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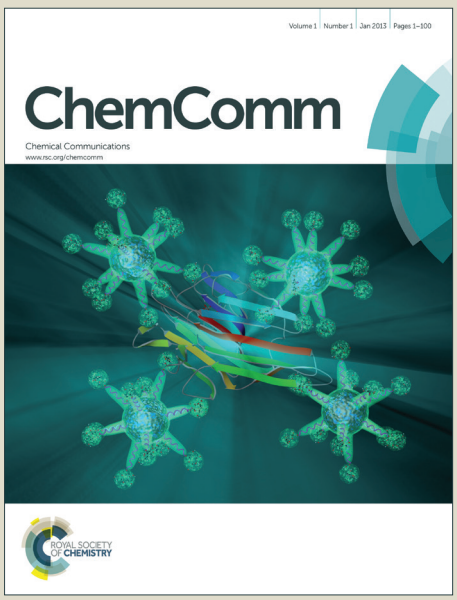

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H_2Me-do2pa: An attractive chelator with fast, stable and inert $^{nat}Bi^{3+}$ and $^{213}Bi^{3+}$ complexation for potential α-radioimmunotherapy applications

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The complexation properties of H_2Me-do2pa towards $^{nat}Bi^{3+}$ reveal a rather fast formation of the $[Bi(Me\text{-}do2pa)]^+$ complex, which is endowed with a very high thermodynamic stability (log K_{BiL} = 34.2) and presents a single non-fluxional structure in solution. X-ray diffraction and solution NMR studies showed an octadentate binding of the ligand to the metal ion. The labelling of H_2Me-do2pa with ^{213}Bi was performed and the resulting complex is stable *in vitro*, sustaining its use as an attractive alternative to H_4dota taken here as reference.

Bismuth(III) salts have been used in medicine for centuries before the advent of antibiotics[1] thanks to their relatively low toxicity as compared to related heavy metal-containing species.[2] Nowadays, radioisotopes of bismuth(III) ions, mainly ^{212}Bi and ^{213}Bi, are under evaluation in nuclear medicine where they are intensively studied as potential α-emitting therapeutic agents.[3] Alpha-radioimmunotherapy (RIT) represents an emerging therapeutic modality for tumour treatment that is currently under active investigation. In contrast with the typical path length of β^- emitting radionuclides (≈ 1 mm to 1 cm depending to their energy), the energy emissions of α-particle decays are directly deposited over a very short distance (40–100 μm), resulting in a high linear energy transfer. The shorter path may also have the advantage of limiting toxicity to normal tissue adjacent to tumour. As such, α-emitting radionuclides are hypothesized as ideal for specific treatment of smaller tumour burden, disseminated disease, and micrometastatic disease.[4]

^{212}Bi ($t_{½}$ = 61 min) and ^{213}Bi ($t_{½}$ = 46 min) are among the few α-particle emitting radionuclides that have suitable properties for developing attractive radiopharmaceuticals; they especially present the advantage over other isotopes (i.e. ^{211}At and ^{225}Ac) of having stable isotopes that allow investigating the coordination chemistry of "cold" derivatives. However, the short half-life time of these radionuclides limits their application. Thereby, the design of bismuth(III) chelators is a challenging task in coordination chemistry, as they must present a high affinity for the cation in terms of thermodynamic stability and kinetic inertness, but also fast complexation kinetics. All these criteria that must be fulfilled have limited the number of chelates investigated to date. As a result, a single chelator dedicated to $^{213/212}$Bi has gone beyond the preclinical stage,[5] and the challenge of finding an ideal ligand still persists.

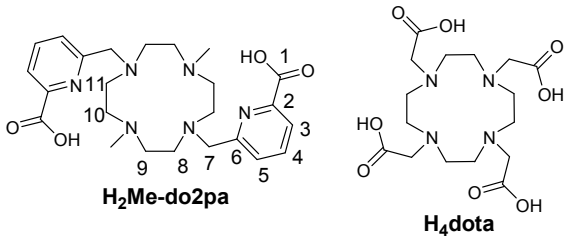

Fig. 1 Structures of ligands H_2Me-do2pa and H_4dota.

Nevertheless, the coordination chemistry of bismuth(III) explored to date is rather scarce.[6] It generally assumes high coordination numbers (6–10) with irregular coordination geometries depending upon the character of the polydentate ligand, of the donor atoms and the nature of the solvent. In addition, bismuth(III) hydrolyses very easily in aqueous solutions even in strong acid media.[7] As a consequence, the study of bismuth(III) complexes in aqueous solution is often difficult due to the formation of hydrolysis products. Tetraazacycloalkane derivatives, particularly cyclen-based ones containing four additionally chelating groups by functionalization of the cyclic nitrogen atoms, are well known for their affinity for lanthanide(III), transition or heavy metal ions. They have proved to be ligands of choice for bismuth(III) complexation, although complexes based on triaza macrocycles have been also investigated.[8] The stability, solution structure and dynamics of the bismuth(III) complex of the well-known H_4dota (Fig. 1) are reported,[9] but the slow complexation rate prevented any RIT application. Nonetheless, a H_4dota derivative has recently been successfully applied for bismuth(III) chelation but has yet to be validated for clinical uses.[10] Recent studies revealed that 2-

pyridylmethyl substituent is a good chelating function for bismuth(III),[11] but to our knowledge cyclen-based ligands bearing picolinate pendant arms have never been used for the complexation of Bi^{3+} cations. A chelator such as H_2**Me-do2pa** (Fig. 1) should offer a N_6O_2 coordination sphere to the metal centre as already demonstrated for lanthanide ions.[12] We, therefore, found this ligand also attractive for the Bi^{3+} sequestration.[13]

The H_2**Me-do2pa** ligand was synthesized according to our previous contribution,[12a] while its Bi^{3+} complex was isolated in good yield (80%) and characterised in solution by NMR (^{1}H and ^{13}C) and HRMS (ESI†). Slow evaporation of an aqueous solution of the [Bi(**Me-do2pa**)]$^+$ complex gave single crystals suitable for X-ray diffraction analysis. The structure is composed of the [Bi(**Me-do2pa**)]$^+$ cation, one disordered nitrate anion and one water molecule involved in hydrogen bonding interaction with the anion and an oxygen atom of a carboxylate group of the ligand. A view of the structure of the complex and bond distances of the metal coordination environment are presented in Fig. 2 (all data are given in Table S1, ESI†). The metal ion is directly bound to the eight donor atoms of the ligand, which adopts a *syn* conformation with the two pendant arms disposed on the same side of the macrocyclic unit. The distance between the Bi centre and the nitrogen atoms of the cyclen unit are *ca.* 0.15 Å longer than the Bi(1)–N(3) distance. The Bi(1)–O(1) distance of 2.385(2) Å is considerably shorter than the Bi–N distances [2.50–2.64 Å], reflecting the tendency of this metal ion to bind to the oxygen donor atoms.[6] The distances between Bi and the donor atoms of the cyclen unit are *ca.* 0.1 Å longer than the average Bi–N distance observed for [Bi(dota)]$^-$ of 2.53 Å.[10]

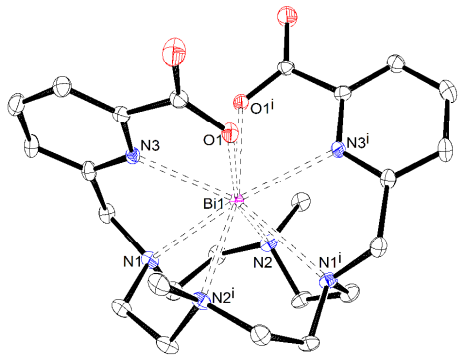

Fig. 2. View of the crystal structure of [Bi(**Me-do2pa**)](NO$_3$)·H$_2$O with atom labelling; hydrogen atoms, the anion and solvent molecule are omitted for simplicity. The ORTEP plot is drawn at the 30% probability level. Bond distances (Å): Bi1—N1 2.643(3), Bi1—N2 2.627(3); Bi1—N3 2.497(3); Bi1—O1 2.385(2).

As described in detail for different complexes of cyclen-based ligands,[14] the *syn* conformation of the ligand in the H_2**Me-do2pa** complexes implies the occurrence of two helicities (one belonging to the crown moiety and the other associated with the layout of the pendant arms).[15] The analysis of the crystal structure shows that the [Bi(**Me-do2pa**)]$^+$ complex adopts a Δ(δδδδ)/Λ(λλλλ) configuration with two enantiomers being present in the crystal lattice, which results in a twisted-square antiprismatic coordination polyhedron around the metal ion. The average twist angle of the two square faces of the polyhedron amounts to -21.2°. A similar coordination polyhedron was observed for [Bi(dota)]$^-$.[9]

The ^{1}H and ^{13}C NMR spectra of the [Bi(**Me-do2pa**)]$^+$ complex were obtained in D_2O solution at pD ~ 7.0 and 298 K. The proton spectrum (Fig. 3) consists of 14 signals corresponding to 14 magnetically non-equivalent proton environments in the ligand (see Fig. 1 for labelling), which points to an effective C_2 symmetry of the complex in solution. This is confirmed by the ^{13}C NMR spectrum, which shows 12 signals for the 24 carbon nuclei of the ligand backbone. The assignments of the proton signals (Table S2, ESI†) were based upon HMQC and HMBC 2D heteronuclear experiments as well as standard 2D homonuclear COSY experiments. The sharp and clear diasterotopic nature of the ^{1}H NMR resonances suggests the presence of a single isomer in solution with no fluxional behaviour being observed at RT. This is in contrast with the behaviour of [Bi(dota)]$^-$, which showed fluxionality due to a Δ(δδδδ) ↔ Λ(λλλλ) enantiomerization process.[9] An ideal candidate for a receptor-targeted radiopharmaceutical should not present static or fluxional isomerization in solution, as fluxional interconversion has been thought to be detrimental for *in vivo* stability, while static isomerization may cause divergent pharmacokinetics and biodistribution.[16]

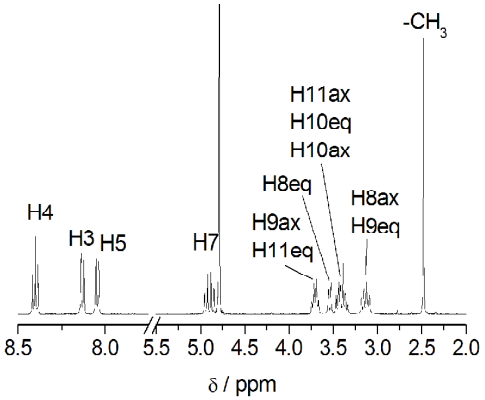

Fig. 3. ^{1}H NMR spectra of the [Bi(**Me-do2pa**)]$^+$ complex at 298 K (pD ~ 7.0, 500 MHz).

The thermodynamic protonation constants of H_2**Me-do2pa** and its stability constants with Bi^{3+} were determined at 25 ºC in 0.5 M KCl aqueous solution (Table S3, Fig. S1, ESI†) using potentiometric or UV spectrophotometric titrations. The [Bi(**Me-do2pa**)]$^+$ complex is fully formed in most of the pH range, therefore titrations at very high pH were used to evaluate the stability constant of the complex, taking advantage of the complex dissociation yielding the bismuth(III) hydroxides $Bi(OH)_3$ and $Bi(OH)_4^-$, as previously described.[17] Accurate values of the hydrolysis constants of bismuth(III) are available and were used for the stability constants determination.[7a] Competition titrations were thus performed using an out-of-cell method also in 0.5 M KCl aqueous solutions, as described in ESI. Our results could be fitted to a model containing

[Bi(**Me-do2pa**)]$^+$ and [Bi(**Me-do2pa**)(OH)], as well as the competing $Bi(OH)_3$ and $Bi(OH)_4^-$ species.

The stability constant obtained for [Bi(**Me-do2pa**)]$^+$ (log K_{BiL} = 34.2) is significantly higher than the one reported for [Bi(dota)]$^-$ (log K_{BiL} = 30.3).[9] However, pBi values, defined as –log $[Bi^{3+}]_{free}$, give a more accurate picture of ligand affinity for the metal when comparing complexes of ligands with differing basicity. Thus, pBi determined for the complexes of H_2**Me-do2pa** and H_4dota led to values of 28.6 and 27.0, respectively, at pH = 7.4 and C_{ligand} = $2\times C_{Bi^{3+}}$ = 20 μM; for H_4dota the reported protonation and stability constants were used.[9] The value for the H_2**Me-do2pa** complex is still higher than the one calculated for H_4dota. Furthermore, the bismuth(III) complex of H_2**Me-do2pa** is fully formed in a large range of pH going from 2 to 11, with the [Bi(**Me-do2pa**)]$^+$ observed as the exclusive species at the pH range 5 to 9 (Fig. S2, ESI†).

The fast formation of the complex is essential for a metallic radiopharmaceutical. Indeed, for any feasible application, the chelator should be able to coordinate rapidly the Bi^{3+} and the corresponding "hot" metal in mild conditions with very high yield. The kinetics of complexation of Bi^{3+} with H_2**Me-do2pa** was determined by UV spectrophotometry in buffered aqueous medium at 25 ºC, under pseudo-first order conditions with 10:1 L:Bi^{3+} ratio. Complexation proceeds very quickly at pH = 5 (acetate buffer) being completed (> 98%) before 2 min, while it is still fast at pH = 3 (citrate buffer) with a half-time of complex formation of 12.5 min and being finished after about 1 h (Fig. S3, ESI†). This result is particularly promising given the slow kinetics of complexation of Bi^{3+} with other ligands such as H_4dota,[8,10] which usually renders its derivatives unsuitable for RIT applications. Additionally, to have an insight of the kinetic inertness of the complex, the dissociation of [Bi(**Me-do2pa**)]$^+$ was studied at 25 °C in 1.0 M HCl solution (Fig. S4, ESI†). The determined $t_{½}$ of 23.9 min reveals that the complex can resist for some time in acidic media with competing donors such as chloride anions. To our knowledge, no comparable studies have been reported for [Bi(dota)]$^-$.

The H_2**Me-do2pa** labelling with ^{213}Bi was performed to evaluated the *in vitro* stability of the [^{213}Bi(**Me-do2pa**)]$^+$ complex using thin layer chromatography (TLC) and reverse phase high-performance liquid chromatography (HPLC) with a gamma NaI scintillator.. To a solution of H_2**Me-do2pa** (0.115 to 3.5 nmol) buffered at pH 7.4 (2 M TRIS) were added a ^{213}Bi solution (3 to 12 MBq in 0.1 HCl / 0.1 M NaI 1:1). Solutions were incubated 15 min at RT or at 90 °C before being analysed. The radiolabeled ligand has a R_f of 0.5 on TLC in the used conditions and the free ^{213}Bi a R_f of 0. With a ratio activity/ligand of 30–40 MBq/nmol, radiochemical yields from 91 to 96% were obtained at 90 ºC and 60% at RT, which is explained by the slower kinetic process under these experimental conditions (Fig 4 A). In similar conditions, the radiolabelling rate of H_4dota is only about 7 % at RT and 80% at 90 °C,[18] which confirm the previous assumptions.[6,8] The good complexation of ^{213}Bi with H_2**Me-do2pa** at 90 °C was confirmed by HPLC, whose chromatogram only showed the presence of [^{213}Bi(**Me-do2pa**)]$^+$ (*retention time*, rt = 5.5 min) but no free ^{213}Bi that has no affinity for the C_{18} column (Fig. 4 B).

Serum stability was studied with a 1 MBq H_2**Me-do2pa** radiolabelled solution (95% of radiochemical purity) mixed with human plasma pool. Plasma stabilities were controlled, by comparison with free ^{213}Bi, after 1 and 2 h of incubation (about 1 and 2 decay periods) at RT by gel permeation chromatography of the plasma and by TLC analysis of the supernatant after plasma proteins precipitation with ammonium sulphate (Fig. 4 C,D). The two gel-filtration chromatograms exhibit almost the same profile with the presence of low molecular weight molecules (< 10000 Da). The only difference observed was the formation of a small fraction of high molecular weight species (rt = 7 min) in the case of free ^{213}Bi, suggesting a slight metal association to blood proteins (Fig. 4 C). Similarly, after precipitation with ammonium sulphate, no significant difference was found between the reference and the complex regarding the radioactivity of precipitate and supernatant (78.8% ± 3.8% in supernatant). TLC experiments done with the supernatants, when compared to the analyses described above (Fig. 4 A), demonstrate the absence of free radionuclide. In the case of free ^{213}Bi, the major part of the recovered metal ion was associated to small size molecules while for [^{213}Bi(**Me-do2pa**)]$^+$ more than 90 % of the radioactivity was found at R_f = 0.5, demonstrating the major presence and the suitable stability in serum of [^{213}Bi(**Me-do2pa**)]$^+$ over the two studied decay periods. These stability tests strengthen those performed in acidic medium with the "cold" analogue [Bi(**Me-do2pa**)]$^+$.

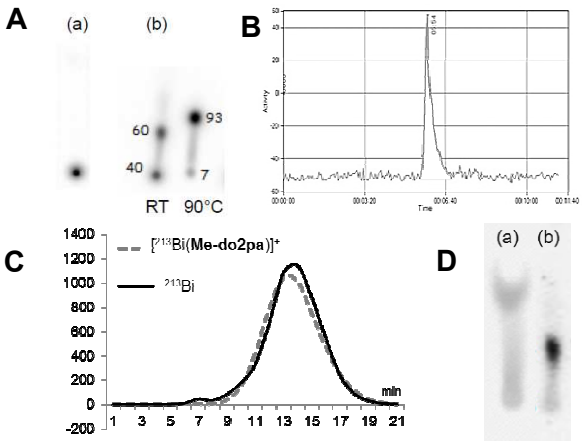

Fig. 4. (A) Silicagel TLC in MeOH / NH_4Cl (20%) 1:1. (a) free ^{213}Bi: R_f = 0. (b) Radiolabelling of H_2**Me-do2pa** (R_f = 0.5) at RT (left), and 90 °C (right). (B) HPLC chromatogram of [^{213}Bi(**Me-do2pa**)]$^+$ (complexation at 90 °C) with rt = 5.5 min (C_{18} Kromasil column, 20 min linear gradient of 0.01 % aqueous TFA-CH_3CN). (C) Gel-filtration chromatogram of plasma containing free ^{213}Bi and [^{213}Bi(**Me-do2pa**)]$^+$. (D) TLC of serum after protein precipitation; silicagel; MeOH/NH_4Cl 20%; 1:1. (a) free ^{213}Bi in plasma, (b) [^{213}Bi(**Me-do2pa**)]$^+$.

In summary, taking into account the very promising use of bismuth(III) in α-radioimmunotherapy, a new chelator H_2**Me-do2pa** was designed to find alternatives to H_4dota, which does not fulfill all the criteria for practical applications. The "cold" [Bi(**Me-do2pa**)]$^+$ complex forms quickly and is highly stable and inert, as proved by several thermodynamic and kinetic studies in solution, clearly appearing as an attractive chelate. Moreover, its existence in solution as a single diastereoisomer, for which fluxional interconversion processes were not

observed, is also thought to be beneficial for radiopharmaceutical applications in terms of *in vivo* stability and non-divergent biodistribution. All these properties were confirmed by the fast complexation of ^{213}Bi with free H_2**Me-do2pa** and the inertness of the radiolabelled complex in serum. The ligand design also allows easy introduction of bioconjugation functions either by replacement of a *N*-methyl group or by substitution on the 4-position of the picolinic moiety, to obtain Bifunctional Chelating Agents (BCAs). Finally, one should bear in mind the short $t_{½}$ value of the α-emitting isotopes of bismuth. Thus, we are currently studying the complexation of Pb^{2+} by H_2**Me-do2pa**, as ^{212}Pb ($t_{½}$ = 10.6 h) is the longer-lived parent nuclide of ^{212}Bi and can serve as an *in situ* generator of ^{212}Bi,[19] well known to be additionally safer (decrease of the γ emissions).

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Notes and references

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†Electronic Supplementary Information (ESI) available: Experimental section, crystallographic data, ^{1}H NMR shifts of the complex, thermodynamic protonation and complex stability constants, speciation diagrams, time course of complex formation and dissociation assays. See DOI: 10.1039/c000000x/

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