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A pyrazolylamine-phosphonate monoester chelator for the *fac*-$[M(CO)_3]^+$ core (M = Re, ^{99m}Tc): Synthesis, coordination properties and biological assessment

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Abstract: Aiming to develop new strategies for the labeling of hydroxyl-containing biomolecules with the organometallic core *fac*-$[^{99m}Tc(CO)_3]^+$, we have prepared a new model bifunctional chelator, **L4** (ethyl hydrogen (2-{[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]amino}ethyl)phosphonate), combining a pyrazolyl-amine chelating group and a monophosphonate ethyl ester function (–P(O)OHOEt). The phosphonate group allows metal stabilization, and, simultaneously, can be considered as a potential attachment site for a biomolecule. Reaction of **L4** with the precursor $[^{99m}Tc(H_2O)_3(CO)_3]^+$ gave the model radiocomplex $[^{99m}Tc(CO)_3(k^3\text{-}L4)]$ (**6a**). This radiocomplex was identified by comparing its chromatographic profile with that of the corresponding Re analog (**6**) under the same conditions, also prepared and fully characterized by the usual analytical techniques. Radiocomplex **6a** is moderately lipophilic ($\log P_{o/w} = 1.07$), presenting high stability *in vitro* without any measurable decomposition or ligand exchange, even in the presence of strong competing chelators such as histidine and cysteine (37°C, 24 h). Biodistribution studies of the complex in CD-1 mice indicated a rapid blood clearance, and a rapid clearance from main organs, occurring primarily through the hepatobiliary pathway. Complex **6a** presents also a high robustness *in vivo*, demonstrated by its resistance to metabolic degradation in blood, and intact excretion into the urine, after RP-HPLC analysis of blood and urine samples. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Tc-99m; rhenium; phosphonate; tricarbonyl; pyrazolyl-amine; phosphazolidine

Introduction

The development of new organometallic radioactive complexes based on the *fac*-$[^{99m}Tc(CO)_3]^+$ moiety for probing complex biochemical processes (e.g. receptor binding and antigen expression), which proceed various diseases such as cancer, is a subject of intense research.[1–4] One of the most important key issues is the availability of adequate bifunctional chelating agents (BFCAs) with coordinating units for stabilization of the radioactive metal core and, simultaneously, pendant amino or carboxylic acid groups for the linkage of important targeting molecules (e.g. peptides, central nervous system receptor ligands, enzyme substrates, etc.), while retaining their biological properties.[3] Promising BFCA includes tridentate ligands, which comprise aliphatic or aromatic amines, carboxylate groups, phosphines, thiols, thioethers and others, giving rise to chelators with different charges and donor-atom sets.[1–5] Therefore, depending on the combination of donating groups, cationic, neutral or anionic complexes with different physicochemical properties and pharmacokinetic profiles are formed.[6] Moreover, the stability of the radioactive complexes *in vivo* is of paramount importance, as dissociation of the radiometal through transchelation processes or reoxidation can lead to poor image quality, unnecessary radiation exposure to the patient and faulty image interpretation. This issue gains utmost importance when dealing with radionuclides for therapy.[4] Thus, the choice of the most adequate chelator for a particular targeting molecule must take into consideration not only the physicochemical and biological properties of the radioactive complexes, but also their stability *in vivo*. Regarding phosphorus-containing ligands for biomedical applications, Katti *et al.* have

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developed tri- and tetradentate chelators combining nitrogen or sulfur as donor atoms, and highly stable tertiary phosphines, namely hydroxymethyl phosphines, which allowed the labeling of tumor-seeking peptides with ^{99m}Tc for potential use in nuclear medicine.[7–10] Most recently, a new BFCA containing a picolylamine-methylphosphonic acid ester group for the labeling of hydroxyl-containing biomolecules with the *fac*-$[^{99m}Tc(CO)_3]^+$ core has been developed.[11] Aiming to develop new site-specific radiopharmaceuticals based on the organometallic approach, we have introduced in the past few years a new family of model ligands for the stabilization of the *fac*-$[M(CO)_3]^+$ moiety (R = ^{99m} Tc, Re), which contains a common pyrazolyl-amine chelating unit (Figure 1), and an aromatic (**L1**) or aliphatic (**L2**) amine, or a carboxylic acid group (**L3**).[12–15]

The model complexes *fac*-$[^{99m}Tc(CO)_3(k^3\text{-L})]^{+/0}$ (L = **L1**, **L2** and **L3**), obtained by reaction of the pyrazolyl-containing ligands **L1**–**L3** with *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$, display different physicochemical and biological properties, and are remarkably stable *in vitro* and *in vivo*, being adequate for the labeling of different types of biologically active molecules.[16,17] To enable fine tuning of the physicochemical and biological properties of the ^{99m}Tc model complexes and to broaden the range of biomolecules that could be labeled with pyrazolyl-containing ligands, we tried to develop new ligands combining a pyrazolyl-amine chelating unit and a phosphonic acid or an hydroxymethyl phosphine group. The main advantage of the phosphonic acids in BFCA is their potential ability to coordinate to the metal center and, simultaneously, functionalize alcohol-containing biomolecules, forming a stable phosphonic acid ester bond (Figure 2).

Herein, we describe the synthesis and characterization of a new model chelator with a pyrazolyl-amine framework and a monophosphonate ethyl ester group (–P(O)OHOEt, **L4**) as well as the attempts undertaken to prepare a chelator with a hydroxymethyl phosphine motif (–$P(CH_2OH)_2$). The preparation, characterization and biological evaluation of the organometallic complexes *fac*-$[M(CO)_3(\kappa^3\text{-L4})]$ (M = Re, **6**; ^{99m}Tc, **6a**) are also reported.

Results and discussion

Synthesis and characterization of diethyl (2-{[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl]amino}ethyl)phosphonate (3)

The synthetic pathway for the preparation of the precursor compound **3** involves two main steps (Scheme 1).

In the first general step, the pthalimide anion is reacted with 1-(2-bromoethyl)-3,5-dimethyl-1H-pyrazole (**1**) to give the intermediate N-substituted pthalimide, which upon treatment with hydrazine monohydrate, followed by acid hydrolysis, gives the corresponding primary amine 2-(3,5-dimethyl-1H-pyrazol-1-yl)ethanamine (**2**) in 82% yield. Selective monoalkylation of the primary amine in **2** with diethyl (2-bromo-ethyl)phosphonate under appropriate reaction conditions gave (2-{[2-(3,5-dimethyl-1*H*-pyrazol-1-yl) ethyl]amino}ethyl)phosphonate (**3**) in moderate yield (32%) after purification by column chromatography.

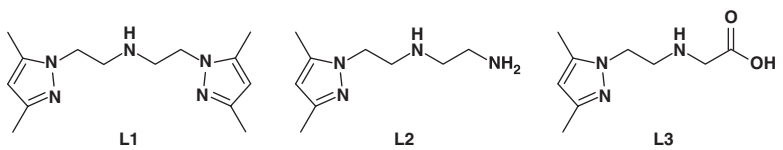

Figure 1 Ligands containing a pyrazolyl-amine chelating unit for the stabilization of the *fac*-$[M(CO)_3]^+$ core (R = ^{99m} Tc, Re).

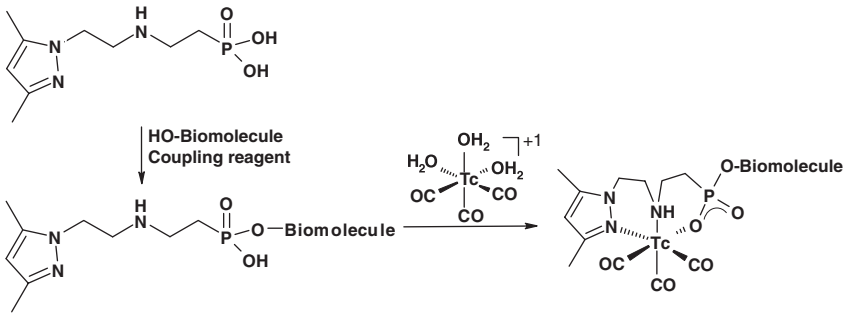

Figure 2 Labeling of alcohol-containing biomolecules with ^{99m}Tc-tricarbonyl.

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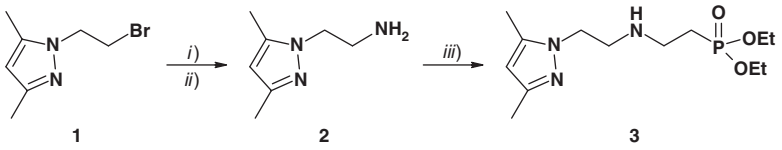


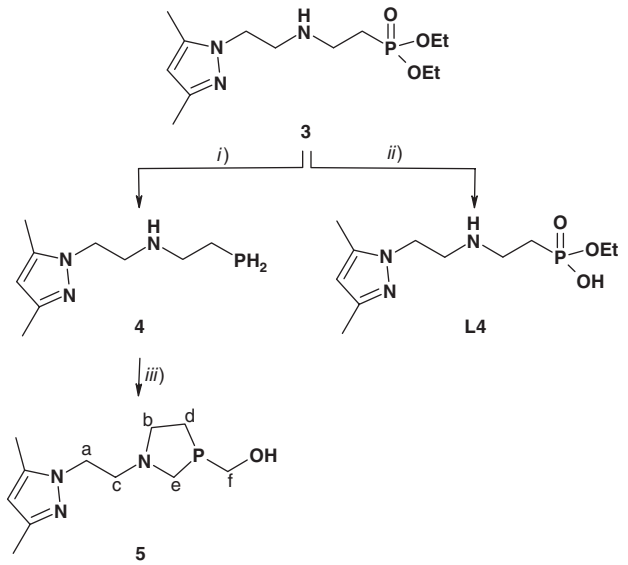
Scheme 1 Synthesis of compound **3**. (i) Potassium phtalimide/CH_3CN, reflux, 72 h. (ii) Hydrazine monohydrate/MeOH, reflux, 3 h, 37% HCl. (iii) Diethyl (2-bromoethyl)phosphonate/CH_3CN, K_2CO_3, reflux, 15 days.

Compound **3** has been characterized by ^{1}H and ^{31}P NMR. The ^{1}H-NMR spectrum ($CDCl_3$) presents the typical sharp singlet peaks for the H(4) proton (δ 5.75) and the methyl groups of the pyrazolyl ring (δ 2.21 and δ 2.18), as well as resonances for the methylenic protons and the diethylphosphonate group. A single peak (δ 30.5) for the phosphorus nucleus present in the molecule is found in the ^{31}P-NMR spectrum in the same solvent.

Synthesis and characterization of {1-[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl]-1,3-azaphospholidin-3-yl}-methanol (5) and ethyl hydrogen (2-{[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl]amino}ethyl)-phosphonate (L4)

The preparation of a chelator combining a pyrazolylamine framework and a stable hydroxymethyl phosphine from precursor **3** has been attempted as outlined in Scheme 2.

Reduction of the phosphonate group in **3** with a 1 M diethyl ether solution of lithium aluminum hydride gave the intermediate compound **4**, which contains a phosphine hydride. Similar to what has been previously described by Katti *et al.* for this type of compounds, **4** presents a high degree of oxidative stability upon contact with atmospheric oxygen and water, and lack of reactivity toward solvents and different type of chemical functionalities.[7–10] The formylation of the P–H bonds, carried out in degassed ethanol with 37% aqueous formaldehyde gave, instead of the hydroxymethyl phosphine groups, the rare heterocyclic phosphazolidine compound **5** (yield: 92%). This intramolecular cyclization is not surprising since it is known that the condensation of primary phosphines with formaldehyde in the presence of secondary amines leads, in a Mannich-type reaction, to tertiary phosphines containing dialkylaminoethyl groups or to phosphazolidine derivatives.[18,19] Compound **5** was obtained as an air-stable, pale-yellow clear oil, and has been thoroughly characterized by FTICR-MS (Fourier transform ion cyclotron resonance mass spectrometry) and $^{1}H/^{13}C/^{31}P$-NMR spectroscopy (see Experimental part). The use of 2D-NMR experiments (^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC) has been



Scheme 2 Synthesis of compounds **L4** and **5**. (i) $LiA1H_4$, diethyl ether. (ii) 1M NaOH solution in EtOH, reflux, 3 h. (iii) HCHO, EtOH, 30 min, room temperature (*Identification system for NMR assignments is displayed for 5*).

crucial for peak assignment in the NMR spectra, and for full characterization of the compound.

Partial alkaline hydrolysis of the diethyl phosphonate ester group in **3** using a standard procedure (1 M NaOH ethanolic solution, reflux) gave **L4**, after purification by Sep-pak, as a white solid (Scheme 2), soluble in water and alcohols. The chelator **L4** has been thoroughly characterized by the usual analytical techniques in chemistry. The most characteristic peaks in the ^{1}H-NMR spectrum (CD_3OD) for this molecule appear at δ 5.88 and δ 2.27/δ 2.17, assigned to the H(4) proton and the methyl groups of the pyrazolyl ring, respectively. As expected, the chemical shifts, multiplicity and relative intensity of the peaks at δ 3.97–3.87 (m, CH_2, 2H) and δ 1.25 (t, CH_3, 3H), assigned to one ethyl group, are in full agreement with the presence of a monophosphonate ethyl ester function (–P(O)OHOEt) in the molecule. The ^{13}C spectrum shows 11 peaks, which correspond to all the carbon nuclei of the ligand. A single peak in the ^{31}P-NMR spectrum is found at δ 20.8. The purity of the isolated compound (>95%) has been ascertained by analytical reversed phase high-performance liquid chromatography (RP-HPLC).

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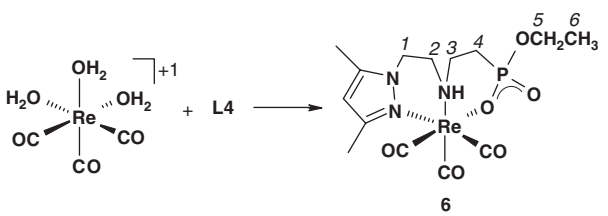
Synthesis and characterization of the Re tricarbonyl complex fac-[Re(CO)$_3$(k^3-L4)] (6)

The bifunctional chelator **L4** reacts with the organometallic precursor *fac*-[Re(CO)$_3$(H$_2$O)$_3$]$^+$ in refluxing water or methanol yielding the neutral complex **6** (ca. 46%) after purification by semi-preparative RP-HPLC (Scheme 3). This complex has been synthesized as a 'non-radioactive' analog of the corresponding ^{99m}Tc complex (**6a**).

Complex **6** is an air-stable white solid, which has been characterized by electrospray ionization mass spectrometry (ESI-MS; *m/z*: 546 [M + H]$^+$, 568 [M + Na]$^+$) and NMR spectroscopy (^{1}H/^{13}C/^{31}P-NMR, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HSQC). The NMR studies and the RP-HPLC chromatograms clearly indicated the formation of two isomers (*isomer* **a**: ret. time 18.6 min, *isomer* **b**: ret. time 18.9 min) in the ratio 4:6, most likely formed due to the different positions assumed by the ester group on the coordinating phosphonate unit. The coordination mode (k^3-*N,N,O*) of **L4** toward the 'Re(CO)$_3$' moiety, and the presence of the rare P–O–Re^I(CO)$_3$ coordination, has been established based on the NMR spectroscopic data of **6**. This coordination mode compares well with the one found in the organometallic compound *fac*-[Re(CO)$_3$(k^3-L)] (L = picolylamine–mehylphosphonic acid ester), which has been characterized both in solution by NMR, and in the solid state by X-ray crystallography.[11] In this complex, the crystal structure revealed the presence of a delocalized electron pair shared by the endstanding P–O bond and the P–O bond involved in the coordination to the metallic center.

In the ^{1}H-NMR spectra (CD$_3$OD) of enriched mixtures in each isomer, obtained by RP-HPLC purification, the most striking features are the different chemical shifts observed for the H(4)pz signal (*isomer* **a**: δ 6.12, *isomer* **b**: δ 6.09), consistent with the involvement of the azole ring in the coordination to the metal, and for the protons of the phosphonate ethyl ester (*isomer* **a**: δ 3.79, O<u>CH$_2$</u>CH$_3$; δ 1.15, OCH$_2$<u>CH$_3$</u>. *isomer* **b**: δ 4.04, O<u>CH$_2$</u>CH$_3$; δ 1.37, OCH$_2$<u>CH$_3$</u>) in both isomers. Another important feature of the spectrum is related with the diastereotopic character of some of the methylenic protons of the ligand backbone upon coordination to the metallic center (δ 4.54, CH$_2$-1, δ 4.04, CH$_2$-1′; δ 3.04, CH$_2$-2; δ 2.74, CH$_2$-2′). This behavior, already observed in other tricarbonyl complexes, is typical for asymmetric ligands containing a pyrazolylamine backbone, being a strong evidence of ligand coordination.[12–14,20] The ^{13}C-NMR spectra showed splitting due to the C–P coupling for some of the aliphatic carbon atoms. Coupling to the C-4 ($^1J_{PC\text{-}4}$ = 7.5 Hz) and to the C-5 (*isomer* **a**: $^2J_{PC\text{-}5}$ = 7.4 Hz *isomer* **b**: $^2J_{PC\text{-}5}$ = 12.1 Hz) is strong for both isomers. The ^{31}P-NMR spectrum in CD$_3$OD shows singlets at δ 25.6 and δ 23.5 assigned to *isomers* **a** and **b**, respectively. These downfield shifted signals (Δ = 4.8 ppm, *isomer* **a**; Δ = 2.7 ppm, *isomer* **b**) relative to free **L4** ligand, is an evidence for P–O–Re^I(CO)$_3$ coordination.



Scheme 3 Preparation of the rhenium tricarbonyl complex *fac*-[Re(CO)$_3$(k^3-L4)] (6). (*Numbering system for NMR assignments is displayed*).

Synthesis and characterization of fac-[^{99m}Tc(CO)$_3$ (k^3-L4)] (6a)

The complex *fac*-[^{99m}Tc(CO)$_3$(k^3-L4)] (**6a**) was obtained in ca. 75% yield at 100°C, using ligand concentrations in the 10^{-5}–10^{-4} M range. The only radiochemical impurities identified by RP-HPLC were [^{99m}TcO$_4$]$^-$ and [^{99m}Tc(CO)$_3$]$^+$. The chemical identity of complex **6a** after RP-HPLC purification was confirmed by comparing its HPLC profile (retention time: 18.8 min) with that of the corresponding rhenium complex **6** (Figure 3). Surprisingly, no evidence for the formation of isomers of the radioactive complex **6a** was found.

Complex **6a** is moderately lipophilic (log $P_{o/w}$ = +1.07), and displays high *in vitro* stability in the presence of excess of cysteine, histidine or PBS pH 7.4, since HPLC

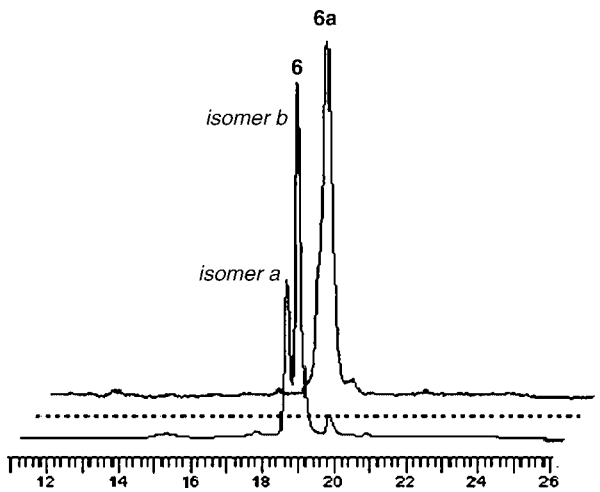


Figure 3 RP-HPLC analytical chromatograms of **6** (UV detection, ret. time = 18.6 min, *isomer* **a**; 18.9 min, *isomer* **b**) and **6a** (γ detection, ret. time = 18.8 min).

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analysis revealed that no *trans*-chelation or reoxidation to pertechnetate occurred, even after 24 h at 37°C. The stability profile is similar to that of other complexes anchored on pyrazolyl-diamine chelators previously reported.[12–17,20]

Biodistribution of 6a. The organ distribution of purified **6a** in female CD-1 mice as a function of time is summarized in Table 1.

Complex **6a** shows a rapid blood clearance as only 0.87 ± 0.20% ID of the administered dose remained in whole blood at 1 h p.i., and a rapid clearance from main, non-excretion, organs. Comparatively, **6a** cleared more rapidly than the analogous complex *fac*-[^{99m}Tc(CO)$_3$(k^3-L3)] stabilized by the pyrazolyl-containing ligand **L3**, which presents similar physico-chemical properties (moderate lipophilicity and neutral charge).[15] As expected from the lipophilic character of both complexes (log $P_{o/w}$: 1.07 and 1.10 for **6a** and *fac*-[^{99m}Tc(CO)$_3$(k^3-L3)], respectively) they were mainly excreted by the hepatobiliary pathway. Consequently, the overall excretion rate was relatively slow, in accordance with what is normally found for lipophilic radiocomplexes. However, the radiocomplex **6a** was excreted faster (54.5% after 4 h p.i.) than *fac*-[^{99m}Tc(CO)$_3$(k^3-L3)] (29.7% after 4 h p.i.). Indeed, after 4 h, the hepatic uptake observed for **6a** was relatively low (4.88 ± 0.38% ID per organ), most of the radioactivity being already in the intestine (37.83 ± 13.28% ID per organ). There was no significant uptake or retention of radioactivity in the stomach (0.10 ± 0.03%/g, 4 h, p.i.), demonstrating that **6a** does not undergo *in vivo* reoxidation to [^{99m}TcO$_4$]$^-$.

To further verify the *in vivo* stability of **6a**, samples of murine serum and urine collected at sacrifice time (1 h p.i.) were analyzed by radiometric RP-HPLC. The murine serum, isolated from blood does not show any traces of pertechnetate, and more than 98% of the radioactivity could be assigned to the complex. Analysis of urine collected at the same time demonstrated again high *in vivo* stability for the complex, because no metabolites could be detected (Figure 4). The resistance to metabolic degradation in blood and the excretion of the ^{99m}Tc tricarbonyl complex **6a** intact into the urine demonstrate its high *in vivo* robustness.

Table 1 Biodistribution data of complex **6a** in female CD-1 mice (n = 5) at 1 and 4 h postinjection (p.i.) (% ID g^{-1} ± standard deviation)

Tissue	**6a**	
	1 h	4 h
Blood	0.63 ± 0.15	0.20 ± 0.02
Liver	7.74 ± 1.76	3.82 ± 0.41
Intestine	22.99 ± 2.34	15.54 ± 4.90
Spleen	0.19 ± 0.03	0.07 ± 0.01
Heart	0.30 ± 0.06	0.12 ± 0.04
Lung	0.46 ± 0.10	0.18 ± 0.02
Kidney	2.77 ± 0.45	1.12 ± 0.24
Muscle	0.22 ± 0.02	0.07 ± 0.01
Bone	0.15 ± 0.02	0.06 ± 0.01
Stomach	0.48 ± 0.16	0.10 ± 0.03
Pancreas	0.43 ± 0.15	0.15 ± 0.03
Total excretion (%)	29.7 ± 2.9	54.5 ± 14.1

Conclusion

We have synthesized and characterized a new chelator containing a pyrazolyl-amine backbone and a phosphonate monoester (**L4**) for stabilization of the *fac*-[M(CO)$_3$]$^+$ core (M = Re, ^{99m}Tc). Reaction of this novel bifunctional chelating ligand with the corresponding organometallic precursor afforded the model radiocomplex *fac*-[^{99m}Tc(CO)$_3$(k^3-L4)] (**6a**) showing that **L4** can be potentially useful for the labeling of biomolecules containing an alcohol function. Complex **6a** has been identified by comparing with the corresponding Re surrogate (**6**), which has also been synthesized and fully characterized. Biodistribution studies of radiocomplex **6a** in female CD-1 mice indicated a rapid blood clearance, and a relatively rapid clearance from main organs, being excreted mainly through the hepatobiliary pathway. The radiocomplex is stable both *in vitro* and *in vivo*, without any measurable decomposition or reoxidation. It is still necessary to optimize the labeling yields of the chelator in order to expand the use of the promising bifunctional chelator **L4** to the labeling of a large variety of hydroxyl-containing biomolecules.

Materials and methods

All chemicals and solvents were of reagent grade and were used without purification unless stated otherwise.

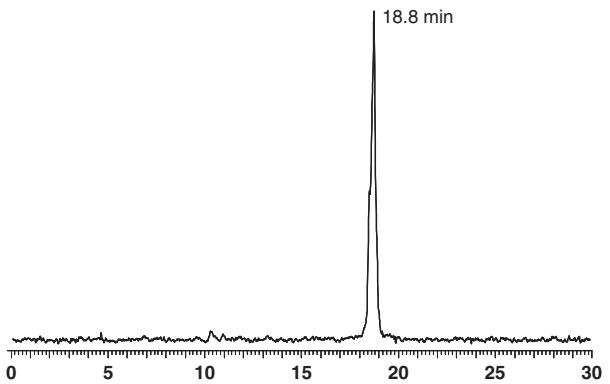

Figure 4 RP-HPLC radioactive trace (γ-detection) for an urine sample collected at 1 h p.i.

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Diethyl (2-bromoethyl)phosphonate was obtained in high yield (>85%) using the classical Michaelis–Arbuzov reaction, i.e. by refluxing dry triethyl phosphite in a 30-fold excess of dry dibromoethane overnight, followed by vacuum distillation.[21] The precursors 1-(2-bromoethyl)-3,5-dimethyl-1*H*-pyrazole (**1**) and $[Re(CO)_3(H_2O)_3]Br$ were prepared according to published methods.[22,23] $Na[^{99m}TcO_4]$ was eluted from a $^{99}Mo/^{99m}Tc$ generator, using 0.9% saline. The radioactive precursor *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared using a IsoLink® kit (Malinckrodt, Inc.). ^1H, ^{11}C NMR and ^{31}P spectra were recorded at room temperature on a Varian Unity 300 MHz spectrometer. ^1H and ^{11}C chemical shifts were referenced with the residual solvent resonances relative to tetramethylsilane, and the ^{31}P-NMR chemical shifts with external 85% H_3PO_4 solution. NMR spectra were run in $CDCl_3$, CD_3OD and D_2O. HPLC analyses were performed on a Perkin Elmer LC pump 200 coupled to a Shimadzu SPD 10AV UV/Vis detector and to a Berthold-LB 507A radiometric detector, using an analytic Macherey–Nagel C18 reversed-phase column (Nucleosil 100-10, 250 × 4 mm) and a gradient of aqueous 0.1% CF_3COOH/MeOH with a flow rate of 1 mL/min.

Gradient: t = 0–3 min: 0% MeOH; 3–3.1 min: 0 → 25% MeOH; 3.1–9 min: 25% MeOH; 9–9.1 min: 25 → 34% MeOH; 9.1–19 min: 34 → 100% MeOH; 19–21 min: 100 → 0% MeOH; 21–30 min: 0% MeOH. Purification of the inactive compounds was achieved on a semi-preparative Macherey–Nagel C18 reversed-phase column (Nucleosil 100-7, 250 × 8 mm) using a gradient of aqueous 0.1% CF_3COOH/CH_3CN as eluent and a flow rate of 2 mL/min. *Gradient*: t = 0–5 min: 20% CH_3CN; 5–30 min: 20 → 100% CH_3CN; 30-34 min: 100% CH_3CN; 34–35 min: 100 → 20% CH_3CN; 35–40 min: 20% CH_3CN.

Synthesis of 2-(3,5-dimethyl-1H-pyrazol-1-yl)ethanamine (2)

Synthesis of 2-[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl]-1H-isoindole-1,3(2H)-dione: To a solution of 1-(2-bromoethyl)-3,5-dimethyl-1*H*-pyrazole (**1**, 9.56 g, 0.047 mol) in dry CH_3CN (200 mL) potassium phtalimide (26.14 g, 0.141 mol) was added. After 72 h under reflux and a nitrogen atmosphere, the solution was decanted and filtered. The clear solution obtained was evaporated to dryness and the residue recrystallized from MeOH, giving the compound as a white solid. Yield: 8.68 g, 69%. ^1H-NMR ($CDCl_3$): δ 7.86–7.76 (m, CH aromatic, 2H); 7.74–7.67 (m, CH aromatic, 2H), 5.75 (s, H(4)pz, 1H); 4.26 (t, CH_2, 2H); 4.01 (t, CH_2, 2H); 2.18 (s, CH_3, 3H), 2.06 (s, CH_3, 3H).

Synthesis of 2-(3,5-dimethyl-1H-pyrazol-1-yl)ethanamine (**2**): Hydrazine monohydrate (9.8 mL, 0.20 mol) was added to a suspension of 2-[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]-1H-isoindole-1,3(2H)-dione (5.21 g, 0.019 mol) in MeOH (150 mL). After refluxing for 3 h, the reaction mixture was allowed to cool to room temperature, and 37% HCl (43.5 mL) was added dropwise. The white solid formed was removed by filtration and the pH of the resulting solution was adjusted to pH 9 with 3 M NaOH. The aqueous phase was extracted with $CHCl_3$ (3 × 100 mL). The organic phases were collected, dried over $MgSO_4$, filtered, and concentrated to dryness, giving an yellow viscous oil which crystallized on standing. Yield: 2.21 g, 82%. ^1H-NMR ($CDCl_3$): δ 5.75 (s, H(4)pz, 1H); 3.97 (t br., CH_2, 2H); 3.06 (t br., CH_2, 2H); 2.20 (s, CH_3, 3H), 2.17 (s, CH_3, 3H), 1.63 (s br., NH_2, 2H).

Synthesis of diethyl (2-{[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl]amino}ethyl)phosphonate (3)

Diethyl (2-bromoethyl)phosphonate (1.092 g, 4.46 mmol) and 2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethanamine (0.414 g, 2.97 mmol) were dissolved in CH_3CN (25 mL) and an excess of K_2CO_3, (0.821 g, 5.94 mmol), KI (catalytic amount) were added. After 15 days under reflux, the suspended solids were removed by filtration, and water (ca. 30 mL) was added to the solution. The reaction mixture was extracted with CH_2Cl_2 (3 × 75 mL). The organic phases were collected, dried over $MgSO_4$, filtered and concentrated to dryness. The residue was purified by column chromatography (MeOH (5–50%)/CH_2Cl_2). The product was obtained as a pale-yellow oil. Yield: 0.62 g, 69%. ^1H-NMR ($CDCl_3$): δ 5.75 (s, H(4)pz, 1H); 4.09–4.02 (m, CH_2, 6H); 3.00 (t, CH_2, 2H); 2.93–2.84 (m, CH_2, 2H) 2.21 (s, CH_3, 3H), 2.18 (s, CH_3, 3H), 1.99–1.88 (m, CH_2, 2H); 1.28 (t, CH_3, 6H). ^{31}P NMR ($CDCl_3$): δ 30.5.

Synthesis of ethyl hydrogen (2-{[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl]amino}ethyl)phosphonate (L4)

The monoethyl ester was obtained by alkaline hydrolysis of **3** (0.105 g, 0.35 mmol) in a 1 M NaOH/EtOH solution (2 mL). After 3 h of reflux, the solution was cooled and the reaction concentrated to half of the initial volume. The solution was then neutralized to pH 7 with 1 M HCl and the solvents removed under reduced pressure until a white solid was obtained. The solid was extracted with three portions of EtOH. After evaporation of the solvent, **L4** was obtained as a white solid. The compound was further purified by Sep-pak: the cartridge was washed with water (2 mL) and the product was eluted with 2:1 methanol:water (2 mL).

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Evaporation of the eluate afforded the product as a white solid. Yield: 0.08 g, 83%. ^{1}H-NMR (CD_3OD): δ 5.88 (s, H(4)pz, 1H); 4.26 (t, CH_2, 2H); 3.97–3.87 (m, CH_2, 2H); 3.44 (t, CH_2, 2H); 3.26–3.17 (m, CH_2, 2H); 2.27 (s, CH_3, 3H), 2.17 (s, CH_3, 3H), 1.96–1.85 (m, CH_2, 2H); 1.25 (t, CH_3, 3H). ^{13}C NMR (CD_3OD) δ 149.9 (C(3/5)pz), 141.7 (C(3/5)pz), 106.7 (C(4)pz), 61.1 (d, $J = 5.7$ Hz, $\underline{POCH_2}CH_3$), 45.1 (d, $J = 26.2$ Hz, $H_2\underline{CP}$), 25.8 (CH_2), 25.2 (CH_2), 24.0 (CH_2), 17.1 (d, $J = 6.6$ Hz, P$\underline{OCH2CH_3}$), 13.4 (CH_3), 10.7 (CH_3). ^{31}P NMR (CD_3OD): δ 20.8. Anal. Calc. for $C_{11}H_{21}N_3O_3PNa$: C, 44.44; H, 7.12; N, 14.13. Found: C, 45.11; H, 6.57; N, 14.07. ESI-MS (+): *m/z* 276 $[M + H]^+$, 298 $[M + Na]^+$. Retention time (RP-HPLC): 9.7 min.

Synthesis of 2-(3,5-dimethyl-1H-pyrazol-1-yl)-N-(2-phosphinoethyl)ethanamine (4)

The compound has been prepared according to a slightly modified reported method[9]: To a stirred cold (0°C) solution of $LiAlH_4$ (2.2 mL, 1 M solution in diethyl ether), a suspension of the diethyl phosphonate **3** (0.328 g, 1.08 mmol) in dry diethyl ether (24 mL) in a nitrogen atmosphere was added. The reaction was allowed to react for 4 h at room temperature; the mixture was cooled to 0°C and the excess of $LiAlH_4$ was eliminated by slow addition of cold aqueous brine solution (20 mL), followed by the addition of aqueous KOH solution (10%, 20 mL). The organic layer was separated, the aqueous layer was extracted with ether (1×20 mL) and CH_2Cl_2 (2×25 mL), and the organic phases were collected and washed with brine (80 mL). After drying over anhydrous $MgSO_4$, the solvents were evaporated in the rotary evaporator under reduced pressure and the colorless oil obtained was vacuum dried. The product is stable for some weeks if stored at −20°C. Yield: 0.14 g, 65%. ^{1}H-NMR ($CDCl_3$): δ 5.75 (s, H(4)pz, 1H); 4.03 (t, CH_2, 2H); 2.98 (t, CH_2, 2H); 2.90 (t, PH, 1H); 2.74 (q, CH_2, 2H); 2.25 (t, PH, 1H); 2.20 (s, CH_3, 3H), 2.17 (s, CH_3, 3H), 1.66–1.58 (m, CH_2, 2H). ^{31}P NMR ($CDCl_3$): δ −147.1.

Synthesis of {1-[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl]-1,3-azaphospholidin-3-yl}-methanol (5)

An aqueous solution of formaldehyde (0.040 ml of a 37% solution) was placed in oxygen-free ethanol (2.5 mL) and purged with nitrogen gas for some minutes. This solution was added dropwise with stirring at room temperature to compound **4** (0.048 g, 0.24 mmol). The resulting solution was allowed to react for 30 min at room temperature. Removal of the solvent *in vacuo* afforded compound **5** as a colorless, viscous oil. Yield: 0.052 g, 92%. ^{1}H-NMR (D_2O): δ 5.76 (s, H(4)pz, 1H); 3.98 (t, CH_2-a, 2H); 3.71–3.53 (m, CH_2-e, 2H); 2.96–2.86 (m, CH_2-b, 1H); 2.80 (t, CH_2-c+CH_2-f, 2H+1H); 2.60–2.46 (m, CH_2-f′, 1H); 2.36–2.26 (m, CH_2-b′, 1H), 2.08 (s, CH_3, 3H), 1.98 (s, CH_3, 3H), 1.92–1.76 (m, CH_2-d, 1H); 1.68–1.59 (m, CH_2-d′, 1H); ^{13}C-NMR (D_2O) δ 148.2 (C(3/5)pz), 140.8 (C(3/5)pz), 104.5 (C(4)pz), 61.0 (d, $^{1}J_{PC} = 19$ Hz, C-e), 54.0 (d, $^{3}J_{PC} = 2$ Hz, C-c) 53.4 (d, $^{2}J_{PC} = 4$ Hz, C-b), 51.4 (d, $^{1}J_{PC} = 13$ Hz, C-f), 45.6 (C-a), 19.1 (d, $^{1}J_{PC} = 10$ Hz, C-d), 11.4 (CH_3), 9.5 (CH_3). ^{31}P-NMR (D_2O): δ −28.5. FTICR-MS(+) *m/z*: 242 $[M + H]^+$. See identification system for NMR assignment in Scheme 2.

Synthesis of $[Re(CO)_3(k^3\text{-L4})]$ (6)

To a solution of $[Re(CO)_3(H_2O)_3]Br$ (0.047 g, 0.12 mmol) in MeOH (10 mL) **L4** (0.032 g, 0.012 mmol) was added, and the resulting mixture was refluxed overnight. After evaporation of the solvent, the residue obtained was dried under vacuum and analyzed by RP-HPLC. The crude product revealed a mixture of unreacted Re precursor (retention time: 6.3 min), free **L4** (retention time: 9.7 min) and complex **6** as two isomers (retention time: *isomer* **a** – 18.6 min, *isomer* **b** – 18.9 min). The isomers were separated and isolated by RP-HPLC. Yield (both isomers): 30 mg, 46%.

Isomer **a**: ^{1}H-NMR (CD_3OD): δ 6.22 (s br., 1H, NH), 6.12 (s, 1H, H(4)pz), 4.54 (dd, $J = 4.5$ Hz, $J = 15.4$ Hz, 1H, CH_2-1), 4.04 (m, 1H, CH_2-1′), 3.79 (p, $J = 7.1$ Hz, 2H, OCH_2-5), 3.47 (m br, 2H, CH_2-4), 3.04 (m, 1H, CH_2-2), 2.74 (m, 1H, CH_2-2′), 2.52 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 1.79 (m br., 2H, CH_2-3), 1.15 (t, $J = 7.4$ Hz, 3H, CH_3-6). ^{13}C-RMN (CD_3OD): δ 197.0–194.5 ($3 \times C{\equiv}O$), 154.5 (C(3/5)pz), 143.9 (C(3/5)pz), 108.4 (C(4)Pz), 62.1 (d, $^{2}J_{PC} = 7.4$ Hz, C-5), 52.1 (d, $^{1}J_{PC} = 7.5$ Hz, C-4), 50.6 (C-2), ∼49.0 (C-1, overlapped with CD_3OD as confirmed by HSQC), 23.8 (d, $^{2}J_{PC} = 22.3$ Hz, C-3), 16.9 (C-6); 15.2 (CH_3pz); 11.4 (CH_3pz). ^{31}P-NMR (CD_3OD): δ 25.6.

Isomer **b**: ^{1}H-NMR (CD_3OD): δ 6.22 (s br., 1H, NH), 6.09 (s, 1H, H(4)pz), 4.54 (dd, $J = 4.5$ Hz, $J = 15.4$ Hz, 1H, CH_2-1,), 4.04 (m, 3H, $CH_2 - 1' + OCH_2 - 5$), 3.59 (m br., 2H, CH_2-4), 3.04 (m, 1H, CH_2-2), 2.74 (m, 1H, CH_2-2′), 2.47 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 1.85 (m, 2H, CH_2-3), 1.37 (t, $J = 7.4$ Hz, 3H, CH_3-6). ^{13}C-RMN (CD_3OD): δ 197.0–194.5 ($3 \times C{\equiv}O$), 154.7 (C(3/5)pz), 143.9 (C(3/5)pz), 108.4 (C(4)Pz), 60.7 (d, $^{2}J_{PC} = 12.1$ Hz C-5), 52.6 (d, $^{1}J_{PC} = 7.5$ Hz, C-4), 50.4 (C-2), ∼49.0 (C-1, overlapped with CD_3OD as confirmed by HSQC), 21.9 (d, $^{2}J_{PC} = 22.4$ Hz, C-3), 16.8 (C-6), 15.4 (CH_3pz), 11.4 (CH_3pz). ^{31}P-NMR (CD_3OD): δ 23.5. In both isomers, there is occasional overlapping of some resonances associated with the two isomers, as confirmed by two-dimensional NMR techniques (^{1}H-^{1}H

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COSY and ^{1}H-^{13}C HSQC). See numbering system for NMR assignments in Scheme 3. ESI-MS (+): m/z 546 $[M + H]^+$, 568 $[M + Na]^+$.

Synthesis of the ^{99m}Tc-complex 6a

A volume of 900 μL of the organometallic precursor *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ in saline pH 7.4 and 100 μL of an aqueous solution of **L4** (8×10^{-4} M) were placed in a 5-mL glass vial under nitrogen. The vial was then heated to 100°C for 60 min, cooled on an ice bath and the final solution analyzed by RP-HPLC. Retention time: 18.8 min. Complex **6a** was purified for the biodistribution studies using a semi-preparative Knauer C18 reversed-phase column (Hypersil 120 ODS 10 μm, 250 × 8 mm) and a gradient of aqueous 0.1% CF_3COOH/MeOH with a flow rate of 2 mL/min. *Gradient*: t = 0–3 min: 0% MeOH; 3–3.1 min: 0 → 25% MeOH; 3.1–9 min: 25% MeOH; 9–9.1 min: 25 → 34% MeOH; 9.1–19 min: 34 → 100% MeOH; 19–21 min: 100 → 0% MeOH; 21–30 min: 0% MeOH. The peak corresponding to the complex was collected in a Falcon flask containing 200 μL of saline. The solvent was removed under a stream of nitrogen until a final volume of 200 μL was reached. The solution was diluted to the desired radioactivity with PBS pH 7.4, and the product analyzed again by analytical RP-HPLC. This compound has also been used for the *in vitro* stability studies.

Octanol–water partition coefficient

The $\log P_{o/w}$ values of complexes **6a** were determined by the multiple back extraction method under physiological conditions (*n*-octanol/0.1 M PBS, pH 7.4).[24]

Cysteine and histidine challenge

In total, 100 μl of purified **6a** was added to 900 μL of 10^{-3} M cysteine or histidine solutions in PBS at pH 7.4. The samples were incubated at 37°C and analyzed by RP-HPLC after 24 h.

Biodistribution studies

All animal studies were conducted in accordance with the highest standards of care, as outlined in the National and European Law. The animals were housed in a temperature- and humidity-controlled room with a 12 h light/12 h dark schedule. The *in vivo* behavior of complexes **6a** was evaluated in groups of five female CD-1 mice (randomly bred, Charles River) weighing approximately 20–25 g each. Animals were injected intravenously with 100 μL (25–30 μCi or 50–60 μCi for 1 and 4 h biodistribution timepoints, respectively) of purified complex **6a** via the tail vein, and were maintained on normal diet *ad libitum*. Mice were sacrificed by cervical dislocation at 1 and 4 h p.i. The injected radioactive dose and the radioactivity remaining in the animal after sacrifice were measured in a dose calibrator (Aloka, Curiemeter IGC-3, Tokyo, Japan). The difference between the radioactivity in the injected and sacrificed animal was assumed to be due to total excretion from whole animal body. Blood samples were taken by cardiac puncture at sacrifice. Tissue samples of the main organs were removed, weighed and counted in a gamma counter (Berthold). Biodistribution results were expressed as a percentage of the injected dose per gram tissue (%ID/g). For blood, bone and muscle, total activity was calculated assuming that these organs constitute 6, 10 and 40% of the total weight, respectively. The remaining activity in the carcass was also measured in a dose calibrator.

Assessment of the in vivo stability of 6a by RP-HPLC analysis

The *in vivo* stability of **6a** was assessed by urine and murine serum RP-HPLC analysis, under the same chromatographic conditions used for **6a**. The samples were obtained 1 h p.i. from the biodistribution experiment. The urine collected at sacrifice time was filtered through a Millex GV filter (0.22 μm) before RP-HPLC analysis. Blood collected from mice was immediately centrifuged at 3000 rpm for 15 min at 4°C, and the serum was separated. The serum was treated with ethanol in a 2:1 (v/v) ratio to precipitate the proteins. After centrifugation at 3000 rpm for 15 min at 4°C, the supernatant was filtered through a Millex GV filter (0.22 μm) and analyzed by RP-HPLC.

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