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Phosphonated chelates for nuclear imaging†

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A series of bis-, tris- and tetra-phosphonated pyridine ligands is presented. In view of their potential use as chelates for radiopharmaceutical applications, the physico-chemical properties of the ligands and of their Co(II), Ni(II), Cu(II), and Zn(II) complexes were studied by means of potentiometry and UV-Vis absorption spectroscopy. The pK_a values of the ligands and of the complexes, as well as the stability constants for the formation of the complexes, are presented. The kinetic aspects of the formation of Cu(II) complexes and of their dissociation in acidic media were studied by means of stopped flow experiments, and the stability of the Cu(II) complex toward reduction to Cu(I) was investigated by cyclic voltammetry and by titration with different reducing agents. The different thermodynamic and kinetic aspects of the polyphosphonated ligands were compared with regard to the impact of the number of phosphonic acid functions. Considering the very promising properties for complexation, preliminary SPECT/CT imaging experiments were carried out on mice with ^{99m}Tc using the bis- and tetra-phosphonated ligands **L**² and **L**¹. Finally, a bifunctional version of chelate **L**¹, **L**^{*}, was used to label MTn12, a rat monoclonal antibody with both specificity and relatively high affinity for murine tenascin-C. The labeling was monitored by MALDI/MS spectrometry and the affinity of the labeled antibody was checked by immunostaining experiments. After chelation with ^{99m}Tc , the ^{99m}Tc -**L**^{*}-MTn12 antibody was injected into a transgenic mouse with breast cancer and the biodistribution of the labeled antibody was followed by SPECT/CT imaging.

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Introduction

The development of radiopharmaceuticals is currently receiving much interest for diagnostic imaging and therapy in

nuclear medicine, particularly in two major imaging modalities, Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). Many radioisotopes are used for these two techniques, among them ^{64}Cu ($T_{1/2} = 12.7$ h, β^+ : 17.8%, $E_{\beta^+} = 656$ keV; β^- : 38.4%, $E_{\beta^-} = 573$ keV) is particularly promising for PET,^{1–5} and ^{99m}Tc ($T_{1/2} = 6$ h, pure γ -emitter, $E_{\gamma} = 140$ keV) is the radionuclide of choice for SPECT imaging.^{2,5,6}

Although these radionuclides can be used for their intrinsic properties, as in the case of thyroid imaging with $^{99m}\text{TcO}_4^-$,⁷ the applications of ^{64}Cu and ^{99m}Tc as targeted radiopharmaceuticals generally require the use of bifunctional chelates (BFC) designed to selectively bind the radionuclide and to be covalently linked to a bio-targeting vector such as an antibody, a peptide or a protein. The ideal BFC should form thermodynamically stable and kinetically inert complexes to prevent any ligand exchange reactions or hydrolysis *in vivo*. The complexation should be efficient and rapid at room temperature and at low concentration, at a pH suitable for biological applications.

Among the possible ligands for ^{64}Cu and ^{99m}Tc complexation, hydrophilic phosphonated ligands have proved to be efficient chelating agents.^{8–16} With phosphonated ligands, the thermodynamic stability constants of the Cu(II) complexes are notably higher than those of their acetate analogues.¹⁷

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† Electronic supplementary information (ESI) available: the Experimental section for the physico-chemistry investigations; potentiometric plots of the cupric complexes formed with ligands **L**¹, **L**², **L**³ and **L**⁴ together with the corresponding hyperquad analysis; distribution diagrams as a function of pH of the protonated metal complexes formed with ligands **L**¹, **L**², **L**³ and **L**⁴ (four figures); absorption spectral variation induced by copper(II) complexation as a function of pH for ligands **L**² to **L**⁴ (four figures); electrochemical analysis of the copper(II) complexes (two Figures); variation of the pM values as a function of pH for ligands **L**² to **L**⁴ (three Figures); ESI-MS characterization of the metal complexes (one table); ¹H, ¹³C and ³¹P NMR spectra of the compounds; stability and protonation constants of the metallic complexes with Ni(II), Zn(II) and Co(II) formed with ligands **L**¹-**L**⁴ (one table); ¹H, ¹³C and ³¹P NMR spectra of the compounds. See DOI: 10.1039/c4ob01514b

Scheme 1 Tetra-phosphonated ligand **L**¹, the activated BFC **L**^{*}, and the bis and tris-phosphonated ligands **L**², **L**³ and **L**⁴.

Our efforts to design new chelate systems for subsequent uses in copper-64 PET imaging led us to the synthesis of the tetra-phosphonated ligand **L**¹ (Scheme 1), a very effective and selective Cu(II) chelating agent ($\log K_{\text{CuL}} = 22.7$).^{11,12} We have also shown that **L**¹ can be derivatized into an activated phosphonated bifunctional chelate **L**^{*} (Scheme 1) which can be coupled to small peptides or large antibodies.¹⁸

In order to fully understand the coordination chemistry of phosphonated chelates, we have designed a series of three new ligands bearing two (**L**²) or three (**L**³, **L**⁴) phosphonate functions on a pyridine framework (Scheme 1). The synthesis of these ligands is reported here, together with their complexation properties towards d elements such as Cu(II), Ni(II), Zn(II) (for ligands **L**², **L**³, **L**⁴), and Co(II) ions (for ligand **L**²).

Finally the potential of such chelates for ^{99m}Tc imaging of mice will be presented for ligands **L**¹ and **L**² and for a **L**^{*} labeled monoclonal antibody directed toward breast cancer.

Results and discussion

Synthesis of the ligands

Scheme 2 presents the different synthetic protocols used for the synthesis of the ligands **L**² to **L**⁴. **L**² was obtained according to two different routes. The first one started from bromination of the commercially available 2,6-dihydroxymethylpyridine **1** with PBr₃ at 0 °C, affording 2,6-dibromomethylpyridine **2** (84–95%), which is then converted into 2,6-bis(ethylaminomethyl)pyridine **3**¹⁹ in 23% yield. Phosphorylation of **3** in the presence of diethyl phosphite and 37% aqueous formaldehyde afforded the bis-phosphonated compound **4** in 69% yield. Subsequent hydrolysis of the phosphonate groups was performed using an excess of TMSBr, followed by methanolysis, affording ligand **L**² in 83% yield.

However, due to the difficulties encountered in the purification of compound **3**, a second approach was elaborated for the synthesis of **L**². This protocol started with the preparation of diethyl(ethylamino)methanephosphonate **7** from ethylben-

zylamine **5** in two steps (phosphorylation and catalytic hydrolysis) and with an overall 81% yield. N-alkylation reaction of **2** with **7** resulted in the formation of the bis-phosphonated compound **4** (24%) which led to ligand **L**² after deprotection of the phosphonate groups using an excess of TMSBr, followed by methanolysis (83%).

The synthesis of ligand **L**³ was based on a mono N-alkylation of **2** with tetra-ethyl iminobis(methanephosphonate)²⁰ to give the bis-diethylester phosphonated intermediate **8** in 58% yield. Subsequent N-alkylation of **8** with diethyl(ethylamino)methylphosphonate **7** in acetonitrile afforded the tris-phosphonated precursor **9** (49%). Hydrolysis of phosphonate esters to their corresponding acids in the presence of an excess of TMSBr in CH₂Cl₂ gave, after methanolysis, the ligand **L**³ in a 90% yield.

Finally, the formation of ligand **L**⁴ was started by the phosphorylation of 2,6-dibromomethylpyridine **2** with triethylphosphite to yield intermediate **10** (48%) which, after N-alkylation reaction with tetra-ethyl iminobis(methanephosphonate),²⁰ gave the tris-phosphonated derivative **11** in 32% yield. Hydrolysis of phosphonate groups using 6 M HCl afforded the ligand **L**⁴ in 46% yield.

Coordination chemistry of ligands **L**¹–**L**⁴

Protonated species of ligands **L¹–**L**⁴.** **L**¹ to **L**⁴ constitute a homogeneous series of chelators with varying numbers of terminal PO_3^{2-} phosphonate functions ranging from 2 to 4. If **L**⁴ is closely related to **L**³ (both ligands contain three phosphonated PO_3^{2-} functions), it markedly differs from **L**³ with one methylamino-*N,N*-bis(methanephosphonate) substituent being replaced by an ethylphosphonate moiety, $-\text{CH}_2\text{PO}_3^{2-}$.

Detailed interpretation of physico-chemical data on the metallic complexes (*e.g.* Cu(II), Ni(II), Zn(II), Co(II)) first requires the complete characterization of the ligand protonation diagram, at least in the pH range in which these data have been collected and also with respect to the targeted application (*i.e.* PET and SPECT imaging). We have therefore investi-

Scheme 2 Synthetic protocols for the preparation of ligands $\mathbf{L}^2$ to $\mathbf{L}^4$. (i) PBr_3 , 0°C , DMF, 84–95%. (ii) EtNH_2 , MeCN, reflux to r.t., 23%. (iii) $\text{HPO}(\text{OEt})_2$, 0°C , $(\text{CH}_2\text{O})_n$ (37% in H_2O), r.t. to reflux for $\mathbf{4}$, 69%; 0°C to reflux for $\mathbf{6}$, 83%. (iv) TMSBr , CH_2Cl_2 , r.t., then MeOH, 83% for $\mathbf{L}^2$; 90% for $\mathbf{L}^3$. (v) H_2 , Pd/C, EtOH, r.t., 98%. (vi) K_2CO_3 , MeCN, 50°C , 24%. (vii) $\text{HN}(\text{CH}_2\text{PO}(\text{OEt})_2)_2$, K_2CO_3 , MeCN, 70°C , 58% for $\mathbf{9}$; 32% for $\mathbf{12}$. (viii) $\mathbf{7}$, K_2CO_3 , MeCN, 70°C , 49%. (ix) $\text{P}(\text{OEt})_3$, 135°C , 48%. (x) 6M HCl, reflux, 46%.

gated the acido-basic properties of ligands $\mathbf{L}^1$ – $\mathbf{L}^4$ in the pH range 2.0–11.5 by various techniques. First, potentiometric titrations of ~ 2 mM solutions of $\mathbf{L}^1$ – $\mathbf{L}^4$ followed by statistical processing of the data^{21,22} first yielded six, four, five and four protonation constants, respectively (Table 1). Importantly, some of the protonation constants were difficult to determine with a good accuracy in view of their extremely low values ($\log K^H \ll 2$).^{23,24} A typical example of potentiometric titrations with the corresponding speciation diagram obtained after processing the data is reported for $\mathbf{L}^2$ in Fig. 1.

The most basic protonation constants (K_{LH} and K_{LH_2} for $\mathbf{L}^1$ – $\mathbf{L}^3$ and K_{LH} for $\mathbf{L}^4$) can be attributed to the tertiary amines, in agreement with data available in the literature for related polyaminomethanephosphonate analogues.^{25–31} The following protonation constants are associated with the first protonation constants of each of the phosphonate $-\text{PO}_3^{2-}$ units (i.e. $\text{RPO}_3^{2-} + \text{H}^+ \leftrightarrow \text{RPO}_3\text{H}^-$), while the logarithmic values of the second protonation constants of the protonated phosphonate groups ($\text{RPO}_3\text{H}^- + \text{H}^+ \leftrightarrow \text{RPO}_3\text{H}_2$) as well as the pK_a of the pyridine

Table 1 Protonation constants (logarithmic K_n^H values) of ligands $\mathbf{L}^1$ – $\mathbf{L}^4$ in water^a

Equilibrium	$\log K_n^H(3\sigma)$			
	$\mathbf{L}^1$ ^{b,c}	$\mathbf{L}^2$ ^b	$\mathbf{L}^3$ ^b	$\mathbf{L}^4$ ^b
$\mathbf{L}^{n-} + \text{H}^+ \xrightleftharpoons{K_1^H} \mathbf{LH}^{1-m}$	11.21(2) ^d 11.1(2) ^e	10.46(2) ^d	11.04(1) ^d	11.8(2) ^d
$\mathbf{LH}^{1-n} + \text{H}^+ \xrightleftharpoons{K_2^H} \mathbf{LH}_2^{2-m}$	10.29(2) ^d 10.71(3) ^e	10.07(3) ^d	10.4(1) ^d 10.8(4) ^e	8.3(3) ^d 7.9(3) ^e
$\mathbf{LH}_2^{2-n} + \text{H}^+ \xrightleftharpoons{K_3^H} \mathbf{LH}_3^{3-m}$	8.04(4) ^d 8.5(2) ^e	6.56(4) ^d	6.9(2) ^d 6.8(2) ^e	6.5(5) ^d 6.8(2) ^e
$\mathbf{LH}_3^{3-n} + \text{H}^+ \xrightleftharpoons{K_4^H} \mathbf{LH}_4^{4-m}$	6.49(6) ^d 7.0(3) ^e	4.52(5) ^d	5.7(3) ^d	5.1(6) ^d 5.37(4) ^e
$\mathbf{LH}_4^{4-n} + \text{H}^+ \xrightleftharpoons{K_5^H} \mathbf{LH}_5^{5-m}$	5.53(8) ^d	—	4.5(3) ^d	—
$\mathbf{LH}_5^{5-n} + \text{H}^+ \xrightleftharpoons{K_6^H} \mathbf{LH}_6^{6-m}$	4.19(9) ^d	—	—	—

^a Solvent: H_2O ; $I = 0.1$ M (NaClO_4); $T = 25.0 \pm 0.2^\circ\text{C}$. ^b $m = 8, 4$ and 6 respectively for $\mathbf{L}^1$, for $\mathbf{L}^2$ and for $\mathbf{L}^3$ and $\mathbf{L}^4$. ^c According to ref 11. ^d Potentiometry. ^e Absorption spectroscopy vs. pH. $K_n^H = [\text{LH}_n^{n-}]/[\text{H}^+][\text{LH}_n^{(n-1)-}]$. The uncertainties (3σ) in the $\log K^H$ values correspond to the added standard deviations (σ) in the cumulative constants. All other protonation constants have been assumed to be <2 .

Fig. 1 (a) Potentiometric titration curve of ligand L^2 and (b) distribution diagrams of the protonated species of the free ligand L^2 . Solvent: water; $I = 0.1$ M (NaClO_4); $T = 25.0(2)^\circ\text{C}$; $[L^2]_{\text{tot}} = 2.44 \times 10^{-3}$ M; $2.47 < \text{pH} < 11.85$.

Fig. 2 (a) Absorption spectrophotometric titration of L^3 as a function of pH and (b) variation of the absorbance at 265 nm vs. pH. Solvent: H_2O ; $T = 25.0(2)^\circ\text{C}$; $I = 0.1$ M (NaClO_4); $l = 1$ cm; $[L^3]_{\text{tot}} = 2.29 \times 10^{-4}$ M.

were estimated to be $\ll 2$. If the average basicity of the phosphonate groups does not significantly vary along the L^1 – L^3 series within experimental errors ($\log K_{\text{av}}^{\text{H}} = 6.06$ for L^1 , $\log K_{\text{av}}^{\text{H}} = 5.51$ for L^2 and $\log K_{\text{av}}^{\text{H}} = 5.7$ for L^3), it is increased for L^4 ($\log K_{\text{av}}^{\text{H}} = 6.63$ for L^4) which is lacking one secondary amine. This feature reflects inter-arm hydrogen bond interactions that might exist between the protonated ammonium of one methyl-amino-*N,N*-bis(methanephosphonate) moiety and the amine of the adjacent one.

To assess the spectrophotometric properties of the polyaminophosphonate ligands L^1 – L^4 and of their protonated species and to confirm the $\text{p}K_{\text{a}}$ values determined by the pHmetric method, we also performed spectrophotometric titrations ($200 \text{ nm} < \lambda < 500 \text{ nm}$) of the free ligands as a function of pH. As a typical example, the case of L^3 is presented in Fig. 2. Regardless of the nature of the ligand, the pyridine constitutes the main chromophore unit and a structured absorption band centered at ~ 260 – 270 nm ($\epsilon \sim 3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), which can be ascribed to its π – π^* transitions,³² was observed. Even though the pyridine nitrogen cannot be protonated in the pH range considered, marked spectral variations (hyperchromic shifts) were measured which can be explained by non-covalent interactions (hydrogen bonding) between the protonated side arms

and the pyridine ionizable site. Generally speaking, the most important variations were observed when the secondary amines are protonated (*i.e.* a five membered ring can be formed upon protonation of the amine and subsequent hydrogen bonding with N_{pyr}). The statistical processing^{33–35} of the absorption and pH data allowed us to substantiate some of the protonation constants which were previously determined by the potentiometric method (Table 1). These protolytic data confirm that L^1 – L^4 constitute intricate systems in terms of protolysis and strongly suggest the occurrence of hydrogen bond networks between the pyridinyl unit and the side arms of the ligands.

Characterization of the cupric complexes with L^1 – L^4 . The stoichiometries of the cupric complexes formed with ligands L^1 – L^4 have been assessed by electrospray mass spectrometry (ESI-MS) in the positive mode. ESI-MS mass spectra were recorded for aqueous solutions containing equimolar concentrations of Cu(II) and the ligand (see Fig. 3 for L^1). Regardless of the nature of the ligand, monocupric monochelates were systematically detected which strongly suggests that the ligands considered in this work display appropriate denticity and pre-organization to fulfill the stereochemical requirements of the Cu(II) ion. The ionization of the cupric complexes was

Fig. 3 ESI mass spectra of the cupric complexes formed with ligand L^1 . Solvent: H_2O ; positive mode. (a) $[Cu] = [L^1]_{tot} = 4.10 \times 10^{-4}$ M.

mainly obtained by the addition of protons and sodium ions. Similar results were obtained with $Zn(II)$ and $Ni(II)$. The pseudo-molecular ions of the different species observed are collected in Table S1 (ESI †) and are in good agreement with the simulated mono-isotopic masses.

Stability of the $Cu(II)$ complexes with L^1 – L^4 . Similarly to the free ligands, potentiometric (Fig. S1, ESI †) and absorption vs. pH (Fig. 4) titrations have been carried out to evaluate the stability constants of the cupric complexes with ligands L^1 – L^4 . Fig. S1 (ESI †) first displays the potentiometric data of aqueous solutions containing nearly equimolar concentrations of $Cu(II)$ and of the ligand. These pHmetric data have been statistically processed²¹ and the corresponding stability and protonation constants of the $Cu(II)$ complexes are shown in Table 2. Similarly to most of the polyaminomethanephosphonated chelators described so far, ligands L^1 – L^4 basically form monocupric monochelates. This binding feature is confirmed by the mass spectrometric data conducted on L^1 – L^4 series (Table S1, ESI †). On the other hand, L^1 – L^4 form $Cu(II)$ complexes whose high stability constants are comparable, for instance, to EDTPA ($\log K_{CuEDTPA} = 23.2$)^{36,37} and are much higher than that of *N*-methylamino-*N,N*-bis(methylenephosphonic acid)^{38,39} which constitutes the side arm model of L^1 , L^3 and L^4 . Furthermore, the substitution of the carboxylate functions of the (2,6-bis[bis((carboxymethyl)amino)methyl]pyridine)²⁵ chelator ($\log K_{CuL} = 15.69$) by phosphonic units (L^1) stabilizes considerably the corresponding cupric complexes. Interestingly, L^1 forms more stable $Cu(II)$ complexes ($\log K_{CuL^1} = 22.71(7)$) than its closely related analogues L^2 – L^4 . Indeed, the lack of one (L^3 , L^4) or two (L^2) phosphonic units is reflected by a destabilization of the $Cu(II)$ complexes of about 3 orders of magnitude ($\log K_{CuL^2} = 19.5(9)$, $\log K_{CuL^3} = 19.1(3)$, $\log K_{CuL^4} = 19.3(6)$). These data clearly demonstrate the importance of the four terminal phosphonate units of L^1 for the binding strength with $Cu(II)$.

Regarding the protonation properties of the cupric complexes with L^1 – L^4 , it is noteworthy that the CuL^1 species is able to be successively protonated four times under our experi-

Fig. 4 Absorption vs. pH titrations of the cupric complexes formed with (a) L^2 and (b) L^3 . Solvent: H_2O ; $I = 0.1$ M ($NaClO_4$); $T = 25.0(2)$ °C; $l = 1$ cm. (a) $[L^2]_{tot} = 1.39 \times 10^{-4}$ M; $[Cu(II)]_{tot} = 1.30 \times 10^{-4}$ M; (1) pH = 4.15; (2) pH = 9.19. (b) $[L^3]_{tot} = [Cu(II)]_{tot} = 1.78 \times 10^{-4}$ M; (1) pH = 2.49; (2) pH = 11.19.

Table 2 Stability and protonation constants of the cupric complexes formed with ligands L^1 – L^4 ^a

Equilibrium	$\log K_{CuLHx} (3\sigma)$			
	L^1	L^2	L^3	L^4
$L + Cu \rightleftharpoons CuL$	22.71(9) ^b	19.50(9) ^b	19.1(3) ^b	19.3(6) ^b
$CuL + H \rightleftharpoons CuLH$	8.24(8) ^b	6.30(1) ^b	7.8(3) ^b	6.1(7) ^b
$CuLH + H \rightleftharpoons CuLH_2$	8.20(6) ^c	7.15(9) ^b	7.7(5) ^c	8.5(7) ^c
$CuLH_2 + H \rightleftharpoons CuLH_3$	6.9(5) ^c	4.71(9) ^b	5.8(3) ^b	4.9(8) ^b
$CuLH_3 + H \rightleftharpoons CuLH_4$	5.7(1) ^b	—	4.3(3) ^b	—
$CuLH_4 + H \rightleftharpoons CuLH_5$	4.0(2) ^b	—	—	—

^a Solvent: H_2O ; $I = 0.1$ M; $T = 25.0(2)$ °C]. The uncertainties (3σ) in the $\log K_{CuLHx}$ values correspond to the added standard deviations (σ) in the cumulative constants. $K_{CuLHx} = [CuLH_x]/[CuLH_{(n-1)}][H]$. Charges have been omitted for the sake of clarity. ^b Potentiometry. ^c Absorption vs. pH.

mental conditions, while CuL^3 can lead to three protonated species. Only two protonations were measured for the $Cu(II)$ complexes with both L^2 and L^4 (Table 2). The protonation sites of the cupric complexes most likely correspond to phospho-

nate units, which implies that the secondary amines remain firmly bound to the Cu(II) cation whatever the pH considered in this study. The involvement of the pyridinyl nitrogen cannot be excluded as well since the stability constant of CuL¹ is by far much higher than that of the Cu(II) complex of a closely related analogue for which the pyridine ring has been substituted by a phenyl one ($\log K_{\text{CuL}} = 15.3$).⁴⁰ Other linear or cyclic analogues also bearing a pyridine unit displayed the same binding characteristic.^{41,42}

Lastly, if significant differences can be measured for the phosphonates pK_as in the free ligands and in the corresponding complexes, the average phosphonate basicities measured for L¹–L³ are almost unaffected by the Cu(II) complexation (L¹ $\log K_{\text{av}}^{\text{H}} = 6.06$, CuL¹ $\log K_{\text{av}}^{\text{H}} = 6.27$; L² $\log K_{\text{av}}^{\text{H}} = 5.51$, CuL² $\log K_{\text{av}}^{\text{H}} = 5.51$, L³ $\log K_{\text{moy}}^{\text{H}} = 5.7$, CuL³ $\log K_{\text{av}}^{\text{H}} = 5.96$). This feature is in excellent agreement with the observations made for instance for EDTPA^{36,37} (EDTPA $\log K_{\text{av}}^{\text{H}} = 5.59$, CuEDTPA $\log K_{\text{CuL}}^{\text{H}} = 5.57$). L⁴ stands in interesting contrast since it is lacking a secondary amine unit for Cu(II) binding. It is then proposed that a phosphonate unit is firmly bound to Cu(II), thus influencing both the number of Cu(II) protonated complexes and the average protonation constant of the remaining phosphonates (L⁴ $\log K_{\text{av}}^{\text{H}} = 6.63$, CuL⁴ $\log K_{\text{av}}^{\text{H}} = 5.5$). The distribution diagrams of the cupric complexes as a function of pH were calculated and are available in the ESI† (Fig. S2).

Fig. 4 portrays the absorption spectral variation induced by Cu(II) complexation as a function of the pH of the solution containing L² and L³ (Fig. S6–S9, ESI†). In addition to the pyridine-centered π – π^* transitions at ~260 nm, the cupric complexes with polyaminophosphonated ligands display informative spectroscopic probes such as intense N→Cu(II) charge transfer absorptions^{43,44} as well as weaker Cu(II)-centered d–d transitions.^{45,46} These absorptions allowed monitoring several key events such as the Cu(II) complexation by L¹–L⁴ and the protonation of the cupric complexes and provided information on the coordination geometry of the metal. L¹–L³ are characterized by intense N→Cu(II) CT absorptions at about 300–310 nm which clearly indicate that the two secondary amines are strongly involved in the complexation of the metallic ion whatever be the pH considered (2.5–11). Subsequently, L⁴, which is lacking one secondary amine, displays a much weaker MLCT absorption. For CuL¹ (4 phosphonates) and CuL³ (3 phosphonates), the rise in the pH induces a bathochromic shift of about 40–50 nm of the N→Cu(II) CT transitions as well as the d–d absorption bands, while very weak variations were observed for cupric complexes with L² (2 phosphonates) and L⁴ (3 phosphonates, only one secondary N). This shift is probably associated with the weakening of one to two Cu–N bonds, as observed for the thiophene containing phosphonate ligand.⁴⁷ Above pH 9, no spectral variation was observed for each of the systems. Regardless of the system considered, the binding of Cu(II) weakly influences the pyridine π – π^* transitions. The significant spectral variations experienced by the tetra- (L¹) and tris-phosphonated L³ ligands therefore likely reflect structural and geometrical modifications of the Cu(II) coordination sphere influenced by the deprotonation

of the phosphonic units. The statistical processing^{33–35} of the spectral and pHmetric data sets allowed determining the protonation constants of the Cu(II) complexes, which were found to be in good agreement with those previously measured by the potentiometric method (Table 2).

Selectivity of Cu(II) complexation toward other metal ions: the case of Ni(II), Zn(II) and Co(II). Using potentiometry, it was possible to accurately determine the stability constants (Table 3) of the metallic complexes with the Zn(II), Ni(II) and Co(II) cations with ligands L¹–L⁴. Similarly to Cu(II), protonated metal complexes were evidenced and their corresponding protonation constants are depicted in the ESI† (Table S2 and Fig. S2 to S5). These data were compared to those available in the literature for reference chelators such as the L¹ analogue bearing terminal carboxylates (denoted L¹_C in Table 3) and DO2P (1,4,7,10-tetraazacyclododecane-1,7-bis(methanephosphonate)), which constitutes a golden standard and is one of the most effective Cu(II) chelators described so far.

The complexation of Cu(II) by ligands L¹–L⁴ is selective compared to that of Zn(II), Ni(II) or Co(II). This binding selectivity also exists for other polyaminomethanephosphonate ligands such as DO2P.¹² The sequence of LM stability constants (Table 3) is indeed in agreement with the Irving–Williams series which defines that the classical thermodynamic order for transition-metal complexes is Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II).^{48–50}

For Cu(II), the presence of four phosphonate units clearly stabilizes the Cu(II) complexes, while small differences are observed for the analogous ligands L²–L³. L¹ is the most appropriate system to stabilize Cu(II) in a distorted square planar pyramidal geometry. Due to its versatility, the presence of a square planar geometry cannot be excluded under acidic conditions where the phosphonate units are protonated. Indeed, L¹ forms stable complexes for pH < 4 and competes with DO2P under these experimental conditions. By contrast, L³ and L⁴, both bearing three phosphonate groups, form with Ni(II) the most stable complexes. This would indicate that these two ligands better fulfill the stereochemical preferences of Ni(II) (octahedral geometry) with respect to L¹ or L². L¹ is seemingly

Table 3 Stability constants^a of metallic complexes (Cu(II), Zn(II), Ni(II) and Co(II)) formed with ligands L¹–L⁴ and corresponding pM^b values

$\log K_{\text{ML}}(3\sigma)^a$ pM (pH 7.4) ^b	L ¹	L ²	L ³	L ⁴	L ¹ _C	DO2P
$\log K_{\text{CuL}}$	22.71(7)	19.50(9)	19.1(3)	19.3(6)	15.69	28.7
pCu ^{II}	15.46	12.89	12.08	13.06	12.07	17.77 ^c
$\log K_{\text{NiL}}$	16.50(3)	14.34(6)	18.2(2)	18.1(4)	—	—
pNi ^{II}	11.79	9.54	13.08	13.65	—	—
$\log K_{\text{ZnL}}$	17.84(4)	11.99(6)	17.2(2)	12.1(3)	15.84	21.2
pZn ^{II}	12.37	8.93	11.89	8.44	13.68	11.73
$\log K_{\text{CoL}}$	16.50(9)	13.9(1)	nd	nd	—	—
pCo ^{II}	11.22	8.86	—	—	—	—

^a $\text{L} + \text{M} \rightleftharpoons \text{ML}$, $K_{\text{ML}} = [\text{ML}]/[\text{M}][\text{L}]$. ^b pM = $-\log[\text{M}]_{\text{free}}$ calculated for $[\text{M}]_{\text{tot}} = 10^{-6}$ M and $[\text{L}]_{\text{tot}} = 10^{-5}$ M at pH 7.4. Solvent: H₂O; $I = 0.1$ M; $T = 25.0$ °C. ^c pM values calculated without the protonation constants of the metal complexes which have not been reported. nd = not determined.

not an ideal binding platform for metal ions of strict coordination geometry such as Ni(II). The main differences between Cu(II) and Ni(II)/Zn(II) can therefore be explained by the stereochemical preferences of these two cations.

To further discuss the complexation properties of L^1 – L^4 , we calculated the pM values⁵¹ for each of these systems (Table 3). Fig. 5a depicts the pM values calculated for L^1 with Cu(II), Zn(II), Ni(II) and Co(II) and demonstrates the high selectivity of L^1 for Cu(II) over a wide range of pH (2–12) (for L^2 – L^4 , see Fig. S12 to S14, ESI†). The same feature is observed for ligands L^2 – L^4 . On the other hand, Fig. 5b illustrates the pCu^{II} values of L^1 – L^4 compared to those of DO2P and EDTPA chosen as references in this study. Ligands L^1 – L^4 display good affinity for Cu(II) and the pCu^{II} values systematically increases with the pH of the solution until pH ~ 7–8 (competition under basic conditions with hydrolysis of the metals). The stability sequence at physiological pH is as follows: DO2P > L^1 > EDTPA- L^4 > L^2 > L^3 . Interestingly, L^1 is among the most powerful Cu(II) chelator under acidic conditions and competes with DO2P which is inefficient at pH < 4. In the pH range from 6 to 12, DO2P can be considered as the most efficient Cu(II) chelator with respect to L^1 – L^4 . This study demonstrates that the presence of at least three phosphonated binding units is required to firmly bind

Cu(II) above pH 7. Increasing the number of phosphonated coordinating groups clearly favors the Cu(II) complexation under acidic conditions, which is of fundamental importance when anticipating PET imaging on cancer cells for instance.

Electrochemical properties. The electrochemical properties of a potential ⁶⁴Cu chelate are among the most important physico-chemical parameters to be considered for PET imaging applications. *In vivo* reduction of the cupric complex by biological reducing agents might indeed lead to unstable and extremely labile copper(I) species. Upon reduction, the ⁶⁴Cu(I) cation may undergo dissociation (by solvolysis, acidolysis or ligand exchange processes) and the radioactive cation could then be widespread in the body with subsequent loss of the selectivity with respect to the target to be imaged and appearance of adverse health effects. It was then of importance to establish the electrochemical properties of the Cu(II) chelate with L^1 – L^4 and to assess if they might be subjected to easy reduction by biological reducing agents (*i.e.* $E_{1/2}$ should be ≥ -0.4 V per NHE). Cyclic voltamperograms (CV) were recorded in water (0.1 M NaClO₄) at different pH values ($3.7 \leq \text{pH} \leq 11$, Fig. S10 and S11, ESI†). Fig. S10 (ESI†) displays the CVs measured for ~mM solutions of CuL^{*i*} (*i* = 1–4) at pH 3.7. Under these experimental conditions, the less stable cupric complexes mainly co-exist under negatively multicharged states ([CuL¹H₃]^{3–}/[CuL¹H₄]^{2–}; [CuL²H][–]/[CuL²H₂]⁰; [CuL³H₂]^{2–}/[CuL³H₃][–]; [CuL⁴H₂]^{2–}). For the sake of clarity, the CV of a mM solution of Cu(ClO₄)₂ has also been recorded. Whatever the system considered, we systematically observed the presence of two irreversible cathodic signals as well as a so-called anodic stripping peak (at ~0.1 V and caused by redissolution of metallic copper) that is a clear signature of the formation of Cu⁰.⁵² The presence of Cu⁰ was further assessed by the formation of a thin orange deposit on the surface of the glassy carbon electrode. For L^3 and L^4 , an additional cathodic peak at –0.12 V seemingly corresponds to reduction of free copper(II). The two cathodic peaks (Table 4) may be ascribed to the Cu²⁺L^{*i*} → Cu⁺L^{*i*} and Cu⁺L^{*i*} → Cu⁰ + L^{*i*} (*i* = 1–4) reduction steps, respectively. The presence of the redissolution peak at ~0.1 V indeed suggests that the electrogenerated Cu(I) and Cu(0) complexes derived from ligands L^1 – L^4 are unstable and dissociate. This feature is confirmed by the irreversibility of the two cathodic peaks whose intensity and potential values are markedly influenced by the potential scanning rate.^{53,54} In addition, these electrochemical processes are not governed by diffusion. Taken together, these data support a chemical reaction (dissociation of the cuprous complexes, denoted C) that precedes an electron transfer (reduction of the cupric complexes, denoted E, EC mechanism).⁵⁵

When the pH was raised (pH > 5.6), the CV pattern is markedly simplified for all systems considered with the formation of a quasi-reversible system whose redox potential is shifted at more positive values. The quasi-reversibility is supported by the i_{pa}/i_{pc} intensity ratios which are close to 1 and by the ΔE values (Table 5). The quasi-reversibility persists even after several scans whatever the potential scanning rate, meaning that the electrogenerated Cu(I) complexes are stable enough on

Fig. 5 (a) Variation of the pM values ($M = \text{Cu}^{\text{II}}$, Zn^{II} , Ni^{II} and Co^{II}) as a function of pH for ligand L^1 and (b) variation of the pCu^{II} values for L^1 – L^4 compared to those of DO2P and EDTPA. Solvent: H₂O; $I = 0.1$ M; $T = 25.0(2)^\circ\text{C}$; $\text{pM} = -\log[M^{\text{II}}]_{\text{free}}$ or $-\log[M^{\text{II}}]_{\text{free}}$, $[L]_{\text{tot}} = 10^{-5}$ M and $[M^{\text{II}}]_{\text{tot}}$ or $[M^{\text{II}}]_{\text{tot}} = 10^{-6}$ M.

Table 4 Electrochemical parameters measured for the Cu(II) complexes with L¹–L⁴ in water at pH 3.7

Complex	1 st reduction peak (Pc1)		2 nd reduction peak (Pc2)		Cu ⁰ redissolution peak	
	<i>E</i> _{pc1} (V per SCE)	<i>i</i> _{pc1} (μA)	<i>E</i> _{pc2} (V per SCE)	<i>i</i> _{pc2} (μA)	<i>I</i> _{re} (μA)	<i>E</i> _{re} (V per SCE)
CuL ¹	−0.51	−66	−1.1	−286	144	0.17
CuL ²	−0.26	−45	−1.0	−136	121	0.12
CuL ³	−0.39	−112	−1.13	−389	326	0.19
CuL ⁴	−0.55	−206	−1.06	−213	165	0.12

Solvent: H₂O; *I* = 0.1 M (NaClO₄); *T* = 25.0(2) °C; *ν* = 200 mV s^{−1}. Reference = Ag/AgCl.

the CV timescale. These features are in good agreement with the stronger stabilities of the cuprous complexes which prevent Cu(II) and Cu(I) dissociation and thereby Cu⁰ formation (the anodic redissolution peak is suppressed under basic conditions).

To further assess the electrochemical stability of the cupric complexes with L¹–L⁴, we tested the resistance of these complexes toward biological reductants such as ascorbic acid (DHA/Asc; *E*⁰ = +0.054 V per NHE at pH 7.0; intracellular concentration from 10 000 to 20 000 μM) or NADPH (NAD(P)/NAD(P)H; *E*⁰ = −0.32 V per NHE at pH 7; intracellular concentration from 200 to 500 μM) at pH 7.4 (tris buffer). The N–Cu(II) CT absorptions constituted a valuable probe to evaluate the reductant-decomposition of the cupric complexes (Fig. 6). As expected, the CuL^{*i*} complexes (*i* = 1–4) are more stable with respect to ascorbic acid (*E*⁰ = +0.054 V per NHE) than to NAD(P)H (*E*⁰ = −0.32 V per NHE). On the other hand, CuL² and to a lesser extent CuL¹ appear to be the most resistant cupric complexes in the presence of NADPH, in agreement with their electrochemical properties (Fig. S10 and S11, ESI†).

Formation and dissociation mechanisms of the cupric complexes with L¹–L⁴

Formation kinetics. If a high thermodynamic stability of the Cu(II) complex with a ligand is a prerequisite for its use in ⁶⁴Cu PET imaging, it may also be associated with very slow kinetics for the formation of the complex, as observed for some cross bridged cyclen derivatives,⁸ and a fast complexation may be preferred to avoid a necessary heating step. The

Table 5 Electrochemical parameters measured for the Cu(II) complexes with L¹–L⁴ in water at pH > 10^a

Complex	<i>E</i> _{pc1} (V)	<i>E</i> _{pa1} (V)	<i>i</i> _{pa1} / <i>i</i> _{pc1}
CuL ¹	−0.5	0.01	0.83
CuL ²	−0.5	−0.26	0.54
CuL ³	−0.16	0.25	1.24
CuL ⁴	−0.07	0.29	0.94

^a Solvent: H₂O; *I* = 0.1 M (NaClO₄); *T* = 25.0(2) °C; *ν* = 200 mV s^{−1}. Reference = Ag/AgCl.

Fig. 6 Spectrophotometric titrations of CuL^{*i*} (*i* = 1–4) complexes with (a) ascorbic acid and (b) NADPH. Solvent: aqueous Tris-HCl (0.05 M) at pH 7.4, [CuL^{*i*}]_{tot} = 3 × 10^{−4} M; *l* = 1 cm; *T* = 25.0(2) °C.

formation kinetic studies of the Cu(II) complexes with L¹–L⁴ were first carried out using stopped flow spectrophotometry at pH ~ 2. The complexes [CuL¹H₄]^{2−}, [CuL²H₂]⁰, [CuL³H₃][−], and [CuL⁴H₂]^{2−} are the major species at pH 2.0, while Cu²⁺ is the predominant free copper(II) species under these experimental conditions of acidity (*K*_{Cu(OH)+} = 10^{−6.29} and *K*_{Cu(OH)2} = 10^{−13.1}).⁵⁶ We have chosen to monitor the formation kinetics under pseudo-first order conditions ([Cu²⁺]_{tot} > 10 × [L]_{tot}) at λ > 300 nm (*i.e.* N→Cu CT absorptions), which corresponds to the largest spectrophotometric amplitudes between the absorptions of the reactants (Cu²⁺ + L¹–L⁴) and of the product CuL^{*i*} (*i* = 1–4). Whatever the system considered, the absorbance always experienced an exponential growth *versus* time indicating a first order kinetics (*ν* = −d[L]/dt = *k*_{obs}[L]) with respect to the ligand. The kinetic recordings revealed single rate limiting steps in the millisecond to second time spans and, importantly, spectral amplitudes were lost during the mixing time (~3 ms) of the stopped-flow device. The absorbances at the end of the rate limiting steps satisfactorily correspond to the expected values for the CuL^{*i*} complexes at pH ~ 2, signifying the absence of any slowest steps.

Formation kinetics of metal complexes and, in this particular case, of copper(II) complexes^{44,57,58} with a wide variety of

ligands showed that these metal complexation processes usually follow a dissociative Eigen–Wilkins mechanism:^{59,60}

The rate limiting step (k_{ex}) corresponds to the desolvation of the metal in an outer-sphere complex formed in a fast pre-equilibrium (K_{os}):

$$k_f = K_{os} \times k_{ex} \text{ with } k_f \text{ (M}^{-1} \text{ s}^{-1}\text{)}, K_{os} \text{ (M}^{-1}\text{)} \text{ and } k_{ex} \text{ (s}^{-1}\text{)} \quad (2)$$

Estimating the K_{os} ⁶¹ values for the free ligands at pH ~ 2 and Cu^{2+} and assuming the reported k_{ex} value^{62–64} ($2 \times 10^8 \text{ s}^{-1}$ in water at 25 °C) for the desolvation of the Cu^{2+} cation, we evaluated the theoretical k_f values (Table 6).

The large values of k_f agree well with the loss of spectral amplitude during the mixing time of the stopped flow apparatus and suggest that the first steps likely correspond to the formation of kinetic intermediates. The pseudo-first order rate constants k_{obs} (s^{-1}) of the rate-limiting step were determined for various Cu^{2+} concentrations (Fig. 7). With the exception of L^4 , the ordinates at the origin were found to be close to zero. Under large excess of $Cu(II)$, the formation of the cupric complexes is indeed favored with respect to the dissociation pathway, thus precluding reliable measurement of the dissociation kinetic k_{CuL}^{-1} parameter. For L^1 (4 phosphonate units) and L^3 (3 phosphonate units), the dependence of k_{obs}

on $[Cu^{2+}]_{tot}$ therefore satisfactorily obeys eqn (3), while a linear dependence was observed for L^2 . Strikingly, no variation of the k_{obs} values with $[Cu^{2+}]_{tot}$ was observed for L^4 :

$$k_{obs} = \frac{k_{CuL} K_{[CuL]^*} [Cu^{2+}]_{tot}}{1 + K_{[CuL]^*} [Cu^{2+}]_{tot}} \quad (3)$$

These features can be rationalized by considering the first step (lost during the mixing time) as a very fast pre-equilibrium (formation of a kinetic $Cu(II)$ intermediate $[CuL]^*$) followed by a rate limiting rearrangement (formation of the final complex CuL) as described by eqn (4) and (5) (*i.e.* as described above, the monomolecular rate constant could not be accurately determined under these experimental conditions):

The kinetic behaviors for L^2 and L^4 correspond to extreme situations depicted in eqn (6) and (7) that intimately depend on the very low (L^2) or very high (L^4) stabilities of the kinetic intermediate $[CuL]^*$, respectively.

$$\text{For } L^4: \text{ if } K_{[CuL]^*} \times [Cu^{2+}]_{tot} \gg 1 \text{ then } k_{obs} = k_{[CuL]} \quad (6)$$

$$\text{For } L^2: \text{ if } K_{[CuL]^*} \times [Cu^{2+}]_{tot} \ll 1 \text{ then } k_{obs} = k_{[CuL]} K_{[CuL]^*} [Cu^{2+}]_{tot} \quad (7)$$

In the case of an intermediate complex such as $[CuL^4]^*$, whose structure is close to the product, the k_{obs} values are independent of $[Cu^{2+}]_{tot}$. The ligand L^4 indeed displays three phosphonated units and only one secondary amine. The stronger implication of the binding units within the kinetic intermediate complex $[CuL^4]^*$ (*i.e.* the stability constant of the kinetic intermediate is expected to be close to that of the final product) would explain the difference with respect to $[CuL^1]^*$ ($\log K_{[CuL^1]^*} = 2.1(2)/k_{[CuL^1]} = 59(9) \text{ s}^{-1}$) and $[CuL^3]^*$ ($\log K_{[CuL^3]^*} = 1.5(2)/k_{[CuL^3]} = 220(40) \text{ s}^{-1}$) for which a saturation kinetics has been evidenced (*i.e.* a much larger difference between the kinetic intermediate and the final complex is anticipated). In the case of the L^2 system (bearing 2 phosphonate units), the much lower stabilities of the intermediate complexes $[CuL^2]^*$ compared to that of $[CuL^1]^*$ (4 phosphonate units) and $[CuL^3]^*$ (3 phosphonate units) enlighten their peculiar formation kinetics. Therefore, only the product $k_{[CuL]} \times K_{[CuL]^*}$ could be evaluated (Table 7). The forward ($k_{[CuL]}$) rate constants as well as the stability constants of the kinetic intermediates are collected in Table 7. The stability constants of $[CuL^2]^*$ and $[CuL^4]^*$ were not determined under our experimental conditions and their values have been estimated to be $>10^{1.5} \text{ M}^{-1}$ for $[CuL^2]^*$ and $\sim \log K_{[CuL^4]^*}$ for $[CuL^4]^*$.

Our kinetic data clearly demonstrated fast formation of kinetic $Cu(II)$ intermediates for L^1 – L^4 followed by a rate-limiting rearrangement to the final more stable cupric complexes. According to the Eigen–Wilkins principle (Table 6), the for-

Table 6 Estimated K_{os} , k_{ex} and k_f average values for ligands L^1 – L^4 in water at pH 2

Ligand	$K_{os} \text{ (M}^{-1}\text{)}$	$k_f \text{ (M}^{-1} \text{ s}^{-1}\text{)}$
$L^1H_6^{2-}$	1.14	2×10^8
L^2H_4	0.16	3.2×10^7
$L^3H_5^-$	0.43	8.6×10^7
$L^4H_4^{2-}$	1.14	2×10^8

Fig. 7 Variation of the pseudo-first order rate constants k_{obs} (s^{-1}) as a function of $[Cu^{2+}]_{tot}$ for the formation of the cupric complexes with L^1 – L^4 . Solvent: H_2O ; $T = 25.0(2) \text{ }^\circ\text{C}$; $l = 1 \text{ cm}$; $I = 0.1 \text{ M}$ ($NaClO_4$); $[L^1]_{tot} = 3.04 \times 10^{-4} \text{ M}$, $[L^2]_{tot} = 1.80 \times 10^{-4} \text{ M}$, $[L^3]_{tot} = 2.06 \times 10^{-4} \text{ M}$ and $[L^4]_{tot} = 1.63 \times 10^{-4} \text{ M}$.

Table 7 Kinetic and thermodynamic constants related to the formation of the cupric complexes with ligands L^1 – L^4 at pH 2^a

Complexes	$\log K_{[CuL]}^b$	$\log K_{[CuL]^*}$	$k_{[CuL]} (\pm\sigma)$ ($M^{-1} s^{-1}$)	$k_{[CuL]} \times K_{[CuL]^*}$ ($\pm\sigma$) ($\times 10^3 M^{-1} s^{-1}$)
CuL^1	6.04 ^b	2.1(2) ^c	59(9) ^c	7.43 ^d
CuL^2	2.29 ^b	$\ll 1.5^d$	$\gg 220^c$	3.32(5) ^c
CuL^3	2.46 ^b	1.5(2) ^c	220(40) ^c	6.96 ^d
CuL^4	2.6 ^b	$\sim \log K_{[CuL^4]}$	4.23(2) ^c	nd

^a Solvent: H₂O; $T = 25.0(2)^\circ C$; $l = 1$ cm; $I = 0.1$ M (NaClO₄). ^b Apparent stability constant recalculated from data available in Tables 1 and 2.

^c Measured values. ^d Estimated or calculated values.

mation of the cupric kinetic intermediates is extremely fast and agrees with the large amplitudes lost during the dead time of the stopped-flow apparatus (*ca.* 3 ms). With the exception of L^4 for which the stability constant of the kinetic intermediate is assumed to be close to that of the final cupric complex, we clearly demonstrated that the stability of the kinetic intermediate and the monomolecular rearrangement rate are intimately dependent on the number of phosphonate units. The stepwise increase of the number of phosphonates for L^1 – L^3 stabilizes the kinetic intermediate and slows down the rearrangement process. The $k_{[CuL]} \times K_{[CuL]^*}$ values are in the same order of magnitude which suggests that the CuL^i ($i = 1$ – 3) complexes are likely sharing similar rearrangement features (the final coordination of the nitrogen binding units).

Dissociation kinetics of the Cu(II) complexes. In addition to a high stability, ⁶⁴Cu chelators have to also display strong kinetic inertness with respect to demetallation in the main biological compartments (*e.g.* blood plasma at pH 7.35–7.45, bile at pH 7.4–7.7, *etc.*). This demetallation reaction can be even more favored in the more acidic compartments (gastric juices, pH 1.2–3) or by exchange processes with other specific endogenous Cu(II) chelators (*e.g.* Cu(II) transporters in the blood plasma and liver: metallothionein, ceruloplasmin, transcuprein or albumin).⁶⁵ For all these reasons, slow dissociation or exchange reaction is an essential property for a ⁶⁴Cu chelator in order to prevent adverse effects in the body and also deterioration in image quality. The proton-assisted dissociation kinetics of the cupric complexes formed with L^1 – L^4 has therefore been carried out using a stopped-flow absorption spectrophotometer and monitored at the maximum of the $N \rightarrow Cu(II)$ CT transitions.

The global decomplexation reactions, under acidic conditions, can be written as follows:

During the dissociation kinetics of the Cu(II) complexes, we observed a large amplitude loss of the LMCT absorbance during the dead time of the stopped flow device (~ 3 ms). This suggests the existence of very fast steps that are not accessible

under our experimental conditions. The LMCT absorbance was monitored *versus* time for several concentrations of perchloric acid in the range $0.02\text{ M} < [H^+] < 0.5\text{ M}$ for CuL^1 and $0.04\text{ M} < [H^+] < 1\text{ M}$ for CuL^i ($i = 2$ – 4). In all cases, the absorbance variation could be fitted with a single exponential function (Fig. 8a) and thereby demonstrates that the rate-limiting dissociation step is first order with respect to the cupric complexes. The corresponding pseudo first-order rate constants are reported in Fig. 8b. The dissociation kinetics of CuL^4 (*i.e.* L^4 lacks a secondary amine with respect to L^3) was too fast to be accurately measured under our experimental conditions.

The pseudo-first-order dissociation rate constants of the Cu(II) complexes linearly vary with $[H^+]$ with a non-zero intercept at the origin. These kinetic data can be described by the following relationship, with k_H ($M^{-1} s^{-1}$) being the bimolecular acidolysis rate constant and k_d (s^{-1}) the monomolecular solvolysis rate constant of the complexes with L^1 – L^4 (eqn (9); Table 8):

$$k_{\text{obs}} = k_H \times [H^+]_{\text{tot}} + k_d \quad (9)$$

Our results show a significant decrease in the $N \rightarrow Cu(II)$ CT absorbance that is in agreement with the fast dissociation of

Fig. 8 (a) Variation of the absorbance at 312 nm for the proton-assisted dissociation of CuL^1 . $[CuL^1]_{\text{tot}} = 7 \times 10^{-5}\text{ M}$; $[H^+]_{\text{tot}} = 5 \times 10^{-3}\text{ M}$. Initial pH = 10. (b) Variation of the pseudo-first order rate constants k_{obs} (s^{-1}) as a function of $[H^+]_{\text{tot}}$ for the proton-triggered dissociation of the cupric complexes with L^1 – L^3 . Solvent: H₂O; $T = 25.0(2)^\circ C$; $l = 1$ cm; $I = 0.1\text{ M}$ (NaClO₄); $[CuL^1]_{\text{tot}} = 7 \times 10^{-5}\text{ M}$, $[CuL^2]_{\text{tot}} = 5.67 \times 10^{-5}\text{ M}$ and $[CuL^3]_{\text{tot}} = 6.84 \times 10^{-5}\text{ M}$. The cupric complexes have been initially prepared at pH ~ 10 .

Table 8 Kinetic constants related to the proton-assisted dissociation of the cupric complexes with ligands L^1-L^3 ^a

Complex	$k_H (\times 10^2 \text{ M}^{-1} \text{ s}^{-1}) (\pm \sigma)$	$k_d (\text{s}^{-1}) (\pm \sigma)$
CuL^1	3.0(2)	81(4)
CuL^2	1.11(1)	0.3(2)
CuL^3	2.8(3)	45(14)

^a Solvent: H_2O ; $T = 25.0(2)^\circ\text{C}$; $I = 0.1 \text{ M}$ (NaClO_4).

the Cu–phosphonate bonds and suggests a rate-limiting step related to the protonation of the bound secondary amines and to the subsequent dissociation of the N–Cu coordination bonds. The large signal amplitude lost during the dead time of the stopped-flow apparatus indeed corresponds to successive fast protonation equilibria prior to the limiting dissociation step. As an example of the L^1 system, the protonated species $[\text{CuL}^1\text{H}_4]^{2-}$ (*i.e.* the 4 phosphonate units are protonated) might correspond to the kinetic intermediate originating from fast protonation steps. The absorption electronic spectra of the $[\text{CuL}^1\text{H}_2]^{4-}$, $[\text{CuL}^1\text{H}_3]^{3-}$ and $[\text{CuL}^1\text{H}_4]^{2-}$ species are similar and indicate that the involved protonations do not alter the chromophore (*i.e.* LMCT). As an example, the suggested mechanism is shown schematically in Fig. 9 for CuL^1 . The limiting step corresponds to the protonation of an amino function and to the concomitant cleavage of the corresponding N–Cu bond to afford the unstable intermediate I_1 which then dissociates very rapidly by the attack of another proton. It is noteworthy that the I_1 kinetic intermediate has a putative structure similar to that which can be anticipated for the CuL^4 system structure in excellent agreement with its very fast dissociation under very acidic conditions.

As far as acidolysis or solvolysis is concerned, the most inert system unexpectedly corresponds to the cupric complexes with L^2 that possesses fewer phosphonate units (Fig. 10). For the acidolysis pathway, increasing the number of phosphonate groups may assist the proton transfer to the nitrogen atoms (relay mechanism) and hence increase the dissociation rate k_H . The number of phosphonate units (Fig. 10) indeed strongly influences the kinetic inertness of the I_1 intermediate complex

that is subjected to a dissociation rate limiting step. For the solvolysis pathway, charge repulsion between the non-bound and negatively charged phosphonates as well as structural constraints applied to the cupric intermediate complexes may explain this peculiar kinetic behavior.

Labeling and characterization of MTn12 antibody with L^*

Tenascin-C (TNC) is a glycoprotein highly expressed during embryogenesis but largely restricted or absent in healthy adult tissues.⁶⁶ Its expression is regained in several pathological situations including many human cancer types.⁶⁷ Many antibodies against TNC have been identified and developed, and some of them are currently used in preclinical or clinical trials.⁶⁸ The MTn12 antibody is a rat monoclonal antibody of the IgG1 type with both specificity and relatively high affinity for murine tenascin-C.⁶⁹ Therefore this antibody was a good candidate to test novel labeling strategies in preclinical murine models of cancer, including breast cancer or insulinoma models,⁷⁰ in which tenascin-C is known to be highly expressed *in vivo*.

Prior to labeling, a MALDI/MS analysis was carried out on MTn12 which was found to have a molecular mass of 148 100 Da. The L^* labeled MTn12 ($L^*\text{-MTn12}$) was also analyzed by MALDI/MS (Fig. 11). The mass spectrum shows a shift of the monocharged peak $[\text{M} + \text{H}]^+$ to the higher masses due to the addition of the BFC. At the maximum, this shift is of about 1000 Da which would roughly correspond to two ligands per antibody.

Immunofluorescence staining

In order to verify that the labeling protocol did not perturb the immunoaffinity of the MTn12 antibody towards tenascin-C, immunofluorescence staining experiments were performed in which MTn12 and $L^*\text{-MTn12}$ were incubated on insulinoma tissue sections from RIP1-Tag2 mice.^{70,71} The presence of the MTn12 antibody was then revealed by staining with an anti-rat secondary antibody labeled with a Cyanine3 dye (Cy3). A control experiment was performed in which no MTn12 was added prior to the addition of the secondary antibody. As can

Fig. 9 Suggested proton-promoted dissociation mechanism of CuL^1 .

Fig. 10 Variation of the acidolysis (k_H) and solvolysis (k_d) rate constants measured for CuL^1 , CuL^2 and CuL^3 complexes as a function of the number of phosphonates.

be seen in Fig. 12, both MTn12 and its L^* labeled version could be detected in tumors of the pancreata sections, confirming that the labeling with L^* did not affect the immunoaffinity of MTn12.

SPECT/CT imaging studies with ^{99m}Tc complexes of ligands L^1 , L^2 and $L^*-MTn12$

The chelating affinity of ligands L^1 and L^2 towards ^{99m}Tc was first checked using radiochromatography experiments consisting in the comparison of the retention factors of ^{99m}Tc in the presence and absence of the ligands. The radiochemical purity for L^1 and L^2 was above 95%. The biodistribution of the complexes formed with the two ligands was then analyzed by SPECT imaging of mice injected with the radioactive complexes. As can be seen in Fig. 13, the biodistribution profile was very different in each case.

Fig. 11 (A) MALDI-TOF MS spectra of MTn12 (upper) and $L^*-MTn12$ (lower). (B) Zoom of the m/z region from 142 000 to 155 000 showing the 1000 units shift in mass due to the added chelate.

Fig. 12 Immunofluorescence staining on RIP1-Tag2 insulinoma tissue sections using L*-MTn12 (a), MTn12 (b) or no secondary antibody as a control (c). Antibody binding was revealed with a Cy3-coupled anti-rat secondary antibody (red) and cell nuclei were stained with DAPI (blue).

In the case of L¹, the complex rapidly accumulates in the kidneys after 5 minutes, and an important part of it can be found in the bladder after 2 h. But it could be noticed that a large part of the complex accumulated in bone tissues of the knees, shoulders, the nose and the spine, as can be observed after 4 h (Fig. 13). This behavior has already been observed for other phosphonate based ^{99m}Tc complexes and is clearly

related with the phosphonate contents of the chelate.^{15,72} In contrast, 7 minutes after injection of the L² complex, a large part of the complex could be found in the kidneys, and it was transferred to the bladder after 1 h 10 min. After 4 h, radio-labeled L² could still be found in the kidneys and the spleen, but also at the joints (knees and shoulders). It is surmised that the rapid clearance of the complex of L² may be related to the presence of the two ethyl chains replacing two phosphonate moieties and giving the complex a more lipophilic character.

Finally, the targeting efficiency of ^{99m}Tc-L*-MTn12 was questioned by injecting it into a transgenic mouse with breast cancer (a transgenic MMTV-NeuNT breast cancer mouse).⁷³ As can be seen in Fig. 14, the radiolabeling was very rapidly found to accumulate in the liver followed by its excretion through the bladder. The same distribution could be observed after 5 h 07 min and 22 h 50 min.

In living mice, the rapid accumulation of the ^{99m}Tc-L*-MTn12 antibody in the liver may be explained by the fact that IgG containing a Fc (constant domain) domain is known to be primarily endocytosed and degraded by liver parenchymal, endothelial and Kupffer cells thanks to the specific expression of the cognate receptors by these liver cell types.⁷⁴

Conclusion

We have synthesized and characterized a new family of ligands based on a pyridine scaffold functionalized with varying numbers of phosphonated functions. The physico-chemical properties of the ligands and their complexes have been studied. Whatever the ligands, monometallic species were observed by mass spectrometry for equimolar solutions of metal and ligands. All the ligands showed a conventional trend for complexation, following the Irving-Williams order of affinity.^{48–50} Nevertheless, ligands L¹ and L² displayed a very good selectivity for Cu(II) ($\Delta \log K_{ML} > 5$) with pCu^{II} values compatible with their potential use for ⁶⁴Cu PET imaging. Interestingly, the loss of a single phosphonate function compared to the tetra-phosphonated ligand L¹ immediately resulted in a drastic loss of affinity towards Cu(II) of three orders of magnitude. More surprising, the loss of a second phosphonate function to lead to L² did not have such an impact on the affinity. These results remain poorly understood in the absence of a clear definition of the coordination polyhedra offered by the ligands. Unfortunately, the presence of numerous protonated forms of the Cu(II) complexes in water highly complicates the interpretations of possible EPR measurements,^{12,47} and was not informative in our hands. Full electrochemical characterization of the cupric complexes was performed, here again showing the availability of the complexes of L¹ and L² to resist biological reduction, a necessary prerequisite for their use *in vivo*. A thorough investigation of the formation and dissociation kinetics of the cupric complexes evidenced very rapid formations, while the proton assisted dissociations were proportional to the number of phosphonate units.

Fig. 13 Top (left) and 100° (right) SPECT/CT images of mice injected with ^{99m}Tc complexes of L^1 and L^2 , after 4 h 16 and 4 h 30 respectively.

Considering their potential interest for *in vivo* imaging, the ability of ligands L^1 and L^2 to coordinate ^{99m}Tc were studied by radiochromatography and by SPECT/CT imaging on healthy mice, showing a preferred fixation of the $^{99m}\text{TcL}^1$ complex on bone tissues, while the complex of L^2 was rapidly excreted through the kidneys and bladder. Finally, a bifunctional version of L^1 was used to label MTn12, a rat monoclonal antibody directed against murine tenascin-C. Although immunofluorescence staining experiments revealed unperturbed selectivity in tumors of the pancreata, the injection of ^{99m}Tc radiolabeled antibody into a transgenic mouse bearing a breast primary cancer showed the antibody to be readily accumulated in the liver, followed by excretion, with no specific signal in the tumor. A possible explanation is that the constant fragments of this IgG antibody were primarily endocytosed by liver cells⁷⁴ and our current efforts are directed towards the development of F(ab)_2 fragments of MTn12 (that lacks the constant fragments) for their labeling and *in vivo* imaging.

Experimental section

Synthesis of the compounds

General methods. Column chromatography and flash column chromatography were performed respectively on silica (0.063–0.200 mm, Merck) and silica gel (40–63 μm , Merck). Solvent mixtures used for TLC and flash column chromatography are reported in v/v ratios. ^1H and ^{13}C NMR spectra were

recorded on an Avance 300 spectrometer at 300 MHz for proton frequency and 75 MHz for carbon frequency. ^{31}P NMR spectra (161.9 MHz) were recorded on Avance 400 apparatus with H-decoupling. Chemical shifts are given in parts per million, relative to the residual protic solvent peak.⁷⁵ IR spectra were recorded on a Nicolet 380 FT-IR spectrometer (Thermo Scientific). Mass spectra (MS) and elemental analyses were obtained by the Service Commun d'Analyse de l'UdS.

Solvents were used as received. Commercially available reagents were used without further purification.

2,6-Bis(ethylaminomethyl)pyridine **3**¹⁹ and tetra-ethyl imino-bis(methanephosphonate)²⁰ were synthesized according to the literature procedures.

2,6-Dibromomethylpyridine 2. To a solution of 2,6-dihydroxymethylpyridine (2.80 g, 20.1 mmol) in DMF (15 mL) was added dropwise, at 0 °C, PBr_3 (4.35 mL, 46.3 mmol). The mixture was stirred at room temperature for 4 h and water was added (20 mL). After extraction with Et_2O (3×100 mL), the organic layer was dried with Na_2SO_4 and the solvent was removed under reduced pressure. 2,6-Dibromomethylpyridine **2** was obtained as a pure white solid (4.81 g, 95%). All analyses correspond to those reported in the literature.⁷⁶

Tetraethyl(((pyridine-2,6-diyl-bis(methylene))-bis(ethylazane-diyl))-bis(methylene))-bis(phosphonate) 4. Procedure 1: A mixture of 2,6-bis(ethylaminomethyl)pyridine¹⁹ **3** (170 mg, 0.88 mmol) and diethyl phosphite (243 mg, 1.76 mmol) was cooled for 10 minutes in an ice-bath and 220 μL of formaldehyde (37% in water) were added slowly maintaining the

Fig. 14 SPECT/CT images of a transgenic mouse with breast primary cancer, 5 minutes (left) and 25 minutes (right) after injection of ^{99m}Tc -L*-MTn12.

temperature below 10 °C. The ice bath was removed and the mixture was stirred at room temperature for 30 minutes and refluxed for 3 h. The solution was concentrated under vacuum and the crude product was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 100/0 to 92/8) to give compound **4** as an oil (302 mg, 69%). ^1H NMR (CDCl_3 , 300 MHz): δ 7.59 (t, $^3J_{\text{H-H}} = 7.7$ Hz, 1H, H_{pyr}), 7.37 (d, $^3J_{\text{H-H}} = 7.6$ Hz, 2H, H_{pyr}), 4.09 (qd, $^3J_{\text{H-H}} = 7.4$ Hz, $^3J_{\text{H-P}} = 7.4$ Hz, 8H, CH_2O), 3.85 (s, 4H, $\text{NCH}_2\text{-Pyr}$), 2.93 (d, $^2J_{\text{H-P}} = 10.5$ Hz, 4H, $(\text{EtO})_2\text{OPCH}_2\text{N}$), 2.70 (q, $^3J_{\text{H-H}} = 7.1$ Hz, 4H, $\text{CH}_3\text{CH}_2\text{N}$), 1.28 (t, $^3J_{\text{H-H}} = 7.1$ Hz, 12H, $\text{CH}_3\text{CH}_2\text{O}$), 1.03 (t, $^3J_{\text{H-H}} = 7.1$ Hz, 6H, $\text{CH}_3\text{CH}_2\text{N}$). ^{13}C NMR (CDCl_3 , 75 MHz): δ 158.8 (C_{pyr}), 136.7 (C_{pyr}), 121.2 (C_{pyr}), 61.9 (d, $^2J_{\text{C-P}} = 6.8$ Hz, CH_2O), 61.2 (d, $^3J_{\text{C-P}} = 8.8$ Hz, $\text{NCH}_2\text{-Pyr}$), 49.4 (d, $^1J_{\text{C-P}} = 163.1$ Hz, $(\text{EtO})_2\text{OPCH}_2\text{N}$), 49.5 (d, $^3J_{\text{C-P}} = 8.8$ Hz, $\text{CH}_3\text{CH}_2\text{N}$), 16.6 (d, $^3J_{\text{C-P}} = 6.1$ Hz, $\text{CH}_3\text{CH}_2\text{O}$), 12.0 ($\text{CH}_3\text{CH}_2\text{N}$). ^{31}P NMR (CDCl_3 , 161.9 MHz): δ 25.6. ESI $^+$ /MS (MeOH): $m/z = 493.2$ ($[\text{M} + \text{H}]^+$, 100%). Calc. for $\text{C}_{21}\text{H}_{41}\text{N}_3\text{O}_6\text{P}_2 \cdot 0.5 \text{H}_2\text{O}$: C, 50.19; H, 8.42; N, 8.36; Found: C, 50.11; H, 8.45; N, 8.52.

Procedure 2: To a mixture of 2,6-dibromomethylpyridine **2** (2 g, 7.55 mmol) and amine **7** (3.1 g, 15.85 mmol) in MeCN (22 mL), under a nitrogen atmosphere, freshly flame-dried K_2CO_3 (4 g, 30.2 mmol) was added. The reaction mixture was heated at 50 °C overnight. After filtration, the solvent was

removed *in vacuo* and the crude oil was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$). Compound **4** was obtained as an oily light yellow product (878 mg, 24%).

(((Pyridine-2,6-diyl-bis(methylene))-bis(ethylazanediy)))-bis-(methylene)diphosphonic acid **L².** To a solution of compound **4** (295 mg, 0.60 mmol) in CH_2Cl_2 (3 mL), TMSBr (1.6 mL, 12.0 mmol) was added and the resulting mixture was stirred at room temperature for 24 h. TMSBr (1.6 mL, 12.0 mmol) was added and the mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure, the resulting residue was taken up with methanol (5 mL) and the mixture was stirred at room temperature for 2 h. The evaporation–dissolution procedure was repeated with methanol (5 mL) and the solution was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue was dissolved in a minimum of methanol. Addition of diethyl ether resulted in the formation of a precipitate, which was collected by centrifugation and dried under vacuum to afford ligand **L**² in 83% (240 mg). ^1H NMR (D_2O , 200 MHz): δ 7.90 (t, $^3J_{\text{H-H}} = 7.7$ Hz, 1H, H_{pyr}), 7.44 (d, $^3J_{\text{H-H}} = 8.0$ Hz, 2H, H_{pyr}), 4.63 (s, 4H, $\text{NCH}_2\text{-Pyr}$), 3.43 (q, $^3J_{\text{H-H}} = 7.3$ Hz, 4H, $\text{CH}_3\text{CH}_2\text{N}$), 3.34 (d, $^2J_{\text{H-P}} = 12.4$ Hz, 4H, $\text{HO}_3\text{PCH}_2\text{N}$), 1.30 (t, $^3J_{\text{H-H}} = 7.1$ Hz, 6H, $\text{CH}_3\text{CH}_2\text{N}$). ^{13}C NMR (D_2O , 75 MHz): δ 149.8 (C_{pyr}), 139.9 (C_{pyr}), 124.2 (C_{pyr}), 57.6 (d,

$^3J_{C-P} = 4.5$ Hz, $NCH_2\text{-Pyr}$), 51.6 (d, $^3J_{C-P} = 3.8$ Hz, CH_3CH_2N), 49.4 (d, $^1J_{C-P} = 136.5$ Hz, HO_3PCH_2N), 8.2 (CH_3CH_2N). ^{31}P NMR (D_2O , 161.9 MHz): δ 8.4. ESI[−]/MS (MeOH/ H_2O): $m/z = 380.1$ ($[M - H]^-$, 100%). Calc. for $C_{13}H_{25}N_3O_6P_2 \cdot H_2O \cdot HBr$: C, 32.51; H, 5.88; N, 8.75; Found: C, 31.92; H, 5.35; N, 8.34.

Diethyl-((benzyl(ethyl)amino)methyl)phosphonate 6. To a mixture of *N*-ethylbenzylamine (5 mL, 33.6 mmol) and diethylphosphite (5.7 mL, 43.6 mmol) at 0 °C, 6 mL (67.2 mmol) of aqueous 37% formaldehyde was added. The resulting mixture was warmed to room temperature and stirring was continued at room temperature for 3 h. The reaction mixture was then heated under reflux for 3 h. The solvents were removed under reduced pressure and the resulting residue was purified by flash chromatography (CH_2Cl_2 /MeOH 97/3) to give compound **6** as a colorless oil (7.95 g; 83%). 1H NMR ($CDCl_3$, 300 MHz): δ 7.24–7.38 (m, 5H, H_{Arom}), 4.13 (qd, $^3J_{H-H} = 6.8$ Hz, $^3J_{H-P} = 6.8$ Hz, 4H, CH_2O), 3.77 (s, 2H, $CH_2\text{-Ph}$), 2.90 (d, $^2J_{H-P} = 10.6$ Hz, 2H, $(EtO)_2OPCH_2N$), 2.71 (q, $^3J_{H-H} = 6.8$ Hz, 2H, CH_3CH_2N), 1.33 (t, $^3J_{H-H} = 6.9$ Hz, 6H, CH_3CH_2O), 1.08 (t, $^3J_{H-H} = 7.0$ Hz, 3H, CH_3CH_2N). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 138.3 (C_{Arom}), 128.3 (C_{Arom}), 127.5 (C_{Arom}), 126.4 (C_{Arom}), 61.1 (d, $^2J_{C-P} = 6.5$ Hz, CH_2O), 58.6 (d, $^3J_{C-P} = 8.8$ Hz, $CH_2\text{-Ph}$), 48.1 (d, $^3J_{C-P} = 8.1$ Hz, CH_3CH_2N), 48.0 (d, $^1J_{C-P} = 161.9$ Hz, $(EtO)_2OPCH_2N$), 15.9 (d, $^3J_{C-P} = 5.6$ Hz, CH_3CH_2O), 11.2 (CH_3CH_2N). ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 25.8. ESI⁺/MS: $m/z = 285.2$ ($[M]^+$, 20%). Calc. for $C_{14}H_{24}NO_3P \cdot H_2O$: C, 55.43; H, 8.64; N, 4.62; Found: C, 54.74; H, 8.35; N, 4.91.

Diethyl-((ethylamino)methyl)phosphonate 7. A solution of **6** (9 g, 31.5 mmol) in ethanol (100 mL) was hydrogenated in the presence of Pd/C catalyst (0.9 g, 10% Pd). After 8 h at reflux, the reaction was complete. The catalyst was filtered off and the filtrate was evaporated to give compound **7** as a pale yellow oil (6 g, 98%). 1H NMR ($CDCl_3$, 300 MHz): δ 4.13 (m, 4H, CH_2O), 2.99 (d, $^2J_{H-P} = 12.1$ Hz, 2H, $(EtO)_2OPCH_2N$), 2.73 (q, $^3J_{H-H} = 6.8$ Hz, 2H, CH_3CH_2N), 1.31 (t, $^3J_{H-H} = 6.8$ Hz, 6H, CH_3CH_2O), 1.10 (t, $^3J_{H-H} = 7.0$ Hz, 3H, CH_3CH_2N). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 61.9 (d, $^2J_{C-P} = 7.2$ Hz, CH_2O), 45.3 (d, $^3J_{C-P} = 16.3$ Hz, CH_3CH_2N), 43.6 (d, $^1J_{C-P} = 153.8$ Hz, $(EtO)_2OPCH_2N$), 16.3 (d, $^3J_{C-P} = 5.4$ Hz, CH_3CH_2O), 14.6 (CH_3CH_2N). ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 25.9. ESI⁺/MS (CH_2Cl_2): $m/z = 196.1$ ($[M + H]^+$, 42%), 413.2 ($[2M + Na]^+$, 100%).

Tetraethyl-(((6-(bromomethyl)pyridin-2-yl)methyl)azanediyl)-bis(methylene))-bis(phosphonate) 8. To a mixture of 2,6-dibromomethylpyridine **2** (3.2 g, 12.1 mmol) and tetra-ethyl iminobis(methanephosphonate)²⁰ (3.07 g, 9.66 mmol) in MeCN under a nitrogen atmosphere, freshly flame-dried K_2CO_3 (2.51 g, 18.15 mmol) was added. The reaction mixture was heated at 50 °C overnight. After filtration, the solvent was removed *in vacuo* and the crude oil was purified by flash chromatography (CH_2Cl_2 /MeOH 100/0 to 92/8). Compound **8** was obtained as an oily light yellow product (2.81 g, 58%). 1H NMR ($CDCl_3$, 300 MHz): δ 7.66 (t, $^3J_{H-H} = 7.8$ Hz, 1H, H_{pyr}), 7.51 (d, $^3J_{H-H} = 8.1$ Hz, 1H, H_{pyr}), 7.31 (d, $^3J_{H-H} = 8.1$ Hz, 1H, H_{pyr}), 4.51 (s, 2H, CH_2Br), 4.11 (m, 10H, $NCH_2\text{-Pyr}$, CH_2O), 3.23 (d, $^2J_{H-P} = 9.9$ Hz, 4H, $(EtO)_2OPCH_2N$), 1.29 (t, $^3J_{H-H} = 7.0$ Hz, 12H, CH_3CH_2O). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 158.7 (C_{pyr}),

155.9 (C_{pyr}), 137.4 (C_{pyr}), 122.9 (C_{pyr}), 121.9 (C_{pyr}), 62.0 (m, $NCH_2\text{-Pyr}$, CH_2O), 50.3 (dd, $^1J_{C-P} = 157.9$ Hz, $^3J_{C-P} = 8.2$ Hz, $(EtO)_2OPCH_2N$), 34.0 (CH_2Br), 16.5 (m, CH_3CH_2O). ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 24.4. ESI⁺/MS (CH_2Cl_2): $m/z = 502.8$ ($[M + H]^+$, 14%), 504.8 ($[M + H]^+$, 14%), 523.1 ($[M + Na]^+$, 100%), 525.1 ($[M + Na]^+$, 100%).

Hexaethyl-(((pyridine-2,6-diyl-bis(methylene))(ethylazanediyl))-tris(methylene))-tris(phosphonate) 9. To a mixture of compound **8** (2.81 g, 5.6 mmol) and amine **7** (1.31 g, 6.7 mmol) in MeCN under a nitrogen atmosphere, freshly flame-dried K_2CO_3 (1.55 g, 11.2 mmol) was added. The reaction mixture was heated at 80 °C for 5 h. After filtration, the solvent was removed *in vacuo* and the crude oil was purified by flash chromatography (CH_2Cl_2 /MeOH 96/4). Compound **9** was obtained as an oily yellow product (1.81 g, 49%). 1H NMR ($CDCl_3$, 300 MHz): δ 7.54 (t, $^3J_{H-H} = 7.5$ Hz, 1H, H_{pyr}), 7.34 (d, $^3J_{H-H} = 8.2$ Hz, 2H, H_{pyr}), 4.08–3.97 (m, 12H, CH_2O), 3.79 (s, 4H, $NCH_2\text{-Pyr}$), 3.13 (d, $^2J_{H-P} = 9.2$ Hz, 2H, $(EtO)_2OPCH_2NET$), 2.87 (d, $^2J_{H-P} = 10.8$ Hz, 4H, $[(EtO)_2OPCH_2]_2N$), 2.62 (q, $^3J_{H-H} = 7.0$ Hz, 2H, CH_3CH_2N), 1.20 (t, $^3J_{H-H} = 6.7$ Hz, 18H, CH_3CH_2O), 0.96 (t, $^3J_{H-H} = 7.3$ Hz, 3H, CH_3CH_2N). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 158.9 (C_{pyr}), 157.7 (C_{pyr}), 136.9 (C_{pyr}), 121.8 (C_{pyr}), 121.6 (C_{pyr}), 62.6 (t, $^3J_{C-P} = 8.0$ Hz, $NCH_2\text{-Pyr}$), 62.0 (d, $^2J_{C-P} = 6.5$ Hz, CH_2O), 62.0 (d, $^2J_{C-P} = 8.6$ Hz, CH_2O), 61.0 (d, $^3J_{C-P} = 8.6$ Hz, $NCH_2\text{-Pyr}$), 50.2 (dd, $^1J_{C-P} = 158.4$ Hz, $^3J_{C-P} = 8.7$ Hz, $[(EtO)_2OPCH_2]_2N$), 49.4 (d, $^3J_{C-P} = 8.9$ Hz, CH_3CH_2N), 49.3 (d, $^1J_{C-P} = 163.3$ Hz, $(EtO)_2OPCH_2NET$), 16.5 (m, CH_3CH_2O), 11.9 (CH_3CH_2N). ^{31}P NMR ($CDCl_3$, 161.9 MHz): δ 24.4, 25.4. ESI⁺/MS (CH_2Cl_2): $m/z = 616.3$ ($[M + H]^+$, 100%). Calc. for $C_{24}H_{48}N_3O_9P_3 \cdot 2H_2O$: C, 44.24; H, 8.04; N, 6.45; Found: C, 43.88; H, 7.79; N, 6.62.

(((Pyridine-2,6-diyl-bis(methylene))(ethylazanediyl))-tris(methylene))triphosphonic acid L³. To a solution of compound **9** (1.81 g, 2.93 mmol) in CH_2Cl_2 (10 mL), TMSBr (11.62 mL, 88 mmol) was added at 0 °C, and the resulting mixture was stirred at room temperature for 24 h. TMSBr (11.6 mL, 88 mmol) was added and the mixture was stirred at room temperature for 24 h. The solvent was removed, the resulting residue was taken up with methanol (10 mL) and the mixture was stirred at room temperature for 2 h. The evaporation–dissolution procedure was repeated with methanol (10 mL) and the solution was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue was dissolved in a minimum of methanol. Addition of diethyl ether resulted in the formation of a precipitate, which was collected by centrifugation and dried under vacuum to afford ligand **L³** (1.71 g, 90%). 1H NMR ($D_2O + NaOD$, 200 MHz): δ 7.68 (t, $^3J_{H-H} = 7.4$ Hz, 1H, H_{pyr}), 7.44 (d, $^3J_{H-H} = 7.4$ Hz, 1H, H_{pyr}), 7.29 (d, $^3J_{H-H} = 6.7$ Hz, 1H, H_{pyr}), 3.90 (s, 2H, $NCH_2\text{-Pyr}$), 3.84 (s, 2H, $NCH_2\text{-Pyr}$), 2.54 (m, 8H, O_3PCH_2N , CH_3CH_2N), 0.85 (t, $^3J_{H-H} = 7.4$ Hz, 3H, CH_3CH_2N). ^{13}C NMR (D_2O , 75 MHz): δ 159.0 (C_{pyr}), 158.2 (C_{pyr}), 138.0 (C_{pyr}), 123.0 (C_{pyr}), 122.6 (C_{pyr}), 62.5 (m, $NCH_2\text{-Pyr}$), 60.2 (d, $^3J_{C-P} = 6.0$ Hz, $NCH_2\text{-Pyr}$), 55.4 (dd, $^1J_{C-P} = 145.9$ Hz, $^3J_{C-P} = 8.8$ Hz, $[HO_3PCH_2]_2N$), 52.6 (d, $^1J_{C-P} = 138.9$ Hz, HO_3PCH_2NET), 48.1 (d, $^3J_{C-P} = 8.0$ Hz, CH_3CH_2N), 10.5 (CH_3CH_2N). ^{31}P NMR

(D₂O, 161.9 MHz): δ 17.1, 16.5. ESI[−]/MS (D₂O): m/z = 446.1 ([M − H][−], 100%). Calc. for C₁₂H₂₄N₃O₉P₃·2H₂O·2HBr: C, 22.34; H, 4.69; N, 6.51; Found: C, 22.80; H, 4.81; N, 6.24.

Diethyl-(((6-bromomethyl)pyridine-2-yl)methyl)phosphonate 10. Compound **2** (480 mg, 1.81 mmol) was dissolved in triethyl phosphite (315 μ L, 1.81 mmol) under a nitrogen atmosphere. The mixture was heated at 50 °C, protected from light, for 4 days. After cooling at room temperature, excess triethyl phosphite was removed under high vacuum. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH 94/6). Compound **10** was isolated as an orange oil (281 mg, 48%). Compound **10** is not stable and is rapidly degraded. ¹H NMR (CDCl₃, 300 MHz): δ 7.55 (t, ³J_{H-H} = 7.8 Hz, 1H, H_{pyr}), 7.21 (d, ³J_{H-H} = 7.8 Hz, 2H, H_{pyr}), 4.42 (s, 2H, CH₂Br), 3.98 (qd, ³J_{H-H} = 7.1 Hz, ³J_{H-P} = 7.1 Hz, 4H, CH₂O), 3.30 (d, ²J_{H-P} = 21.6 Hz, 2H, (EtO)₂OPCH₂), 1.20 (t, ³J_{H-H} = 7.0 Hz, 6H, CH₃CH₂O). ¹³C NMR (CDCl₃, 75 MHz): δ 155.7 (d, J_{C-P} = 2.7 Hz, C_{pyr}), 152.0 (d, J_{C-P} = 8.3 Hz, C_{pyr}), 136.7 (d, J_{C-P} = 2.2 Hz, C_{pyr}), 122.8 (d, J_{C-P} = 5.0 Hz, C_{pyr}), 120.9 (d, J_{C-P} = 2.6 Hz, C_{pyr}), 61.4 (d, ²J_{C-P} = 6.5 Hz, CH₂O), 35.7 (d, ¹J_{C-P} = 134.3 Hz, (EtO)₂OPCH₂), 33.2 (CH₂Br), 15.6 (d, ³J_{C-P} = 5.7 Hz, CH₃CH₂O). ³¹P NMR (CDCl₃, 161.9 MHz): δ 24.4.

Tetraethyl-(((6-(diethylmethylenephosphonate)pyridin-2-yl)-methyl)azanediyl)-bis(methylene))-bis(phosphonate) 11. To a mixture of compound **10** (800 mg, 2.5 mmol) and amine **7** (790 mg, 2.5 mmol) in MeCN under a nitrogen atmosphere, freshly flame-dried K₂CO₃ (684 mg, 4.95 mmol) was added. The reaction mixture was heated at 80 °C, protected from light, for 48 h. After filtration, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography (CH₂Cl₂/MeOH 100/0 to 96/4). Compound **11** was obtained as an oily product (476 mg, 32%). ¹H NMR (CDCl₃, 300 MHz): δ 7.60 (t, ³J_{H-H} = 8.4 Hz, 1H, H_{pyr}), 7.45 (d, ³J_{H-H} = 7.9 Hz, 1H, H_{pyr}), 7.25 (d, ³J_{H-H} = 7.5 Hz, 1H, H_{pyr}), 4.07 (m, 14H, CH₂O, NCH₂-Pyr), 3.36 (d, ²J_{H-P} = 22.0 Hz, 2H, (EtO)₂OPCH₂), 3.20 (d, ²J_{H-P} = 10.4 Hz, 4H, [(EtO)₂OPCH₂]₂N), 1.28 (t, ³J_{H-H} = 7.1 Hz, 12H, CH₃CH₂O), 1.24 (t, ³J_{H-H} = 7.1 Hz, 6H, CH₃CH₂O). ¹³C NMR (CDCl₃, 75 MHz): δ 158.4 (d, J_{C-P} = 2.1 Hz, C_{pyr}), 151.9 (d, J_{C-P} = 8.2 Hz, C_{pyr}), 137.0 (d, J_{C-P} = 2.4 Hz, C_{pyr}), 122.8 (d, J_{C-P} = 4.8 Hz, C_{pyr}), 121.8 (d, J_{C-P} = 3.3 Hz, C_{pyr}), 62.5 (t, ³J_{C-P} = 7.9 Hz, NCH₂-Pyr), 62.2 (d, ²J_{C-P} = 6.6 Hz, CH₂O), 62.1 (m, CH₂O), 50.4 (dd, ¹J_{C-P} = 159.2 Hz, ³J_{C-P} = 8.3 Hz, [(EtO)₂OPCH₂]₂N), 36.6 (d, ¹J_{C-P} = 135.1 Hz, (EtO)₂OPCH₂), 16.5 (m, CH₃CH₂O). ³¹P NMR (CDCl₃, 161.9 MHz): δ 25.1, 24.4. ESI⁺/MS (CH₂Cl₂): m/z = 559.2 ([M + H]⁺, 100%). Calc. for C₂₁H₄₁N₂O₉P₃·2H₂O: C, 42.43; H, 7.63; N, 4.71; Found: C, 42.90; H, 7.03; N, 5.06.

(((6-(Methylene)pyridin-2-yl)methyl)azanediyl)-bis(methylene))triphosphonic acid L⁴. Compound **11** (190 mg, 0.34 mmol) was dissolved in 6 M HCl (6 mL) and the mixture was heated at reflux overnight. The solvent was evaporated under reduced pressure and the residue was dissolved in a minimum of methanol. Addition of diethyl ether resulted in the formation of a precipitate, which was collected by centrifugation and dried under vacuum to give ligand L⁴ (60 mg, 46%). ¹H NMR (D₂O, 200 MHz): δ 8.39 (t, ³J_{H-H} = 7.9 Hz, 1H, H_{pyr}), 7.90 (d, ³J_{H-H} = 7.9 Hz, 1H, H_{pyr}), 7.84 (d, ³J_{H-H} = 7.9 Hz,

1H, H_{pyr}), 4.71 (s, 2H, NCH₂-Pyr), 3.51 (d, ²J_{H-P} = 21.2 Hz, 2H, HO₃PCH₂), 3.35 (d, ²J_{H-P} = 12.0 Hz, 4H, [HO₃PCH₂]₂N). ¹³C NMR (D₂O, 100 MHz): δ 151.9 (C_{pyr}), 148.5 (C_{pyr}), 146.2 (C_{pyr}), 127.7 (d, J_{C-P} = 4.3 Hz, C_{pyr}), 125.7 (C_{pyr}), 56.6 (m, NCH₂-Pyr), 52.6 (dd, ¹J_{C-P} = 144.7 Hz, ³J_{C-P} = 5.8 Hz, [HO₃PCH₂]₂N), 34.8 (d, ¹J_{C-P} = 123.4 Hz, HO₃PCH₂). ³¹P NMR (D₂O, 161.9 MHz): δ 13.7. ESI[−]/MS (D₂O): m/z = 389.0 ([M − H][−], 100%). Calc. for C₉H₁₇N₂O₉P₃·HCl: C, 25.34; H, 4.25; N, 6.56; Found: C, 25.07; H, 4.32; N, 6.40.

MALDI-MS analyses

Prior to analysis, the MTn12 antibodies, either uncoupled or coupled to the BFC chelate, were desalted on a C18 ZipTip (Millipore). Ten μ g of antibody were loaded and then eluted in 2 μ L MeCN 80%/TFA 0.1%. The whole eluate was spotted on the target plate with 0.6 μ L of sinapinic acid (saturated solution diluted 3 times in MeCN 50%/TFA 0.1%). The mass spectrometer (Autoflex, Bruker Daltonics) was operated in positive linear mode, with BSA as a calibrant.

Production and purification of MTn12 antibody. The MTn12 anti-tenascin-C mouse antibody⁶⁹ was purified from hybridoma culture supernatants by affinity chromatography using a HiTrap protein G sepharose column and an AktaPrime purification system (GE Healthcare).

The labeling experiment was carried out on 500 μ g (500 μ L of a 1 mg mL^{−1} solution in PBS) of the antibody MTn12 to which 2 mg of solid BFC L^{*} (2.7 μ mol) was added, corresponding to 800 equivalents of the ligand for the antibody. The reaction medium was adjusted to a pH of 7.4 with 200 mM NH₄HCO₃ and was stirred for 2 h at room temperature. The reaction medium was purified repeatedly to eliminate the excess of ligand with PBS using Vivaspin500 (Sartorius) modules with a 30 kDa cut-off (500 μ L of PBS per cycle; 20 cycles).

Immunostaining experiment. Immunofluorescence analysis of murine insulinoma tumors was performed by staining pancreata sections from RIP1-Tag2 mice as previously described.⁷⁰ The MTn12 antibody, either coupled to the BFC chelate or uncoupled, was incubated overnight at 4 °C on tissue sections (2 μ g mL^{−1}), and a Cy3-coupled anti-rat secondary antibody (1/1600 for 1 h at room temperature; Interchim) was used for detection. Cell nuclei were stained with DAPI. Negative controls were performed by omitting the primary antibody.

SPECT animal imaging. All work performed on animals was in accordance with the local ethics committee following the rules (agreement number AL/05/12/02/13 of the regional ethical committee). Biodistribution of the ^{99m}Tc complexes of ligands L¹ and L² was obtained by tail vein injection of the complexes. Transgenic breast cancer mice (MMTV-NeuNT TG. NK line)⁷³ in the FVB background were obtained by breeding MMTV-NeuNT male with wild-type FVB female mice. Genotyping was performed on DNA extracted from tail as previously described,⁷⁰ using two primer pairs specific to the NeuNT (forward1: GGAAGTACCCGGATGAGGAGGGCATATG, and reverse1: CCGGGCAGCCAGGTCCCTGTGTACAAGCCG; forward2: TCCTGTGTGGATCTGGATGA, and reverse2: GTCAGCGGCTC-

CACTA(CTC). Female mice with macroscopically visible primary tumors were used three months after the palpation of the first primary tumor for intravenous injection of the labeled ^{99m}Tc -L*-MTn12 antibody in the lateral tail vein and *in vivo* imaging by SPECT.

In vivo CT/SPECT analysis. In order to recognize the different organs, prior to SPECT analysis, a CT image was recorded on the AMISSA platform, a home-made multimodality imaging system for small animals combining X-ray, SPECT and PET devices. The μCT delivered a 3D reconstructed volume of the animal in real time.⁷⁷

Targeting of ^{99m}Tc -labeled molecules was monitored *via* the μSPECT imaging technique. Mice were anesthetized by intraperitoneal injection of $10\ \mu\text{L g}^{-1}$ of a solution made with ketamine hydrochloride 10% (Imalgene, Centravet, Velaine en Haye, France) and Xylazine 5% (Rompun, Centravet, Velaine en Haye, France) and placed in prone position in the animal holder after injection in the tail vein. μSPECT imaging was performed 4 minutes post-intravenous injection of 124 MBq, 118 MBq and 59 MBq in 220 μL , 200 μL and 100 μL of ^{99m}Tc molecules for L¹, L² and L*-MTn12, respectively, injected in the tail vein. The μSPECT system consists of a four head detection gamma camera. Each head comprises five separated detection modules arranged along a circle of 58 mm with the pinhole as the center. A detection module consists of a YAP:Ce matrix of 8×8 scintillating crystals $2.3 \times 2.3 \times 28\ \text{mm}^3$ each coupled to a multi-anode photomultiplier 8×8 (Hamamatsu H 8804). The distance from the pinhole to the axis of rotation is 28 mm and the distance between the pinhole and the crystal is 58 mm which results in a magnification factor of 2.07.⁷⁸ Images were reconstructed using the OSEM (Ordered Subset Expectation Maximisation) iterative algorithm adapted for pinhole imaging. Images were viewed and quantified using the Anatomist freeware (http://brainvisa.info/index_f.html).

General protocol for radiolabeling

Ligand L¹ (5 mg) or ligand L² (4.5 mg) were dissolved in water (1 mL). Sodium ascorbate (150 μL , 5 mg mL^{-1}) and stannous chloride (75 μL , 2 mg mL^{-1}) in 0.1 M hydrochloric acid were then added to the above solution. The pH of the resulting solution was adjusted to 6.5–7.0 with 1 M NaOH. Freshly eluted $^{99m}\text{TcO}_4^-$ (1 mL, 600–700 MBq mL^{-1}) was added, and the resulting mixture was then stirred at room temperature for 30 min for optimum labelling yield.

The labeling efficiency was estimated chromatographically using ITLC-SG strips as the stationary phase and acetone as the mobile phase. After developing the TLC, it was cut into two equal halves and radioactivity of each segment was determined in a well-type γ -ray counter. The free $^{99m}\text{TcO}_4^-$ moved with the solvent, while the radiolabeled formulation remained at the point of application.

L* was labeled with ^{99m}Tc using ^{99m}Tc -glucoheptonate as an intermediate. Briefly, $^{99m}\text{TcO}_4^-$ (1.4 GBq in 400 μL) was added to a solution containing 50 μL sodium glucoheptonate (10 mg mL^{-1}) and 150 μL SnCl_2 (2 mg mL^{-1} in 0.1 M HCl). This solution is then added to the vial containing the L*

labeled MTn12 antibody (250 μL at 1.8 mg mL^{-1} in PBS, pH 7.4). The labeling reaction was completed after 30 min with slow stirring at room temperature.

The mixture was purified by centrifugation at 13 000 RPM over 30 000 MW cutoff filtering devices (Amicon Ultra 0.5 mL centrifugal filters, Millipore).

The radiochemical purity of ^{99m}Tc -L*-MTn12 was estimated to be more than 95% by instant thin layer chromatography (ITLC). ^{99m}Tc -L*-MTn12 remains immobile, and other species (^{99m}Tc -glucoheptonate and $^{99m}\text{TcO}_4^-$) are mobile when saturated saline was used as a developing solvent.

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