colourless oil by removal of the solvent from the collected fractions. Yield: 10 mg (0.029 mmol), 16%.

^{1}H NMR ($CDCl_3$, δ ppm): 8.24 (1H, s, C<u>H</u>), 7.62 (3H, s, H-3/5 (pz)), 7.54 (3H, s, H-3/5 (pz)), 4.31 (6H, s, 6.9 Hz, C<u>H</u>$_2$), 3.32 (9H, s, OC<u>H</u>$_3$). ^{13}C NMR ($CDCl_3$, δ ppm): 141.7 (C-3/5 (pz)), 128.7 (C-3/5 (pz)), 120.1 (C-4 (pz)), 83.3 (<u>C</u>H), 65.1 (<u>C</u>H$_2$), 57.9 (O<u>C</u>H$_3$). FTICR-MS *m*/*z*: 346.1747 (M^+, calcd for $C_{16}H_{22}N_6O_3$, 346.1748).

4.2.2. $HC[4\text{-}(EtOCH_2)pz]_3$ (*L2*)

Compound **L2** was synthesized as above described for **L1**, starting from 4-ethoxymethylpyrazole (**9**) (234 mg, 1.86 mmol) and using the same proportions of reagents and solvents. After 3 days of reaction, the solvent was removed under vacuum and the residue was redissolved in diethyl ether, and washed with saturated brine and water. The ethereal phase was treated with decolorizing charcoal, filtered and the solvent removed under vacuum to afford compound **L2** as a white microcrystalline solid. Yield: 176 mg (0.453 mmol), 73%.

^{1}H NMR ($CDCl_3$, δ ppm): 8.25 (1H, s, C<u>H</u>), 7.60 (3H, s, H-3/5 (pz)), 7.55 (3H, s, H-3/5 (pz)), 4.32 (6H, s, 6.9 Hz, C<u>H</u>$_2$), 3.45 (6H, q, OC<u>H</u>$_2$), 1.15 (9H, t, C<u>H</u>$_3$). ^{13}C NMR ($CDCl_3$, δ ppm): 141.6 (C-3/5 (pz)), 128.6 (C-3/5 (pz)), 120.2 (C-4 (pz)), 83.15 (<u>C</u>H), 65.6 (<u>C</u>H$_2$), 63.0 (<u>C</u>H$_2$), 15.0 (<u>C</u>H$_3$). FTICR/MS *m*/*z*: 388.2214 (M^+, calcd for $C_{19}H_{28}N_6O_3$, 388.2217).

4.2.3. $HC[3,5\text{-}(EtOCH_2)_2pz]_3$ (*L3*)

Compound **L3** was synthesized as above described for **L1**, starting from 3,5-bis(ethoxymethyl)pyrazole (**10**) (485 mg, 2.63 mmol). After 3 days of reflux, the organic phase was separated, washed with water and dried over $MgSO_4$. The brown residue oil was applied on a silica gel column which was eluted with a gradient from 100% $CHCl_3$ to 98% $CHCl_3$/2% MeOH. Removal of the solvent from the collected fractions gave **L3** as a pale yellow oil. Yield: 230 mg (0.41 mmol), 47%.

^{1}H NMR ($CDCl_3$, δ ppm): 8.68 (1H, s, C<u>H</u>), 6.32 (3H, s, H-4 (pz)), 4.41 (6H, s, C<u>H</u>$_2$), 4.32 (6H, s, C<u>H</u>$_2$), 3.46 (6H, q, 6.9 Hz, C<u>H</u>$_2$), 3.34 (6H, q, 6.9 Hz, C<u>H</u>$_2$), 1.16 (9H, t, 6.9 Hz, CH_3), 1.06 (9H, t, 6.9 Hz, CH_3). ^{13}C NMR ($CDCl_3$, δ ppm): 149.9 (C-3/5 (pz)), 141.7 (C-3/5 (pz)), 107.2 (C-4 (pz)), 79.0 (C–H), 66.3 (<u>C</u>H$_2$), 65.7 (<u>C</u>H$_2$), 65.5 (<u>C</u>H$_2$), 62.9 (<u>C</u>H$_2$), 15.1, (<u>C</u>H$_3$), 14.8 (<u>C</u>H$_3$). FTICR/MS *m*/*z*: 563.3552 ($[M + H]^+$, calcd for $C_{28}H_{46}N_6O_6$, 563.3553).

4.2.4. $HC[4\text{-}(EtO(O)C)pz]_3$ (*L4*)

Compound **L4** was synthesized as above described for **L1**, starting from ethyl 1*H*-pyrazole-4-carboxylate (**1**) (602 mg, 4.27 mmol). After 3 days of reflux, the organic phase was separated, washed with water and dried over $MgSO_4$. The brown residue oil was applied on a silica gel column which was eluted with a gradient from 100% $CHCl_3$ to 95% $CHCl_3$/5% MeOH. Removal of the solvent from the collected fractions gave **L4** as a pale yellow oil. Yield: 94 mg (0.22 mmol), 15%.

^{1}H NMR ($CDCl_3$, δ ppm): 8.35 (1H, s, C<u>H</u>), 8.13 (3H, s, H-3/5 (pz)), 8.04 (3H, s, H-3/5 (pz)), 4.29 (6H, q, OC<u>H</u>$_2$), 1.31 (9H, t, C<u>H</u>$_3$). ESI/MS *m*/*z*: 429.3 ($[M - H]^-$, calcd for $C_{19}H_{21}N_6O_6$, 429.41).

4.2.5. $\{[4\text{-}(MeOCH_2)pz](CH_2)_2NH(CH_2)_2NH_2\}$ (*L5*)

A solution of **10** (0.25 g, 1.1 mmol) and 1,2-ethylenediamine (1.5 mL, 22 mmol) in ethanol (2 mL) was refluxed for 5 h. The solvent was removed under reduced pressure and the residue extracted with $CHCl_3$. After filtration and removal of the solvent under vacuum **L5** was obtained as a yellow oil. Yield: 190 mg (0.96 mmol), 87%.

^{1}H NMR ($CDCl_3$, δ ppm): 7.48 (1H, s, H-3/5 (pz)), 7.43 (1H, s, H-3/5 (pz)), 4.31 (2H, s, C<u>H</u>$_2$), 4.20 (2H, t, C<u>H</u>$_2$), 3.33 (3H, s, C<u>H</u>$_3$), 3.05 (2H, t, C<u>H</u>$_2$), 2.88 (2H, m, C<u>H</u>$_2$), 2.68 (2H, m, C<u>H</u>$_2$). ^{13}C NMR ($CDCl_3$, δ ppm): 139.6 (C-3/5 (pz)), 129.3 (C-3/5 (pz)), 118.1 (C-4 (pz)), 65.3 (O<u>C</u>H$_2$), 57.7 (<u>C</u>H$_2$), 52.2 (<u>C</u>H$_2$), 51.9 (<u>C</u>H$_2$), 49.2 (<u>C</u>H$_2$), 41.5 (<u>C</u>H$_3$).

4.2.6. $\{[4\text{-}(HOCH_2)pz](CH_2)_2NH(CH_2)_2NH_2\}$ (*L6*)

To a solution of **L5** (289 mg, 1.46 mmol) in CH_2Cl_2 (5 mL) were added dropwise 4 mL of a 1 M solution of BBr_3 in CH_2Cl_2, while keeping the reaction vial at −20 °C. At this temperature, the reaction mixture was stirred for 3 h. After this time, 5 mL of distilled

water were added to the mixture. The aqueous phase was separated and freeze-dried to afford a yellowish residue, which was extracted with $CHCl_3$. Compound **L6** was recovered as a yellow oil, after filtration and removal of the solvent under vacuum. Yield: 109 mg (0.59 mmol), 40%.

^{1}H NMR ($CDCl_3$, δ ppm): 7.38 (1H, s, H-3/5 (pz)), 7.36 (1H, s, H-3/5 (pz)), 4.42 (2H, s, C$\underline{H}_2$), 4.09 (2H, tr, C$\underline{H}_2$), 2.93 (2H, tr, C$\underline{H}_2$), 2.61 (2H, tr, C$\underline{H}_2$), 2.52 (2H, tr, C$\underline{H}_2$). ^{13}C NMR ($CDCl_3$, δ ppm): 138.6 (C-3/5 (pz)), 128.7 (C-3/5 (pz)), 121.9 (C-4 (pz)), 83.3 (C$\underline{H}$), 55.1 (C$\underline{H}_2$), 51.9 (C$\underline{H}_2$), 51.5 (C$\underline{H}_2$), 48.9 (C$\underline{H}_2$), 41.1 (C$\underline{H}_2$). FTICR-MS *m/z*: 207.1196 ([M + Na]$^+$, calcd for $C_8H_{16}N_4ONa$, 207.1216).

4.3. Synthesis of the Re complexes

4.3.1. *fac-[Re(CO)$_3${HC[4-(CH$_3$OCH$_2$)pz]$_3$}]Br (**Re1**)*

A solution of [Re(CO)$_3$(H$_2$O)$_3$]Br (3.5 mg, 8.7 μmol) and **L1** (3 mg, 8.7 μmol) in CD_3OD was heated at 60 °C during 3 h. After this time, ^{1}H NMR analysis of the reaction mixture has shown an almost quantitative formation of **Re1**. The solvent was removed under vacuum and the residue was washed with diethyl ether and dried under vacuum to afford a beige solid which was formulated as compound **Re1**. Yield: 4.9 mg (7.0 μmol), 81%.

IR Data (KBr, ν/cm^{-1}): 2035s, 1943s (C≡O). ^{1}H NMR ($CDCl_3$, δ ppm): 12.62 (1H, s, C$\underline{H}$), 8.92 (3H, s, H-3/5 (pz)), 7.98 (3H, s, H-3/5 (pz)), 4.30 (6H, s, C$\underline{H}_2$), 3.36 (9H, s, C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, δ ppm): 146.6 (C-3/5 (pz)), 132.8 (C-3/5 (pz)), 122.1 (C-4 (pz)), 74.0, (C$\underline{H}$), 64.0 (C$\underline{H}_2$), 58.8 (O$\underline{C}$H$_3$). ESI-MS *m/z*: 616.9 (M$^+$, calcd for $C_{19}H_{22}N_6O_6Re$, 617.1).

4.3.2. *fac-[Re(CO)$_3${HC[4-(CH$_3$CH$_2$OCH$_2$)pz]$_3$}]Br (**Re2**)*

A solution of [Re(CO)$_3$(H$_2$O)$_3$]Br (25 mg, 62 μmol) and **L2** (25 mg, 64 μmol) in methanol was refluxed overnight. The solvent was removed under vacuum and the residue was washed with diethyl ether. Compound **Re2** was recovered as a beige solid after drying under vacuum. Yield: 42 mg (57 μmol), 92%.

IR Data (KBr, ν/cm^{-1}): 2036s, 1944s, 1923s (C≡O). ^{1}H NMR ($CDCl_3$, δ ppm): 12.54 (1H, s, C$\underline{H}$), 8.85 (3H, s, H-3/5 (pz)), 7.98 (3H, s, H-3/5 (pz)), 4.33 (6H, s, C$\underline{H}_2$), 3.52 (6H, q, 6.9 Hz, C$\underline{H}_2$), 1.20 (9H, t, 6.9 Hz, C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, δ ppm): 146.7 (C-3/5 (pz)), 133.0 (C-3/5 (pz)), 122.2 (C-4 (pz)), 73.8 (C$\underline{H}$), 66.6 (C$\underline{H}_2$), 62.0 (C$\underline{H}_2$), 15.0 (C$\underline{H}_3$). ESI-MS *m/z*: 658.9 (M$^+$, calcd for $C_{22}H_{28}N_6O_6Re$, 659.2).

4.3.3. *fac-[Re(CO)$_3${HC[3,5-(CH$_3$CH$_2$OCH$_2$)$_2$pz]$_3$}]Br (**Re3**)*

Compound **Re3** is a beige solid which was obtained according to the procedure described for **Re2**, starting from [Re(CO)$_3$(H$_2$O)$_3$]Br (34 mg, 84 μmol) and **L3** (47 mg, 84 μmol). Yield: 46% (35 mg, 38 μmol).

IR Data (KBr, ν/cm^{-1}): 2038s, 1949s (C≡O). ^{1}H NMR ($CDCl_3$, δ ppm): 9.29 (1H, s, C$\underline{H}$), 6.72 (3H, s, H-4 (pz)), 5.05 (6H, s, C$\underline{H}_2$), 4.67 (6H, s, C$\underline{H}_2$), 3.67 (6H, q, 6.9 Hz, C$\underline{H}_2$), 3.70 (6H, s, C$\underline{H}_2$), 1.25 (18H, t, 6.9 Hz, C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, δ ppm): 157.5 (C-3/5 (pz)), 144.9 (C-3/5 (pz)), 109.4 (C-4 (pz)), 71.9 (C$\underline{H}$), 67.5 (C$\underline{H}_2$), 67.4 (C$\underline{H}_2$), 66.3 (C$\underline{H}_2$), 62.6 (C$\underline{H}_2$), 62.6 (C$\underline{H}_3$). ESI-MS *m/z*: 833.1 (M$^+$, calcd for $C_{31}H_{46}N_6O_9Re$, 833.3).

4.3.4. *fac-[Re(CO)$_3${HC[4-(HOCH$_2$)pz]$_3$}]Br (**Re4**)*

To a solution of **Re1** (26 mg, 0.042 mmol) in CH_2Cl_2 (10 mL) at −20 °C was added 0.38 mL of 1.0 M boron tribromide in CH_2Cl_2. The mixture was allowed to react for 3 h at −20 °C and was subsequently quenched with water. The organic phase was separated, dried over $MgSO_4$ and the solvent removed under vacuum. The residue was purified by gradient HPLC (100% aqueous 0.1% CF_3COOH solution → 100% CH_3CN) using a Nucleosil column (10 μm, 250 mm × 7 mm) and a flow rate of 2 mL/min. Compound **Re4** was recovered as a white solid after removal of the solvent from the collected fractions. Yield: 33% (8 mg, 0.014 mmol).

IR Data (KBr, ν/cm^{-1}): 2042s, 1921s (C≡O). ^{1}H NMR ($CDCl_3$, δ ppm): 11.58 (1H, s, C$\underline{H}$), 8.72 (3H, s, H-3/5 (pz)), 8.08 (3H, s, H-3/5 (pz)), 4.32 (6H, s, C$\underline{H}_2$). ^{13}C NMR ($CDCl_3$, δ ppm): 149.4 (C-3/5 (pz)), 135.6 (C-3/5 (pz)), 124.2 (C-4 (pz)), 78.1 (C$\underline{H}$), 55.1 (C$\underline{H}_2$). ESI-MS *m/z*: 575.2 (M$^+$, calcd for $C_{16}H_{16}N_6O_6Re$, 575.1). RP-HPLC, retention time: t_R = 19.8 min.

4.3.5. *fac-[Re(CO)$_3${HC[4-(EtO(O)C)pz]$_3$}]Br (**Re5**)*

A solution of [Re(CO)$_3$(H$_2$O)$_3$]Br (18.6 mg, 46 μmol) and **L4** (20 mg, 46 μmol) in water was refluxed overnight. The solvent was removed under vacuum and the residue was washed with diethyl ether. The residue was purified by gradient HPLC (100% aqueous 0.1% CF_3COOH solution → 100% CH_3CN) using a Nucleosil column (10 μm, 250 mm × 7 mm) and a flow rate of 2 mL/min. Compound **Re5** was recovered as a beige solid after removal of the solvent from the collected fractions. Yield: 21% (6.9 mg, 9.8 μmol).

IR Data (KBr, ν/cm^{-1}): 2036s, 1944s, 1923s (C≡O). ^{1}H NMR ($CDCl_3$, δ ppm): 12.54 (1H, s, C$\underline{H}$), 9.10 (3H, s, H-3/5 (pz)), 8.46 (3H, s, H-3/5 (pz)), 4.32 (6H, q, C$\underline{H}_2$), 1.32 (9H, t, C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, δ ppm): 159.7 (C$\underline{O}$OEt), 149.1 (C-3/5 (pz)), 143.5 (C-3/5 (pz)), 119.2 (C-4 (pz)), 76.2 (C$\underline{H}$), 62.3 (C$\underline{H}_2$), 14.5 (C$\underline{H}_3$). ESI-MS *m/z*: 701.0 (M$^+$, calcd for $C_{22}H_{22}N_6O_9Re$, 700.7). RP-HPLC, retention time: t_R = 21.1 min.

4.3.6. *fac-[Re(CO)$_3${[4-(MeOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]$^+$ (**Re6**)*

A solution of [Re(CO)$_3$(H$_2$O)$_3$]Br (25 mg, 62 μmol) and **L5** (13 mg, 62 μmol) in methanol (5 mL) was refluxed overnight. The methanol was removed under vacuum and the residue was washed with hexane and chloroform. The recovered insoluble solid was dried under vacuum and formulated as compound **Re6**. Yield: 23 mg (48 μmol), 79%.

IR Data (KBr, ν/cm^{-1}): 2020s, 1916s (C≡O). ^{1}H NMR (CD_3OD, δ ppm): 7.99 (1H, s, H-3/5 (pz)), 7.96 (1H, s, H-3/5 (pz)), 6.88 (1H, m, N–H), 4.58 (2H, m, C$\underline{H}_2$), 4.30 (1H, m, C$\underline{H}_2$), 3.72 (3H, s, C$\underline{H}_3$), 3.65 (2 + 1H, m, C$\underline{H}_2$), 3.07 (1H, m, C$\underline{H}_2$), 2.79–2.80 (1 + 1H, m, C$\underline{H}_2$), 2.64 (1H, m, C$\underline{H}_2$), 2.18 (1H, m, C$\underline{H}_2$). ^{13}C NMR ($CDCl_3$, δ ppm): 196.5 (C$\underline{O}$), 195.9 (C$\underline{O}$), 195.7 (C$\underline{O}$), 146.2 (C-3/5 (pz)), 134.5 (C-3/5 (pz)), 121.7 (C-4 (pz)), 65.4 (O$\underline{C}$H$_2$), 58.3 (C$\underline{H}_2$), 56.5 (C$\underline{H}_2$), 52.5 (C$\underline{H}_2$), 49.4 (C$\underline{H}_2$), 41.6 (C$\underline{H}_3$). ESI-MS *m/z*: 469.4 (M$^+$, calcd for $C_{12}H_{18}N_4O_4Re$, 469.5).

4.3.7. *fac-[Re(CO)$_3${[4-(HOCH$_2$)pz](CH$_2$)$_2$NH(CH$_2$)$_2$NH$_2$}]Br (**Re7**)*

A solution of [Re(CO)$_3$(H$_2$O)$_3$]Br (48 mg, 119 μmol) and **L6** (22 mg, 199 μmol) in distilled water (10 mL) was refluxed overnight. The water was removed under vacuum and the residue was washed with chloroform. The recovered insoluble beige solid was dried under vacuum and formulated as compound **Re7**. Yield: 50 mg (94 μmol), 78%.

IR Data (KBr, ν/cm^{-1}): 2025s, 1906s (C≡O). ^{1}H NMR (CD_3OD, δ ppm): 7.94 (1H, s, H-3/5 (pz)), 7.93 (1H, s, H-3/5 (pz)), 4.52 (2 + 1H, m, C$\underline{H}_2$), 4.25 (1H, m, C$\underline{H}_2$), 3.58 (1H, m, C$\underline{H}_2$), 2.97 (1H, m, C$\underline{H}_2$), 2.79–2.83 (1 + 1H, m, C$\underline{H}_2$), 2.54 (1H, m, C$\underline{H}_2$), 2.16 (1H, m, C$\underline{H}_2$). ^{13}C NMR ($CDCl_3$, δ ppm): 198.1 (C$\underline{O}$), 194.8 (C$\underline{O}$), 194.5 (C$\underline{O}$), 145.2 (C-3/5 (pz)), 134.0 (C-3/5 (pz)), 125.1 (C-4 (pz)), 55.8 (C$\underline{H}_2$), 55.2 (C$\underline{H}_2$), 53.2 (C$\underline{H}_2$), 49.4 (C$\underline{H}_2$), 41.6 (C$\underline{H}_2$). ESI-MS *m/z*: 455.1 (M$^+$, calcd for $C_{11}H_{16}N_4O_4Re$, 455.1). RP-HPLC, retention time: t_R = 11.3 min.

4.4. X-ray diffraction analysis

Single crystals of **Re7** adequate for X-ray diffraction analysis were obtained from a saturated solution of the complex in methanol. The X-ray diffraction analysis has been performed on a Bruker

AXS APEX CCD area detector diffractometer, using graphite monochromatic Mo Kα radiation (0.71073 Å). The crystal data are summarized in Table 3.

Empirical absorption correction was carried out using SADABS [32]. Data collection and data reduction for **1** were done with SMART and SAINT programs [33]. The structure was solved by direct methods with SIR97 [34] and refined by full-matrix least-squares analysis with SHELXL97 [35] using the WINGX [36] suite of programs. Non-hydrogen atoms were refined with anisotropic thermal parameters whereas H-atoms were placed in idealized positions and allowed to refine riding on the parent C atom. Molecular graphics were prepared using ORTEP3 [37]. Crystallographic data for the structure of **1** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-835282. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: int. code +44-1223/336-033; e-mail: deposit@ccdc.cam.ac.uk].

4.5. General procedure for the synthesis of the ^{99m}Tc complexes (*Tc1–Tc3* and *Tc6*)

In a nitrogen-purged glass vial, 900 μL of the organometallic precursor *fac*-[^{99m}Tc(OH$_2$)$_3$(CO)$_3$]$^+$ at pH = 4 (**Tc1–Tc3**) or at pH = 7.4 (**Tc6**) were added to 100 μL of an ethanolic solution (10^{-2}–6.2×10^{-2} M) of **L1–L3** or to an aqueous solution of **L5** (10^{-4} M). The resulting solutions were heated at 100 °C for 30–60 min. After cooling to room temperature, the pH of the solutions was adjusted to pH 7.4 with 1 N NaOH in the case of **Tc1–Tc3**. Complexes **Tc1–Tc3** and **Tc6** have been obtained typically with a radiochemical yield ≥95%, as checked by gradient HPLC analysis, and were used in the biodistribution and metabolism studies without further purification.

4.6. Octanol–water partition coefficient

The log $P_{o/w}$ values of complexes **Tc1–Tc3** (Table 2) were determined by the shake-flask method [38] under physiological conditions (*n*-octanol/0.1 M PBS, pH 7.4).

Table 3
Crystallographic data for complex **Re7**.

Empirical formula	$C_{11}H_{16}BrN_4O_4Re \cdot H_2O$
Molecular weight	552.40
Crystal system	Monoclinic
Crystal size/mm	0.40 × 0.20 × 0.16
Space group	*P*21/*c*
a (Å)	8.7994(2)
b (Å)	8.8891(2)
c (Å)	20.6216(5)
α/°	90
β/°	91.1620(10)
γ/°	90
V/Å^3	1612.66(6)
T/K	150(2)
Z	4
D_c/g cm^{-3}	2.275
Reflections collected	9381
Independent reflections	3047 [*R*(int) = 0.0344]
μ(Mo Kα) (mm^{-1})	10.040
σ range for data collection (°)	3.26–25.68
No. of data	3047
No. of parameters	200
Final R indices [I > 2σ(I)]	
R_1	0.0288
wR_2	0.0690
R indices (all data)	
R_1	0.0350
wR_2	0.0705
GOF	1.077

4.7. Biodistribution studies

The biodistribution of complexes **Tc1–Tc3** and **Tc6** was evaluated in groups of 3–5 female CD-1 mice (randomly bred, Charles River) weighing approximately 20–25 g each, at 1 h and 2 h after intravenous administration with 100 μL (5–11 MBq) of each preparation via the tail vein as previously described. Studies were carried out according the EU guidelines for Animal Care and Ethic for Animal Experiments. Biodistribution results were expressed as percentage of the injected dose per gram tissue (% ID/g) and are shown in Table 2 and Table S1.

4.8. Metabolism studies

Blood and urine samples of mice injected with **Tc1–Tc3** and **Tc6**, collected at sacrifice time, were analyzed by HPLC to check the *in vivo* stability of the complexes. Prior to HPLC analysis urine samples were centrifuged and the serum from the blood samples was separated and treated with ethanol to precipitate proteins. The supernatant from these biological samples was analyzed using the conditions referred above for the HPLC analysis of the Re and ^{99m}Tc complexes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jorganchem.2013.11.013.

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