

Bifunctional Bisphosphonate Complexes for the Diagnosis and Therapy of Bone Metastases

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**Electronic Supplemental Information
(ESI)**

General Procedures. Reagents were obtained from commercial sources and used as received unless otherwise noted. Alendronate monosodium was synthesized following a reported procedure.¹ NMR spectra were obtained using a Bruker Avance 400 at 20 °C in D₂O (Cambridge Isotope Laboratories) unless otherwise noted. ¹H chemical shifts are referenced with respect to the residual solvent peak (δ_{H} 4.79 ppm).² ³¹P resonances were referenced to an external solution of 85% H₃PO₄ (δ_{P} 0 ppm). ¹³C chemical shifts were left unreferenced. High-resolution mass spectra were obtained at the EPSRC National Mass Spectrometry Service Centre at Swansea University using a Thermo Scientific LTQ Orbitrap XL spectrometer coupled to an Advion TriVersa NanoMate using nanoelectrospray ionization. Elemental analyses were carried out at the Elemental Analysis Service at London Metropolitan University. Reverse phase (RP) and size exclusion (SE) HPLC analyses were carried out using an Agilent 1200 series system equipped with a quadruple pump, a UV detector set at 254 nm (RP) or 280 nm (SE) and a radiodetector (Lablogic) optimised for the detection of γ -rays. For RP studies, Agilent Zorbax Eclipse XDB-C18 columns (5 μm , 4.6 x 150 mm (Analytical); 5 μm , 21.2 x 150 mm (Preparative)) were used. For SE chromatography, a TOSOH TSK-GEL G2500PW_{XL} was used. [Re(CO)₃(H₂O)₃]⁺ was synthesised and characterised following a literature reference.³ [^{99m}TcO₄]⁻Na and ^{99m}Tc-MDP were obtained from the Radiopharmacy at Guy's and St Thomas' Hospital NHS trust, London, UK.

RP-HPLC Methods:

The following methods were employed using the stated conditions.

Method A:

- Solvent **A**: H₂O (0.1% TFA)
- Solvent **B**: CH₃CN (0.1% TFA)

Time	Eluate	% A	% B
0 min	1 mL/min (Analytical) 5 mL/min (Preparative)	100	0

3 min	1 mL/min (Analytical) 5 mL/min (Preparative)	100	0
16 min	1 mL/min (Analytical) 5 mL/min (Preparative)	70	30
18 min	1 mL/min (Analytical) 5 mL/min (Preparative)	100	0
20 min	1 mL/min (Analytical) 5 mL/min (Preparative)	100	0

Method B:

- Solvent **A**: 0.05 M TEAP (Triethylammonium phosphate) pH=2.25
- Solvent **B**: MeOH

Time	Eluate	% A	% B
0 min	1 mL/min	100	0
5 min	1 mL/min	100	0
6 min	1 mL/min	75	25
9 min	1 mL/min	66	34
20 min	1 mL/min	0	100
30 min	1 mL/min	0	100
31 min	1 mL/min	100	0
35 min	1 mL/min	100	0

Synthesis and Characterisation

Synthesis and characterisation of dipicolylamine-alendronate (3)

To a suspension of alendronate monosodium (**2**) (389 mg, 1.43 mmol) in 12 mL of distilled water was added NaOH (260 mg, 6.50 mmol) and the mixture stirred at room temperature for 10 min until all the bisphosphonate had dissolved. At this point, 2-picolyl chloride hydrochloride (**1**) (472 mg, 2.86 mmol) was added which resulted in the solution turning slightly orange. The pH of the reaction mixture was 12.1. After 16 h stirring at room temperature the pH of the solution was 9.7 and was increased back to 12.1 by addition of more NaOH (56 mg, 1.43 mmol). The mixture was left stirring at room

temperature for a further 24 h, after which the pH had lowered to 10.7. At this point, the reaction mixture was washed with dichloromethane (3 x 10 mL) and the organic washings discarded. The water layer was evaporated to dryness to yield the crude product as a salmon-pink solid (811 mg). **3** was purified as needed as the mono-TFA salt by dissolving in water (0.1 % TFA) and purifying using preparative RP-HPLC using method A (Rt = 7:06 mm:ss).

^1H NMR (400.1 MHz, D_2O , 298 K): δ_{H} (ppm); 8.41 (2H, d, $J = 4.9$ Hz, Py- H^6), 7.77 (2H, dd, $J_{\text{AB}} \approx J_{\text{AC}} \approx 7.8$ Hz, Py- H^5), 7.42 (2H, d, $J = 7.7$ Hz, Py- H^3), 7.31 (2H, dd, $J_{\text{AB}} \approx J_{\text{AC}} \approx 6.5$ Hz, Py- H^4), 3.93 (4H, s, py- $\text{CH}_2\text{-N}$), 2.71 (2H, br, -N- $\text{CH}_2\text{-CH}_2\text{-}$), 1.97 (2H, br, - $\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-}$), 1.83 (2H, br, - $\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-}$); ^{13}C NMR (100.6 MHz, D_2O , 298 K): δ_{C} (ppm); 152.5 (2C, Py), 147.06 (2CH, Py), 141.45 (2CH, Py), 127.23 (2CH, Py), 116.23 (q, $^1J_{\text{C-F}} = 291$ Hz, $\text{CF}_3\text{-COOH}$), 72.97 (t, $^1J_{\text{C-P}} = 140$ Hz, $\text{CH}_2\text{-C}(\text{PO}_3)_2(\text{OH})$), 55.51 (C, PyCH_2), 55.07 (C, PyCH_2), 38.67 (- $\text{CH}_2\text{-}$), 30.92 (- $\text{CH}_2\text{-}$), 20.64 (- $\text{CH}_2\text{-C}(\text{PO}_3)_2(\text{OH})$); $^{31}\text{P}\{^1\text{H}\}$ NMR (161.9 MHz, D_2O , 298 K): δ_{P} (ppm); 19.09. HR-ESI-MS (- ion): found: 430.0931 (100 %), calc. 430.0938 for $[\mathbf{3}]^-$, and matches theoretical isotope distribution. Found: C, 36.42; H, 3.52; N, 6.30. $\text{C}_{20}\text{H}_{23}\text{F}_6\text{N}_3\text{O}_{11}\text{P}_2$ requires: C, 36.54; H, 3.53; N, 6.39. $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$: 3375br (O- $\text{H}_{\text{stretch}}$), 1111s (P=O $_{\text{stretch}}$), 959vs (P-O $_{\text{stretch}}$).

Synthesis and characterisation of $\text{Re}(\text{CO})_3$ -dipicolylamine-alendronate (**5**)

5 was synthesized in an NMR tube by mixing an equimolar amount of a 2.54 mM solution of **3** in 0.5 mL of 100 mM carbonate buffer pH 9 in D_2O and $[\text{Re}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ from a stock solution in D_2O and heating for 30 minutes at 100 °C. To confirm the formation of **5**, the crude reaction was monitored by RP-HPLC to show a single peak with Rt 15:18 (method A) and 12:58 mm:ss (method B, Fig. S2)

^1H NMR (400.1 MHz, D_2O , 298 K): δ_{H} (ppm); 8.84 (2H, d, $J = 5.2$ Hz, Py- H^6), 7.84 (2H, td, $J = 7.8$ and 1.5 Hz, Py- H^5), 7.46 (2H, d, $J = 7.9$ Hz, Py- H^3), 7.26 (2H, pseudo-triplet, $J \approx 6.5$ Hz, Py- H^4), 4.24 (4H, s, py- $\text{CH}_2\text{-N}$), 2.65 (2H, br, -N- $\text{CH}_2\text{-CH}_2\text{-}$), 1.82 (4H, br, - $\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-}$); $^{31}\text{P}\{^1\text{H}\}$ NMR (161.9 MHz, D_2O , 298 K): δ_{P} (ppm); 18.20;

HR-ESI-MS (- ion): found: 701.0325 (100 %), calc. 701.0310 for $C_{19}H_{22}N_3O_{10}P_2Re$ and matches theoretical isotope distribution. $\nu_{max}(KBr) / cm^{-1}$: 3412br (O-H_{stretch}), 2030vs (C=O_{stretch}), 1906s (C=O_{stretch}), 1064s (P=O_{stretch}), 965vs (P-O_{stretch}).

Dipicolylamine-alendronate titration studies with $[Re(CO)_3]^+$

2.54 mM solutions of **3** in 0.5 mL of 100 mM carbonate buffer at pH 9 in D_2O were incubated with increasing molar equivalents of $[Re(CO)_3]^+$ for 30 minutes at 100 °C. After cooling, each solution was analysed by RP-HPLC using method A. **3** elutes at 7:06 mm:ss, whereas **6** elutes at 15:08 mm:ss. Formation of **5** is seen after the addition of 0.5 equivalents of $[Re(CO)_3]^+$. Unidentified peaks were observed when more than 1.5 equivalents were used, consistent with the formation of BP-bound multimetallic species.

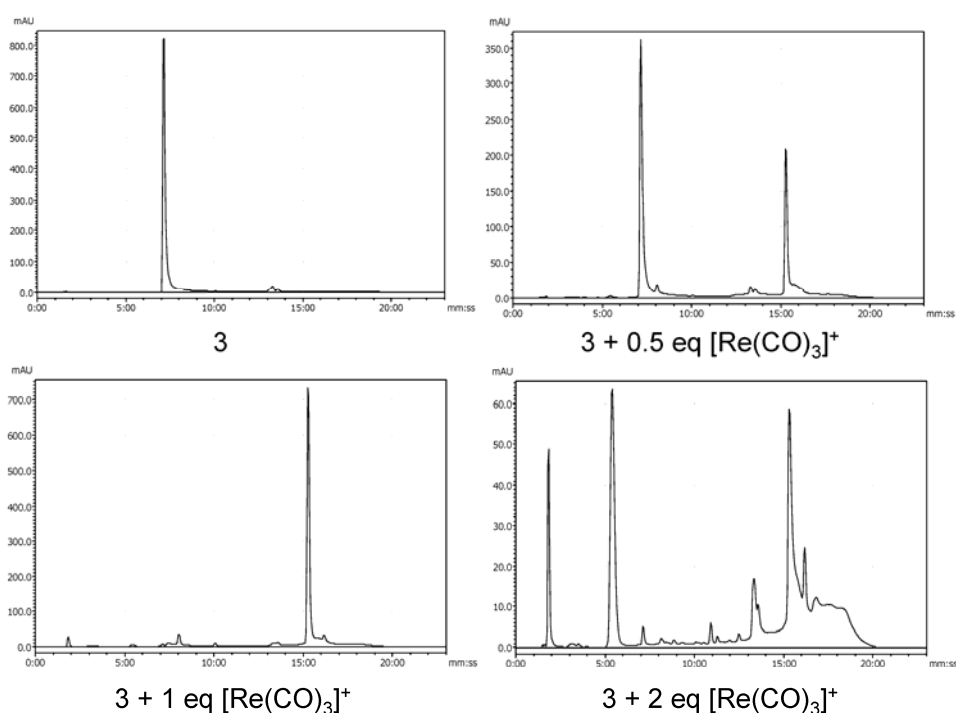


Fig. S1. RP-HPLC chromatograms of **3** (top left) and **3** plus increasing amounts of $[Re(CO)_3]^+$.

Radiolabelling

$[\text{}^{99\text{m}}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ was synthesized from an Isolink™ kit (Mallinckrodt Medical B.V.) as described in the manufacturer's instructions. Briefly, 1 mL of $[\text{}^{99\text{m}}\text{TcO}_4]^-$ (100 MBq) was added to the kit and the vial heated at 100 °C for 20 minutes. After cooling to room temperature, the contents were neutralised with 120 μL of 1M HCl and analysed by HPLC to confirm the purity of the newly formed $[\text{}^{99\text{m}}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$.

To establish the minimum concentration at which **3** could be efficiently labelled with $[\text{}^{99\text{m}}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$, five solutions of different concentrations of **3** were prepared (1.7×10^{-3} M, 1.7×10^{-5} M, 1.7×10^{-7} M and 1.7×10^{-9} M) in 100 mM carbonate buffer at pH 9. An aliquot (100 μL) of each solution was mixed with 100 μL of $[\text{}^{99\text{m}}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ (15 MBq), before being heated in a sealed vial for 30 minutes at 100 °C. After cooling the solutions in a water bath, each sample was analyzed by analytical RP-HPLC using method A. Very efficient labelling (22×10^3 GBq/g, radiochemical yield >98 %) was found when concentrations higher than 1.7×10^{-5} M were used and no labelling when the concentration was lower than 1.7×10^{-7} M. Using HPLC method B, **6** elutes at 13:26 mm:ss (γ detection) and **5** at 13:14 mm:ss (UV detection) (Fig. S2). $[\text{}^{99\text{m}}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ and $[\text{}^{99\text{m}}\text{TcO}_4]^-$ elute at 03:20 and 8:00 mm:ss, respectively (γ detection, Fig. S3).

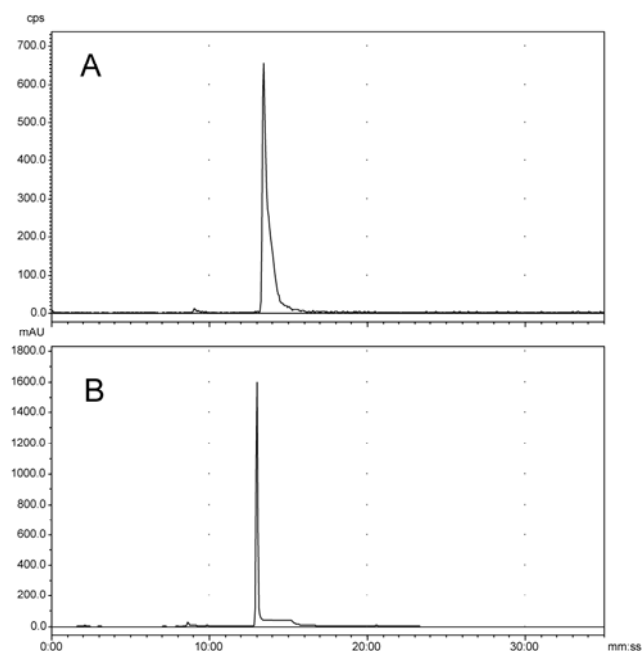


Fig. S2 RP-HPLC chromatograms (method B) of **6** (A, γ detection, 13:26 mm:ss) and **5** (B, UV detection, 13:14 mm:ss). The difference in R_t observed (12 s) is due to the lag time between the in-line γ and UV detectors.

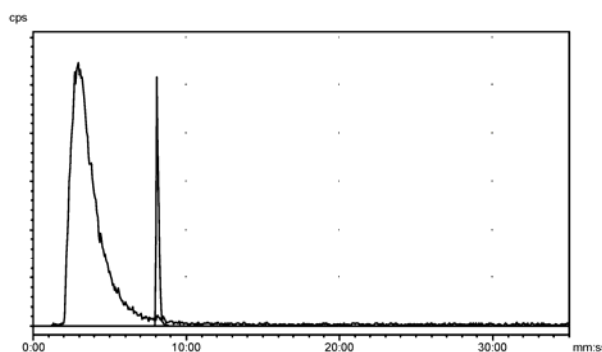


Fig. S3 Overlaid RP-HPLC radiochromatograms (γ detection) using method B of $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ (03:20 mm:ss) and $[^{99m}\text{TcO}_4]^-$ (08:00 mm:ss).

For *in vivo* studies, the same procedure as above was followed but mixing 200 μL of **3** (1.7×10^{-3} M) and 200 μL $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ (200 MBq) and heating at 100 $^\circ\text{C}$ in a sealed vial for 20 minutes. **6** (1.4 GBq/g, radiochemical yield >98 %) was then diluted with saline as needed and the solution was made sterile prior to injection by using a 0.2 μm sterile syringe filter.

***In vitro* calcium salt binding studies**

In order to assess the binding of **6** and ^{99m}Tc -MDP to different calcium salts, 1 mg/mL suspensions of hydroxyapatite (HA), β -tricalcium phosphate, calcium phosphate dibasic, calcium oxalate, calcium carbonate and calcium pyrophosphate in 50 mM TRIS pH 6.9 were prepared. Immediately after, 10 μL (0.8 MBq) of either **6** or ^{99m}Tc -MDP were added to each suspension and vortexed for 1 h at room temperature. The solutions were then centrifuged (5 min, 10,000 rpm) and a 100 μL aliquot of the supernatant of each sample analyzed using a gamma counter. A control sample lacking calcium salt was also prepared and counted as a control. The results are expressed as % binding using the following equation:

$$\% \text{ binding} = \left(1 - \frac{\text{CPM}_s}{\text{CPM}_c} \right) \times 100$$

where CPM_s are the counts per minute of each sample and CPM_c are the counts per minute of the control.

Serum binding and stability studies

Human serum samples (1 mL, Sigma-Aldrich) were incubated with 200 μ L (14 MBq) of **6** in a 5% CO₂/95% air atmosphere at 37 °C for 24 h. Aliquots (20 μ L) were taken at 0, 3, 6 and 18 hours after mixing and analysed by SE-HPLC using a flow of 1 mL/min of PBS buffer as eluent. Serum proteins eluted at 5 minutes, whereas **6** eluted at 10 minutes. The percentages were obtained by measuring the area under the peaks for both species in the gamma chromatogram.

^{99m}Tc-MDP eluted with similar Rt as serum proteins (consistent with its polymeric nature) ruling out the use of SE-HPLC. An established alternative method for measuring serum binding was used. Thus, human serum samples (1 mL) were incubated with 200 μ L (14 MBq) ^{99m}Tc-MDP in a 5% CO₂/95% air atmosphere at 37°C for 24 h. Aliquots (50 μ L) were taken at 0, 3, 6 and 18 hours after mixing and the proteins of each time point were precipitated by addition of EtOH (70 μ L). The samples were centrifuged and the supernatant separated. The precipitated proteins were washed (2 times) with another 70 μ L of EtOH. The radioactivity of supernatant and precipitated proteins was counted using a gamma counter.

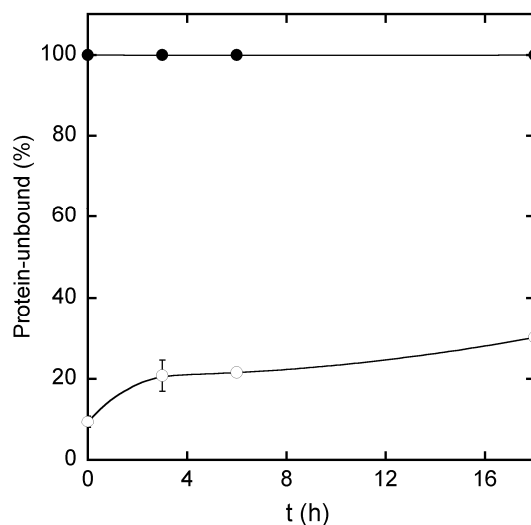


Fig. S4. Serum protein binding study of **6** (black circles) and ^{99m}Tc-MDP (empty circles).

In order to assess the stability of the binding of **6** to HA in serum, an identical experiment was set in the presence of 10 mg of HA. In this case, at each time point, 50 μ L of the supernatant were withdrawn after centrifugation of the mixture and analyzed using a gamma counter. **6** remained bound to HA in serum for at least 18 h.

To assess the chemical stability of **6**, the serum samples were centrifuged after 18 h using a Vivaspin filter with a molecular cut-off of 5000. The entire radioactivity transferred to the filtrate and was analysed by analytical RP-HPLC (Method B) to confirm that **6** had not decomposed.

Biodistribution Studies

Biodistribution studies were carried out in accordance with British Home Office regulations governing animal experimentation. Adult female Balb/C mice were injected intravenously with approximately 50 MBq of **6** or ^{99m}Tc-MDP in 200 μ l (n=3, ^{99m}Tc-MDP; n=4, **6**). After 6.5 hours, the mice were culled by cervical dislocation and the following organs were dissected; femur, pancreas, kidneys, heart, stomach, spleen, intestine, liver, lung, muscle, tail and a sample of blood. Each sample was weighed and counted with a gamma counter (LKB compugamma), together with standards prepared

from a sample of the injected material. The percent of injected dose per gram of tissue was calculated for each tissue type.

Imaging Studies

Adult female Balb/C mice were injected i.v. in the tail vein with approximately 50 MBq of ^{99m}Tc -MDP or **6** in 200 μL (n=3). At 1, 2 and 6 hour timepoints (**6**) and 30 minutes, 1, 2, 4 and 6 hour timepoints (^{99m}Tc -MDP), the animals were anesthetised using isoflurane and imaged using a NanoSPECT/CT animal scanner (Bioscan Inc.). Whole-body SPECT images were obtained in 20 projections over 30 min using a 4-head scanner with 4 x 9 (2 mm) pinhole collimators in helical scanning mode and CT images with a 45 kVP X-ray source, 1000 ms exposure time in 180 projections over 9 min. Images were reconstructed in a 256 x 256 matrix using HiSPECT (Scivis GmbH), a reconstruction software package, and images were fused using proprietary Bioscan InVivoScope (IVS) software.

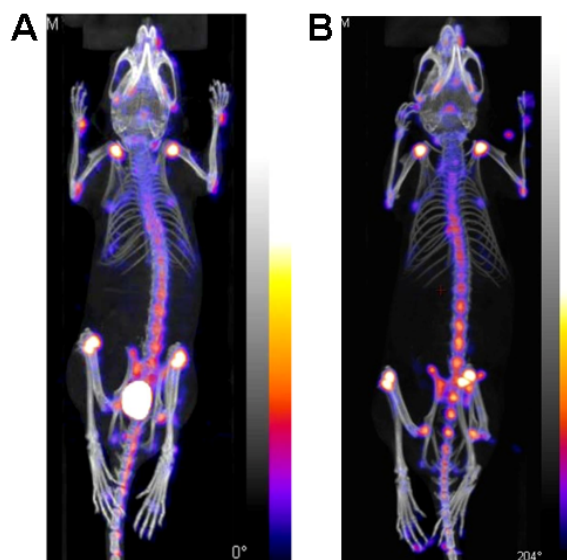


Fig. S5 SPECT/CT images showing the essentially identical bone uptake of ^{99m}Tc -MDP (A) and **6** (B) in mice.

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