Synthesis of novel bisphosphonate polyamine conjugates and their affinity to hydroxyapatite

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Dedicated with respect to Professor Pawel Kafarski

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Abstract

Bisphosphonates are negatively charged molecules, widely used as bone drugs since they have high affinity to bone surface. Polyamines are small organic bases that are positively charged under the physiological conditions and they participate in the regulation of important cellular functions. In this study, we report a general synthesis strategy for bisphosphonate-polyamine conjugates starting from ethenylidene bisphosphonates and N-Boc protected polyamines. Four novel conjugates were prepared and characterized with spectroscopic methods. The affinity of these novel compounds to hydroxyapatite was also determined by using $^{99m}$Tc procedure.

Keywords: Ethenylidene bisphosphonate, polyamine, synthesis, hydroxyapatite, technetium-99m labelling

Introduction

Bisphosphonates (BPs) are enzymatically stable negatively charged structural analogs of naturally occurring pyrophosphates (Figure 1). During their lifetime of 50 years, BPs have been used for many purposes starting from water softeners by inhibiting the crystallization of calcium salts and ending up to drug applications. The clinical efficiency of BPs is based on the high affinity for Ca$^{2+}$ ions in bone mineral hydroxyapatite.$^1$ BPs are used in treatment of calcium metabolism disorders, e.g. osteoporosis, because they are effective inhibitors of bone resorption,$^1$ and also as bone scanning agents if linked to a gamma-emitting technetium isotope.$^2$
Polyamines (PAs) are aliphatic organic bases that are positively charged under the physiological conditions. They exist in millimolar concentrations in all eukaryotic cells and participate in the regulation of important cellular functions, such as proliferation, differentiation and functioning of ion channels like NMDA-receptors and inward rectifier potassium channels. Natural PAs, putrescine, spermidine and spermine (see Figure 1) and their analogues, have been investigated as chemopreventative and antiparasitic agents, NMDA receptor modulators, polyamine-based venoms, metal chelators and as potential carriers for drug delivery.\textsuperscript{3-5} However, while both BPs and PAs are well known compounds there is only some studies related to BP-PA conjugates, in which m=n=2 and R=H (see Figure 1).\textsuperscript{6,7}

Here we report the first general synthesis strategy to prepare novel bisphosphonate polyamine (BP-PA) conjugates starting from easily available tetralkyl ethenylidenebisphosphonate. Preparation method allows producing either tetraacidic BPs or partially esterified BPs, which possibly have longer half-life in blood circulation compared to tetraacidic BPs.\textsuperscript{8} The prepared molecules are potential SPECT imaging agents for calcium plaques in brains, since the BP part is an excellent $^{99m}$Tc chelator and PA moiety is expect to cross the blood-brain-barrier.\textsuperscript{9,10}

Results and Discussion

Synthesis strategies to BP-PA are dependent on the chain length m (see Figure 1) between the BP bridging carbon and the first nitrogen atom in PA chain. According to literature search the easiest starting point to prepare BP-PA conjugates are ethenylidenebisphosphonates, which allow direct Michael addition to activated double bond as shown in Scheme 1, leading to compounds in which m=1.\textsuperscript{11} In the case m=0, a three-component reaction is needed between a primary amine, trialkyl orthoformate and dialkyl phosphate, but low yields and unexpected by-products are obtained.\textsuperscript{12} Longer chains (m > 1) are typically prepared by adding first appropriately substituted alkyl halide either to 1 or 2, but additional reaction steps are needed to obtain the target molecule.\textsuperscript{13}

Our approach to synthesize these novel BP-PA conjugates (Scheme 1) follows the easiest strategy starting from BP tetraesters 1a or b, which were first converted into ethenylidene derivatives 2 by the known method.\textsuperscript{11} End products 4 and 6 were obtained without difficulties,
when 2a was purified chromatographically, and 2b was distilled under reduced pressure before use. This strategy also allows to prepare P,P'-diesters of BP-PA conjugates (4c), since selective demethylation of 2a is obtained with NaI in acetone to give 2c in good yield.

Scheme 1. Synthesis of bisphosphonate-polyamine compounds 6, 4a, 4b and 4c. Reagents: (i) 1. (H₂CO)ₙ, Et₂NH, MeOH; 2. p-TsOH, toluene; (ii) H₂N(CH₂)ₙNHBoc, toluene; (iii) H₂N(CH₂)₃NH(CH₂)₄NH(CH₂)₃NH₂, K₂CO₃, toluene; (iv) NaI, acetone; (v) H₂N(CH₂)₄NHBoc, methanol; (vi) 4 M HCl.

In the next step two Boc protected PAs (N-Boc-propane-1,3-diamine, N-Boc-butane-1,4-diamine) were conjugated to 2a by using Michael type addition. The intermediates 3a and 3b were easily obtained after refluxing in toluene, but intermediate 3c was more complicate to prepare from P,P'-diester 2c due to solubility problems. The best 84% yield was obtained after 7 days of refluxing in methanol. In the last step both isopropyl and Boc protective groups were simultaneously removed with acid catalysis hydrolysis to give the end products 4a and 4b. In the case of 4c only Boc protection group was removed when 3c was treated with 4M HCl and 4c was obtained with quantitative yield. This method was also tested to prepare more complicated BP-PAs, since N,N'-bis(3-aminopropyl)butane-1,4-diamine (Spm) afforded without difficulties mostly intermediate 5 and according to NMR and mass spectrometry results the only impurity was unreacted Spm starting material.

The prepared compounds were identified by using ¹H, ¹³C and ³¹P NMR spectroscopy and the results are given in the experimental part. Assignments of the signals were verified by using 2D ¹H-¹H COSY (homonuclear correlated spectroscopy), 2D ¹³C-¹H HSQC (heteronuclear single quantum
correlation spectroscopy), and 2D \(^{13}\text{C}^\text{1}^\text{H}\) HMBC (heteronuclear multiple bond correlation spectroscopy) spectra. Moreover, some interesting spin systems were analyzed by using PERCH software.\(^1\)\(^6\)

In the case of ethenylidene BPs 2, \(^1\text{H}\) NMR spectra were rather complicated due to AA’XX’ spin system in H\(_2\text{C}=\text{CP}_2\) fragment and four different coupling constants (\(2J_{\text{HH}}\), \(3J_{\text{HP}}\), \(3J_{\text{HP}}\) and \(2J_{\text{PP}}\)) were analyzed for 2a by using PERCH software. Moreover, \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra for compounds 2a, 3a, 3b, and 5 showed two separated CH\(_3\) doublets due to the different electronic environments of the CH\(_3\) groups.\(^1\)\(^7\) The same phenomenon was observed also for OCH\(_2\text{Me}\) carbon.

The last peculiarity of the studied compounds was observed in \(^{13}\text{C}\) NMR spectra for symmetric ethenylidenes 2b and 2c. The symmetrical structures of these molecules resulted in an AA’X (P’-C-P-O-C) spin system and OCH\(_3\) signal was expected to give a second-order quintet in the carbon spectra.\(^1\)\(^8\) However, only a triplet was observed due to large \(2J_{\text{PP}}\) coupling (ca. 45 Hz). Successful addition of PAs to 2 was easily detected from \(^1\text{H}\) NMR spectra, since characteristic doublet triplet (dt) and triplet triplet (tt) couplings were observed for N-CH\(_2\)-CHP\(_2\) protons, respectively. End products were also characterized with accurate mass spectrometric studies. In some cases (e.g. 4c) sodium adduct, like [M+Na]\(^+\) and [M+Na\(_2\)]\(^+\) give rise to major signal over typical [M+H]\(^+\) signal.

The relative binding affinities of the \(^{99m}\text{Tc}\) labelled bisphosphonate molecules were studied using hydroxyapatite precipitation tests.\(^1\)\(^9\) Compounds 4a-c, and 6 and etidronate (1-hydroxy-ethane-1,1-diphosphonic acid) were first labeled with \(^{99m}\text{Tc}\). Radiochemical purity was approximately >99%, with the exception of compound 4c, of which the purity was 90%. This indicates in average high technetium chelating properties of all the compounds. The affinity of the studied compounds with hydroxyapatite (Table 1.) was determined by measuring the remaining activity of the supernatant after centrifugation.\(^1\)\(^9\) As expected the affinity to the hydroxyapatite was highest with the compound 4a without any methylation. Also the length of the polyamine spacer affected to the binding efficiency. The affinity of the all polyamine conjugates (4a-c and 6) was higher than the etidronate control. This was expected, as amino-containing bisphosphonates have been shown to have a higher affinity to hydroxyapatite compared to non-amino BPs.\(^2\)\(^0\)

Table 1. The relative affinity of etidronate and BP-PA conjugates to hydroxyapatite after one hour incubation at room temperature. The binding activity of the all BP-PA conjugates was higher than the control compound

| Compound | Affinity to hydroxyapatite (%) |
|----------|--------------------------------|
| Etidronate | 30±9                       |
| 4a        | 83±6                       |
| 4b        | 72±3                       |
| 4c        | 53±8                       |
| 6         | 72±19                      |
Conclusions

A general method to prepare bisphosphonate polyamine conjugates was developed starting from ethenylidene bisphosphonates and Boc protected polyamines containing one primary amine group. This method allows preparing either tetraacid or partially esterified bisphosphonate polyamine conjugates. Bisphosphonate moieties were labeled with technetium-99m by using conventional stannous chloride reduction under the nitrogen atmosphere. All the conjugates showed radiochemical purity of over 99% except compound 4c (90%).

Experimental Section

General. \(^1\)H, \(^{13}\)C, and \(^{31}\)P NMR spectra were recorded at 500.1, 125.8, and 202.4 MHz on a Bruker Avance 500 DRX (Germany) spectrometer using tetramethylsilane (TMS) in CDCl\(_3\) and MeOD or sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP) in D\(_2\)O as internal standards. High-resolution electrospray mass spectra were obtained on Applied Biosystems/MDS Sciex QSTAR XL. Radioactivity was measured with LKB-Wallac CliniGamma-gamma counter (WALLAC OY, Finland). Tubes for gamma counter and microcentrifuge tubes were purchased from Sarstedt. Centrifuge that was used in the binding assay was Heraeus Biofuge pico. \(^{99m}\)Tc-pertechnetate (\(^{99m}\)TcO\(_4^-\)) in saline solution was obtained as a donation from the Kuopio University Hospital. Thin layer chromatography was accomplished using Merck TLC aluminium sheets (silica gel 60 F\(_{254}\)) and development was done with potassium permanganate. Column chromatography was carried out on Merck silica gel (70-230 mesh). Instant thin layer chromatography (ITLC) plates were purchased from PALL Life Sciences. Hydroxyapatite powder had been purchased from Sigma. Compounds are named based on ChemOffice name generator.

**Tetraisopropyl ethenylidenebisphosphonate (2a).** Prepared by the known method\(^{11,14}\) from tetraisopropyl methylenediphosphonate. \(^1\)H NMR (CDCl\(_3\)): \(\delta_H 6.92\) (2H, AA’XX’ spin system, \(^2J_{HH'} = 2.7\) Hz, \(^3J_{HP} = 25.5\) Hz, \(^2J_{PP} = 46.4\) Hz, \(^2J_{FP} = 49.7\) Hz, CH\(_2\)), 4.77- 4.70 (4H, m, CH\(_2\Me_2\)), 1.35- 1.31 (24H, m, CHMe\(_2\)). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta_C 147.34\) (CH\(_2\)), 134.81 (t, \(^1J_{CP} = 168.6\) Hz, P\(_2\)C), 71.42 (4C, t\(^{18}\), \(^2J_{CP} = 3.0\) Hz, OCH\(_2\)), 24.04 (4C, CMe\(_2\)), 23.82 (4C, CMe\(_2\)). \(^{31}\)P NMR CDCl\(_3\)): \(\delta_P 12.25\).

**Tetramethyl ethenylidenebisphosphonate (2b).** Prepared by the known method\(^1\) from tetramethyl methylenediphosphonate.. \(^1\)H NMR (CDCl\(_3\)): \(\delta_H 7.13-6.92\) (2H, m, CH\(_2\)), 3.84-3.78 (12H, m, OCH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta_C 150.09\) (CH\(_2\)), 129.98 (t, \(^1J_{CP} = 167.5\) Hz, P\(_2\)C), 53.09 (4C, t\(^{18}\), OCH\(_3\)). \(^{31}\)P NMR (CDCl\(_3\)): \(\delta_P 16.65\).

**Dimethyl ethenylidenebisphosphonate disodium salt (2c).** Tetramethyl ethenylidenebisphosphonate 2b (1.0 g, 0.004 mol, 1.0 equiv) and sodium iodide (1.23 g, 0.008 mol, 2.0 equiv) were dissolved in dry acetone (20 ml) and the reaction mixture was kept in 60 °C for 18 h. The white solid was filtered and washed first with acetone (20 ml) and then with ethyl acetate (10 ml).
The product was dried under reducer pressure to give disodium salt of 2c (857 mg, 82%) as a white solid. $^1$H NMR (D$_2$O): $\delta_\text{H}$ 6.61-6.46 (2H, m, CH$_2$), 3.55-3.50 (6H, m, OCH$_3$). $^{13}$C NMR (D$_2$O): $\delta_\text{C}$ 144.96 (CH$_2$), 140.94 (t, $J_{\text{CP}}$ = 152.4 Hz, PCP), 54.69 (2C, t$^{18}$, OCH$_3$). $^{31}$P NMR (D$_2$O): $\delta_\text{P}$ 14.56.

**Tert-butyl [3-[[2,2-bis(diisopropoxyphosphoryl)ethyl]amino]propyl]carbamate (3a).** To a stirred suspension of tetraisopropyl ethenylidenebisphosphonate 2 (300 mg, 0.84 mmol, 1.0 equiv) in dry toluene (15 ml), was added a solution of N-Boc-propane-1,3-diamine (150 mg, 0.84 mmol, 1.0 equiv) in dry toluene (5 ml). The reaction mixture was refluxed for 2 h, concentrated under reduced pressure, dissolved in DCM (10 ml), washed with saturated aqueous NaHCO$_3$ solution (3 x 5 ml) and water (3 x 5 ml), dried (MgSO$_4$) and concentrated under reduced pressure to give 3a (350 mg, 78%) as a yellow oil. $^1$H NMR (CDCl$_3$): $\delta_\text{H}$ 4.85-4.71 (4H, m, CHMe$_2$), 3.24-3.16 (2H, m, CH$_2$CH$_2$NH), 3.11 (2H, dt, $J_{\text{HP}}$ = 16.4 Hz, $J_{\text{HH}}$ = 6.0 Hz, CHCH$_2$NH), 2.67-2.61 (2H, m, NHCH$_2$), 2.49 (1H, tt, $J_{\text{HP}}$= 23.5 Hz, $J_{\text{HH}}$ = 6.0 Hz, P$_2$CH), 1.70-1.61 (2H, m, CH$_2$CH$_2$CH$_2$), 1.37 (9H, brs, CMe$_3$), 1.40-1.29 (24H, m$^1$, CHMe$_2$). $^{13}$C NMR (CDCl$_3$): $\delta_\text{C}$ 156.15 (C=O), 71.41 (2C, m$^1$, OCH), 71.22 (2C, m$^1$, OCH), 46.86 NHCH$_2$), 46.12 (t, $J_{\text{CP}}$ = 4.0 Hz, CHCH$_2$), 39.19 (CH$_2$NH), 38.74 (t, $J_{\text{CP}}$ = 133.7 Hz, PCP), 29.74 (CH$_2$CH$_2$CH$_2$), 28.55 (3C, CMe$_3$), 24.28 (4C, m$^1$, CHMe$_2$), 24.03 (4C, m$^1$, CHMe$_2$). $^{31}$P NMR (CDCl$_3$): $\delta_\text{P}$ 21.81.

(2-[(3-Aminopropyl)amino]ethane-1,1-diyl)diphosphonic acid (4a). Intermediate 3a was dissolved in 4 M HCl (20 ml) and the mixture was refluxed for 2 h and concentrated under reduced pressure. Crystallization from water with ethanol gave 4a (156 mg, 71%) as a white solid. $^1$H NMR (D$_2$O): $\delta_\text{H}$ 3.41 (2H, dt, $J_{\text{HP}}$ = 14.2 Hz, $J_{\text{HH}}$ = 7.3 Hz, CHCH$_2$NH), 3.21-3.14 (2H, m, NHCH$_2$), 3.10-3.02 (2H, m, CH$_2$NH$_2$), 2.37 (1H, tt, $J_{\text{HP}}$ = 21.0 Hz, $J_{\text{HH}}$ = 7.3 Hz, P$_2$CH), 2.11-2.02 (2H, m, CH$_2$CH$_2$CH$_2$). $^{13}$C NMR (D$_2$O): $\delta_\text{C}$ 45.75 (CHCH$_2$), 44.61 (NHCH$_2$), 36.90 (CH$_2$NH), 36.80 ($J_{\text{CP}}$ = 118.4 Hz, PCP), 24.12 (CH$_2$). $^{31}$P NMR (D$_2$O): $\delta_\text{P}$ 16.12. HRMS [M+H]$^+$ calculated for P$_2$O$_4$C$_5$H$_7$N$_2$: 263.0562. Found: 263.0564.

**Tert-butyl (4-[[2,2-bis(diisopropoxyphosphoryl)ethyl]amino]butyl]carbamate (3b).** Preperad as 3a from 2 (500 mg, 1.4 mmol, 1.1 equiv) to give 3b (529 mg, 81%) as a yellow oil. $^1$H NMR (CDCl$_3$): $\delta_\text{H}$ 4.87-4.70 (4H, m, CHMe$_2$), 3.19-3.01 (4H, m, CHCH$_2$NH, CH$_2$CH$_2$NH), 2.62-2.56 (2H, m, NHCH$_2$), 2.49 (1H, tt, $J_{\text{HP}}$ = 23.5 Hz, $J_{\text{HH}}$ = 6.0 Hz, P$_2$CH), 1.57-1.47 (4H, m, CH$_2$CH$_2$CH$_2$CH$_2$), 1.44 (9H, brs, CMe$_3$), 1.39-1.30 (24H, m$^1$, CHMe$_2$). $^{13}$C NMR (CDCl$_3$): $\delta_\text{C}$ 156.12 (C=O), 79.03 (CMe$_3$), 71.41 (2C, m$^1$, OCH), 71.20 (2C, m$^1$, OCH), 48.81 (NHCH$_2$), 46.12 (t, $J_{\text{CP}}$ = 3.9 Hz, CHCH$_2$), 40.62 (CH$_2$NH), 38.94 (t, $J_{\text{CP}}$ = 133.7 Hz, PCP), 28.57 (3C, CMe$_3$), 27.97 (CH$_2$), 27.39 (CH$_2$), 24.33 (4C, m$^1$, CHMe$_2$), 24.07 (4C, m$^1$, CHMe$_2$). $^{31}$P NMR (CDCl$_3$): $\delta_\text{P}$ 21.94.

(2-[(4-Aminobutyl)amino]ethane-1,1-diyl)diphosphonic acid (4b). Preperad as 4a from intermediate 3b (529 mg, 0.97 mmol) to give 4b (253 mg, 76%) as a white solid. $^1$H NMR (D$_2$O): $\delta_\text{H}$ 3.42 (2H, dt, $J_{\text{HP}}$ = 14.1 Hz, $J_{\text{HH}}$ = 7.3 Hz, CHCH$_2$NH), 3.17-3.08 (2H, m, NHCH$_2$), 3.05-2.97 (2H, m, CH$_2$NH$_2$), 2.45 (1H, tt, $J_{\text{HP}}$ = 21.4 Hz, $J_{\text{HH}}$ = 7.3 Hz, P$_2$CH), 1.82-1.67 (4H, m, CH$_2$CH$_2$CH$_2$CH$_2$). $^{13}$C NMR (D$_2$O): $\delta_\text{C}$ 47.13 (NHCH$_2$), 45.39 (CHCH$_2$), 39.19 (CH$_2$NH), 36.61 (t,
\( {^1}J_{CP} = 120.0 \text{ Hz, } P_{CP}, \) 24.22 (CH\(_2\)), 23.10 (CH\(_2\)). \(^{31}\)P NMR (D\(_2\)O): \( \delta_p 16.65 \). HRMS [M+H]\(^+\) calculated for P\(_2\)O\(_6\)C\(_8\)H\(_{19}\)N\(_2\): 277.0718. Found: 277.0718.

Tert-butyl (4-((2,2-bis(hydroxy(methoxy)phosphoryl)ethyl)amino)butyl)carbamate (3c). P,P’-dimethyl ester 2c (200 mg, 0.763 mmol, 1.0 equiv) and N-Boc-butane-1,4-diamine (222 mg, 1.186 mmol, 1.6 equiv) were dissolved in methanol and refluxed for seven days. The reaction mixture was concentrated under reduced pressure, dissolved in water (10 ml), washed with DCM (3 x 5 ml), and concentrated again under reduced pressure to give 3c (258 mg, 84%) as a white solid. \(^1\)H NMR (500.1 MHz, MeOD): \( \delta_h 3.61 \) (6H, d, \(^3\)J\(_{HP} = 10.4 \text{ Hz, } OCH\(_3\)), 3.11-2.97 (4H, m, CH\(_2\)NH), 2.66-2.55 (2H, m, NHCH\(_2\)), 2.33 (1H, tt, \(^2\)J\(_{HP} = 21.3 \text{ Hz, } \delta_HH = 6.5 \text{ Hz, } P\(_2\)CH), 1.59-1.47 (4H, m, CH\(_2\)CH\(_2\)), 1.43 (9H, s, CMe\(_3\)). \(^{13}\)C NMR (125.8 MHz, MeOD): \( \delta_c 157.85 \) (C = O), 79.14 (CMe\(_3\)), 51.46 (2C, OCH\(_3\)), 49.05 (NHCH\(_2\)), 47.55 (t, \(^2\)J\(_{CP} = 3.0 \text{ Hz, } CH\(_2\)CH\(_2\)), 40.58 (CH\(_2\)NH\(_2\)), 36.79 (t, \(^1\)J\(_{CP} = 122.6 \text{ Hz, } P\(_2\)CP)), 28.21 (3C, CMe\(_3\)), 26.99 (2C, CH\(_2\)CH\(_2\)). \(^{31}\)P NMR (202.4 MHz, MeOD): \( \delta_p 22.01 \).

Dimethyl (2-(4-aminobutylamino-1,1-diy1)bis(hydrogen phosphonate) (4c). To remove the protective groups 3c (100 mg, 0.3 mmol) was dissolved in 4 M HCl (5 ml) and the mixture was stirred in room temperature for 45 min and concentrated under reduced pressure to give 4c (96 mg, 100 %) as a white solid. \(^1\)H NMR (MeOD): \( \delta_h 3.68 \) (6H, d, \(^3\)J\(_{HP} = 10.7 \text{ Hz, } OCH\(_3\)), 3.45 (2H, dt, \(^3\)J\(_{HH} = 7.0 \text{ Hz, } \delta_HH = 14.5 \text{ Hz, } CH\(_2\)H), 3.22-3.12 (2H, m, NHCH\(_2\)), 3.10-3.01 (2H, m, CH\(_2\)NH\(_2\)), 2.67 (1H, tt, \(^2\)J\(_{HP} = 21.9 \text{ Hz, } \delta_HH = 7.0 \text{ Hz, } P\(_2\)CH), 1.87-1.72 (4H, m, CH\(_2\)). \(^{13}\)C NMR (MeOD): \( \delta_c 55.50 \) (2C, OCH\(_3\)), 49.88 (NHCH\(_2\)), 47.48 (CH\(_2\)CH\(_2\)), 41.70 (CH\(_2\)NH\(_2\)), 36.59 (t, \(^1\)J\(_{CP} = 125.0 \text{ Hz, } P\(_2\)CP)), 26.71 (CH\(_2\)CH\(_2\)), 25.52 (CH\(_2\)CH\(_2\)). \(^{31}\)P NMR (MeOD): \( \delta_p 19.38 \). HRMS [M+H]\(^+\) calculated for P\(_2\)O\(_6\)C\(_8\)H\(_{19}\)N\(_2\): 305.1031. Found: 305.1031.

Tetraisopropyl (2-((3-(4-aminopropyl)amino)butyl)amino)propyl)amino)ethane-1,1-diy1)bis(phosphonate) (5). To a stirred suspension of tetraisopropyl ethylnylidenebisphosphonate 2 (300 mg, 0.84 mmol, 1.0 equiv) in dry toluene (15 ml), was added a solution of N,N’-di(3-aminopropyl)butane-1,4-diamine (170 mg, 0.84 mmol, 1.0 equiv) in dry toluene (5 ml). The reaction mixture was refluxed for 2 h, filtered, and concentrated under reduced pressure to give 5 (390 mg, 83%, purity ca. 80%) as a pale yellow oil. \(^1\)H NMR (CDCl\(_3\)): \( \delta_h 4.84-4.71 \) (4H, m, CHMe\(_2\)), 3.11 (2H, dt, \(^3\)J\(_{HP} = 16.4 \text{ Hz, } \delta_HH = 5.9 \text{ Hz, } P\(_2\)CHCH\(_2\)), 2.79-2.74 (2H, m, CH\(_2\)NHC\(_{11}\)H\(_3\)), 2.69-2.57 (10H, m, 5xCH\(_2\)N), 2.49 (1H, tt, \(^2\)J\(_{HP} = 23.5 \text{ Hz, } \delta_HH = 5.9 \text{ Hz, } P\(_2\)CH), 1.70-1.59 (4H, m, CH\(_2\)CH\(_2\)CH\(_2\)), 1.55-1.47 (4H, m, CH\(_2\)CH\(_2\)CH\(_2\)), 1.37-1.32 (24H, m\(_{17}\), CHMe\(_2\)). \(^{13}\)C NMR (CDCl\(_3\)): \( \delta_c 70.04 \) (2C, m\(_{17}\), OCH), 70.24 (2C, m\(_{17}\), OCH), 48.99 (2C, CH\(_2\)(CH\(_2\)CH\(_2\))), 47.16 (NH(CH\(_2\)CH\(_2\)))\(_{12}\)), 47.02 (CH\(_2\)(CH\(_2\))\(_{12}\)), 46.89 (CH\(_2\)NH\(_2\)), 45.11 (t, \(^2\)J\(_{CP} = 4.0 \text{ Hz, } CH\(_2\)H), 39.56 (CH\(_2\)NH\(_2\)), 37.84 (t, \(^1\)J\(_{CP} = 133.7 \text{ Hz, } P\(_2\)CP), 32.90 (CH\(_2\)NH\(_2\)), 26.98 (CH\(_2\)), 27.01 (CH\(_2\)), 23.20 (4C, m\(_{17}\), CHMe\(_2\)), 22.93 (4C, m\(_{17}\), CHMe\(_2\)). \(^{31}\)P NMR (CDCl\(_3\)): \( \delta_p 21.98 \).

(2-((3-(4-(Aminopropyl)amino)butyl)amino)propyl)amino)ethane-1,1-diy1)diphosphonic acid (6). To remove the protective groups 5 was dissolved in 4 M HCl (20 ml) and the mixture was refluxed for 2 h and concentrated under reduced pressure. Crystallization from water with ethanol gave 6 (228 mg, 70%, purity ca. 80%) as a brown solid. \(^1\)H NMR (D\(_2\)O): \( \delta_h 3.50-3.38 \) (2H, m, CH\(_2\)CH\(_2\)NH\(_2\)), 3.24-2.98 (12H, m, 6xCH\(_2\)N), 2.56-2.42 (1H, m, P\(_2\)CH), 2.16-1.98 (4H, m,
CH₂CH₂CH₂), 1.80-1.66 (4H, m, CH₂(CH₂)₂CH₂). ¹³C NMR (D₂O): δc 47.44 (2C, CH₂(CH₂)₂CH₂), 45.34 (m, CHCH₂), 44.99 (2C, CH₂NH, NHCH₂), 44.77 (CHCH₂NHCH₂), 37.01 (1C, CH₂NH₂), 36.43 (t, ¹JC₃P = 122.0 Hz PCP), 24.17 (2C, CH₂CH₂CH₂), 23.19 (2C, CH₂CH₂). ³¹P NMR (202.4 MHz, D₂O): δP 16.88.

**HRMS [M+H]⁺ calculated for P₂O₆C₁₂H₃₃N₄: 391.1881. Found: 391.1875.**

**Labeling with technetium-99m and hydroxyapatite-binding assay**

All the bisphosphonate compounds were labeled with ⁹⁹ᵐTc using stannous chloride reduction method. Briefly 350 µl of the bisphosphonate compound (1 mg/ml in water), 60 µl ascorbic acid (2.5 mg/ml in saline) and 60 µl stannous chloride (1 mg/ml in saline) were mixed in a glass vial and the atmosphere was replaced with nitrogen. Technetium-99m (app. 2 MBq) was added through the stopper. The solution was incubated at room temperature for 1 h. After the labeling the radiochemical purity was determined by instant thin layer chromatography (ITLC) with acetone as an eluent.

The hydroxyapatite-binding assay was performed according to the procedure described by Ogawa etc.¹⁹ with some modifications. 50 µl of a labeled compound and 200 µl of Tris-buffered saline (50 mmol/l pH 7.5) were pipetted into microcentrifuge tube contained 1 gram of hydroxyapatite powder, the empty tube being as a control. Tubes were gently shaken at the room temperature for 30 min and centrifuged (15 min, 13000 rpm). 50 µl of the supernatant was collected and the radioactivity was measured by a gammacounter.

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