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A one step/one pot synthesis of *N,N*-bis(phosphonomethyl)amino acids and their effects on adipogenic and osteogenic differentiation of human mesenchymal stem cells

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ABSTRACT

The one pot reaction of amino acids with diethylphosphite and formaldehyde yielded *N,N*-bis(phosphonomethyl)amino acids. This synthetic route does not require harsh reagents to cleave the ester group. The molecular structures of the new compounds were determined by X-ray diffraction methods. By employing DFT calculations the hydrolysis of the intermediate phosphonic esters to the respective acids could be explained by the decreasing P–OEt bond strength for C_α-bisalkylated amino acids. Biological evaluation on the adipogenic and osteogenic differentiation of mesenchymal stem cells revealed no modification of the adipocyte differentiation, but inhibition of osteoblast formation at concentrations without detectable cytotoxicity.

1. Introduction

Compounds containing phosphonate groups were found to exhibit important pharmacological properties and are widely used in the treatment of various diseases.[1,2] The big majority of phosphonates and bisphosphonates are applied in the treatment of osteoporosis (e.g., sodium alendronate) and hypercalcemia (caused by cancer-related increased bone resorption), for calcium regulation (e.g., disodium clodronate, etidronic acid), and inhibition of bone resorption (e.g., sodium ibandronate, sodium risedronate, disodium tiludronate),[3–10] which is caused by the affinity of the bisphosphonates to hydroxylapatite and Ca^{2+} ions. Osteoporosis is a wide-spread disease in the elder population and affects up to 50% of females and up to 15% of males.[11] The fragility of the bones leads to a high risk of fractures and the quality of life decreases significantly.

Furthermore, drugs with antiviral (e.g., sodium foscarnet, Cidofovir), antibiotic (e.g., Fosfomycin) and antineoplastic (e.g., Fotemustine) activity are known.[10] Bisphosphonates were also developed with activity against malaria, toxoplasmosis and leishmaniasis in vitro and in vivo and some were observed to inhibit specifically biosynthetic routes in plants and protozoans.[12,13] Other examples are *N*-(phosphonomethyl)glycine (Glyphosate), and *N,N*-(bisphosphonomethyl)glycine (Glyphosine) which are used worldwide as plant growth regulators and herbicides.[14] They typically affect the shikimate biosynthesis pathway for the formation of aromatic amino acids and flavonoids; Glyphosate inhibits the conversion shikimat-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimat-3-phosphate and inorganic phosphate, and the biosynthesis of proteins.[15]

The idea of exploiting the high affinity of bisphosphonates to Ca^{2+} led to the development of platinum coordination compounds conjugated to bisphosphonates via a chelating group. Platinum coordination compounds hold an important position in cancer chemotherapy,[16,17] and phosphorus-containing ligands were used for linking metal centers to organic moieties.[18–21] Osteotropic bisphosphonomethyl platinum(II) complexes showed remarkable activity against osteosarcoma and bone metastases.[22] The type of DNA adducts formed by this compound are similar to cisplatin and carboplatin, due to the release of the

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bisphosphonates and interaction with nucleobases, which is fastened by Ca^{2+} ions.[23,24] In vivo experiments revealed that the Pt complexes in combination with the antimetastatic agent razoxane suppressed the tumor growth of an osteosarcoma and reduced metastases.[25]

Herein, we present a new way to synthesize aminobisphosponic acids bearing a bisalkyl-substituted C_α atom in a one step/one pot procedure. The compounds have been characterized by standard methods, including X-ray diffraction analysis for establishing the structures. DFT calculations explain the hydrolysis of phosphonic esters to the corresponding acids. The compounds were tested on their biological activity on the adipogenic and osteogenic differentiation of mesenchymal stem cells isolated from human adipose tissue (hMADS cells).

2. Experimental

2.1. General

Analytical grade materials were obtained from commercial suppliers and used without further purification. Melting points were determined with a Büchi B-540 apparatus and are uncorrected. NMR spectra were recorded on a Bruker Avance DPX-400 spectrometer at 400.13 MHz (^{1}H), 100.63 MHz (^{13}C{^{1}H}) and 161.98 MHz (^{31}P{^{1}H}) or on a Bruker FT-NMR spectrometer Avance III^{TM} 500 MHz at 500.10 MHz (^{1}H), 125.75 MHz (^{13}C{^{1}H}) and 202.44 MHz (^{31}P{^{1}H}) at 298 K in D_2O or DMSO-d_6. Electrospray ionization mass spectra were recorded on a Bruker esquire$_{3000}$ in negative ion mode. Elemental analyses were performed by the Laboratory for Elemental Analysis of the Faculty of Chemistry, University of Vienna, with a Perkin Elmer 2400 CHN Elemental Analyzer. The elemental analysis of P was performed using the following procedure:[26] Molybdate forms a complex with *ortho*-phosphate which can be reduced to a polymeric species exhibiting a characteristic blue color ('molybdenum blue') suitable for colorimetric analysis. In a first step the sample (2–5 mg) is digested by addition of 750 μl of concentrated sulfuric acid and heating for an hour. After cooling, 750 μl of fuming nitric acid are added and the mixture is kept boiling for another half an hour until no more NO_x fume is released. After cooling, the digest is diluted to ca. 25 ml and titrated against phenolphthaleine, filled up to the volumetric mark and 5.0, 10.0 or 25.0 ml of this analyte solution are used for colorimetric determination. An aliquot of the analyte solution is transferred to a 50.0 ml volumetric flask. 2.5 ml of 2.5% ammonium molybdate in 5 M sulfuric acid and 5.0 ml of 0.25% *p*-methylaminophenol in 15% sodium pyrosulfite are added and the flask is filled up close to the volumetric mark. Molybdenum blue forms at 40 °C in a water bath within 7 min. Then the solution is cooled down to room temperature and at a maximum of 30 min prior to the analysis the volumetric flask is filled up to the mark with water. Absorption was measured with an HP 8253 diode array UV–vis spectrophotometer (λ = 820 nm) using water as reference. Calibration is done using triphenylphosphine as analytical standard which is treated in the same way as the samples.

2.2. General procedure for synthesis

The amino acid (1 equiv) was suspended in diethylphosphite (2 equiv) and 36% aqueous formaldehyde solution (4 equiv) was added dropwise at room temperature. The suspension was stirred for 1 h at room temperature and refluxed for 17 h. The acid crystallized from the mother liquor within 24 h after cooling to room temperature. The crystals were collected, washed with ethanol and recrystallized from water.

2.2.1. *N,N*-Bis(phosphonomethyl)-2-amino-2-methylpropionic acid 1a

Using the general procedure, 2-amino-2-methylpropionic acid (1.44 g, 14 mmol) was reacted with diethylphosphite (3.61 mL, 28 mmol) and formaldehyde solution (4.28 mL, 56 mmol). Yield: 2.14 g (52%), colorless crystals. Mp 194–196 °C; ^{1}H NMR (D_2O, 400.13 MHz) δ = 1.57 (s, 6H, CH_3), 3.43 (d, $^2J_{HP}$ = 13.0 Hz, 4H, CH_2) ppm; ^{13}C NMR (D_2O, 100.63 MHz) δ = 20.0 (CH_3), 50.8 (J = 133.0 Hz, CH_2), 73.9 (^3J = 5.0 Hz, $C(CH_3)_2$), 174.2 (CO) ppm; ^{31}P NMR (D_2O, 161.98 MHz) δ = 9.6 ppm. Anal. Calcd for $C_6H_{15}NO_8P_2{\cdot}H_2O$: C, 23.31; H, 5.54; N, 4.53; P, 20.04. Found: C, 23.37; H, 5.27; N, 4.40; P, 19.77. ESI-MS (neg): *m/z* 289.9 $[M-H]^-$.

2.2.2. *N,N*-Bis(phosphonomethyl)-1-amino-1-cyclohexane carboxylic acid 1b

Using the general procedure, 1-amino-1-cyclohexanecarboxylic acid (2.00 g, 14 mmol) was reacted with diethylphosphite (3.61 mL, 28 mmol) and formaldehyde solution (4.28 mL, 56 mmol). Yield: 1.53 g (33%), colorless crystals. Mp 187–189 °C; ^{1}H NMR (D_2O, 500.10 MHz) δ = 1.05–1.20 (m, 1H, CH_2), 1.36–1.50 (m, 2H, CH_2), 1.58 (m, 1H, CH_2), 1.65–1.80 (m, 4H, CH_2), 2.17 (m, 2H, CH_2), 3.44 (d, $^2J_{HP}$ = 13.1 Hz, 4H, $N(CH_2)_2$) ppm; ^{13}C NMR (DMSO-d_6, 125.75 MHz) δ = 23.5 (CH_2), 25.7 (CH_2), 34.0 (CH_2), 51.5 (^3J = 4.0 Hz, J = 159.0 Hz), 67.0 (^3J = 8.8 Hz, ^3J = 9.7 Hz), 174.4 (CO) ppm; ^{31}P NMR (D_2O, 202.44 MHz) δ = 8.9 ppm; Anal. Calcd for $C_9H_{19}NO_8P_2{\cdot}H_2O$: C, 30.95; H, 6.06; N, 4.01. Found: C, 31.03; H, 5.64; N, 3.94. ESI-MS (neg): *m/z* 330.0 $[M-H]^-$.

2.2.3. *N,N*-Bis(phosphonomethyl)-1-amino-1-cyclopentane carboxylic acid 1c

Using the general procedure, 1-amino-1-cyclopentanecarboxylic acid (1.00 g, 7.7 mmol) was reacted with diethylphosphite (2.00 mL, 15.4 mmol) and formaldehyde solution (2.35 mL, 30.8 mmol). Yield: 0.80 g (33%), colorless crystals. Mp 184–186 °C; ^{1}H NMR (D_2O, 400.13 MHz) δ = 1.72–1.86 (m, 4H, CH_2), 2.08–2.18 (m, 2H, CH_2), 2.19–2.29 (m, 2H, CH_2), 3.45 (d, 4H, $^2J_{HP}$ = 11.0 Hz, $N(CH_2)_2$) ppm; ^{13}C NMR (D_2O, 100.63 MHz) δ = 25.6 (CH_2), 33.6 (CH_2), 52.7 (J = 130.0 Hz, $N(CH_2)_2$), 83.2 (^3J = 5.0 Hz, $C(CH_2)_2$), 174.5 (CO) ppm; ^{31}P NMR (D_2O, 161.98 MHz) δ = 9.9 ppm; Anal. Calcd for $C_8H_{17}NO_8P_2{\cdot}H_2O$: C, 28.67; H, 5.71; N, 4.18; P, 18.48. Found: C, 28.77; H, 5.48; N, 4.07; P, 18.70. ESI-MS (neg): *m/z*: 316.3 $[M-H]^-$.

2.3. Crystal structure data

X-ray diffraction measurements were performed on a Nonius Kappa CCD diffractometer (**1b**) or on a Bruker X8 APEX II CCD diffractometer (**1a** and **1c**). A single crystal was mounted on a goniometer head at 40, 30 and 40 mm from the detector, and 841, 455, and 1009 frames were measured, each for 30, 10, and 60 s over 1, 1.5 and 1° scan width. The data were processed using the DENZO-SMN and SAINT software packages.[27] Crystal data, data collection parameters, and structure refinement details are given in Table 1. The structures were solved by direct methods and refined by full-matrix least-squares techniques. Non-hydrogen atoms were refined with anisotropic displacement parameters. H atoms were inserted at calculated positions and refined with a riding model. The atoms C4 and C5 of the cyclopentane ring, and the water molecule in **1c** were found to be disordered over two positions with S.O.F. 0.86 to 0.14. The following computer programs were used: structure solution, SHELXS-97;[28] refinement, SHELXL-97;[28] molecular diagrams, ORTEP-3;[29] computer, Pentium IV; scattering factors.[30] The crystal structures have been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition numbers CCDC 708525 (**1a**), 708526 (**1b**) and 708527(**1c**).

Table 1
Selected crystallographic data for **1a–c**

	1a	1b	1c
Empirical formula	$C_6H_{15}NO_8P_2{\cdot}H_2O$	$C_9H_{19}NO_8P_2{\cdot}H_2O$	$C_8H_{17}NO_8P_2{\cdot}H_2O$
fw (g mol^{-1})	309.15	349.21	335.18
Temperature (K)	296(2)	120(2)	296(2)
Crystal size	0.19 × 0.06 × 0.04	0.14 × 0.26 × 0.34	0.57 × 0.48 × 0.31
Color, habit	Colourless, plate	Colourless, block	Colourless, block
Crystal system	Monoclinic	Monoclinic	Monoclinic
Space group	$P2_1/n$	$P2_1/n$	$P2_1/n$
a (Å)	11.1277(5)	11.188(2)	11.1652(2)
b (Å)	6.7162(3)	7.043(1)	6.8742(1)
C (Å)	17.1227(7)	18.576(4)	18.2364(4)
β (°)	99.303(3)	90.96(3)	93.650(1)
V (Å^3)	1262.85(10)	1463.5(5)	1396.84(4)
Z	4	4	4
D_{calcd} (g cm^{-3})	1.626	1.585	1.594
μ (cm^{-1})	3.84	3.41	3.54
F(0 0 0)	648	736	704
θ range for data collection (°)	2.41–31.78	3.09–26.00	3.17–30.00
h Range	−16, 16	−13, 13	−15, 15
k Range	−9, 6	−8, 8	−9, 9
l Range	−25, 24	−22, 22	−25, 25
No. refls. used in refinement	4277	2877	4078
No. parameters	173	209	214
R_{int}	0.0340	0.0121	0.0225
R_1[a]	0.0389	0.0288	0.0291
wR_2[b]	0.1133	0.0853	0.0921
GOF[c]	1.032	1.036	1.064

[a] $R_1 = \sum||F_o| - |F_c||/\sum|F_o|$.

[b] $wR2 = \{\sum[w\,(F_o^2 - F_c^2)^2]/\sum[w(F_o^2)^2]\}^{1/2}$.

[c] GOF = $\{\sum[w(F_o^2 - F_c^2)^2]/(n-p)\}^{1/2}$, where n is the number of reflections and p is the total number of parameters refined.

2.4. Adipocytes/osteoblast differentiation

hMADS3 cells[31] have been induced to undergo differentiation in a culture medium allowing differentiation into both adipocytes and osteoblasts, as previously described by Zaragosi et al.[32] in the absence (control adipocytes/osteoblasts differentiation) or in the presence of 10^{-7} M or 3×10^{-7} M of **1a–c** or the reference substance sodium alendronate trihydrate (Sigma). Fourteen days after, cells were fixed and stained with Oil red O to reveal adipocytes and with Von Kossa to reveal calcium deposition secreted by osteoblasts. Adipocyte differentiation has been quantified by GPDH activity (a specific marker of adipocytes) and osteoblast differentiation by ALP activity (an osteogenic-specific marker).

2.5. Computational details

The full geometry optimization of all structures has been carried out at the DFT level of theory using Becke's three-parameter hybrid exchange functional[33] in combination with the gradient-corrected correlation functional of Lee, Yang and Parr[34] (B3LYP) with the help of the GAUSSIAN-03[35] program package. Symmetry operations were not applied. The geometry optimization was carried out using the 6-31G(d) basis set followed by single-point calculations of the total energies at the 6-311+G(d,p) basis set. The Hessian matrix was calculated analytically for all optimized structures in order to prove the location of correct minima (no 'imaginary' frequencies). The starting geometries for the optimizations were based on the experimental X-ray structures of **1a–c** (this work). Vertical bond energies were calculated as a difference of the sum of the total energies of fragments with unrelaxed geometry and the total energy of the corresponding amino acid, aminophosphonic acid or ester of aminophosphonic acid. For aminophosphonic acids and their esters, energies of a single P–OH and P–OEt bond, respectively, have been calculated.

3. Results and discussion

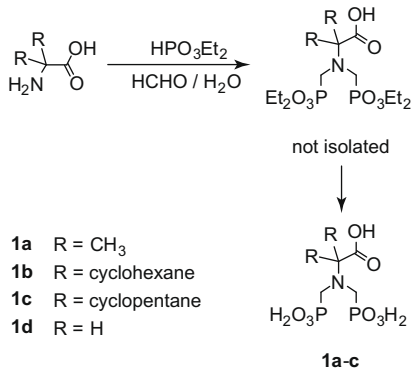
3.1. Synthesis

Different synthetic routes to aminophosphonic acids have been reported in the literature. The most frequently used are the Mannich-type Moedritzer–Irani-reaction and the Kabachnik–Fields-reaction,[36–38] the former involving the reaction of formaldehyde, phosphonic acid and α-amino acids in aqueous hydrochloric acid to yield α-aminomethylphosphonic acids.[36] Kabachnik and Fields introduced the synthesis of α-aminomethylphosphonate from aldehydes or ketones, amines and dialkylphosphites in a one pot-reaction.[39,40] The phosphonic acids, required for binding to bone material and for exhibiting activity on osteoclasts,[41,42] can be obtained by cleavage of the ester groups.[43,44]

The conversion of different α-amino acids to the corresponding α-aminobis(methylphosphonic acids) was reported in the literature,[45,46] but only the reaction with glycine yielded pure product.[36] Even changing from glycine to alanine provides mainly a decomposition product due to N–C_α cleavage. Such unique behavior of glycine in this process compared to other amino acids may be accounted for by the highest N–C_α bond energy in glycine. Indeed, quantum chemical calculations at the DFT level of theory (see Computational Details) indicate that the vertical N–C_α bond energy in glycine (310.8 kcal/mol) is by 28.4 and 48.1 kcal/mol higher than in alanine and 2-amino-2-methyl-propionic acid, respectively.

Meanwhile, when changing to amino acids having no protons at the C_α, such as 1-amino-1-cyclohexane-carboxylic acid, 1-amino-1-cyclopentane-carboxylic acid or 2-amino-2-methyl-propionic acid, and using diethylphosphite as P source the corresponding acids **1a–c** have been obtained in a one pot/one step procedure involving the hydrolysis of the ester groups, probably catalyzed by the carboxylic acid functionality (Scheme 1). Notably, no by-products were observed and the compounds were isolated in good yields after crystallization from the reaction mixture and characterized by means of ^{1}H, ^{13}C and ^{31}P NMR spectroscopy and single crystal X-ray diffraction analysis.

The reaction progress was followed by ^{31}P NMR and several groups of signals were observed (Fig. 1), which were assigned to esters (20–26 ppm), monophosphonates (13–20 ppm), diethylphosphite and its hydrolysis products (9–11 ppm) and the bisphosphonic acids (ca. 10 ppm). Normally the Kabachnik–Fields-reaction leads to the synthesis of phosphonic esters which can be further converted into the acids, with the mildest method being microwave-assisted hydrolysis with trimethylsilyl bromide.[44] This procedure should lead to a single signal assignable to the ester, however in the present case the ester bonds were cleaved which leads initially to a mixture of different di- and monoesters and finally to the acid as the main product. Exceptions are such amino



Scheme 1. Synthetic route to *N*,*N*-bis(phosphonomethyl) amino carboxylic acids.

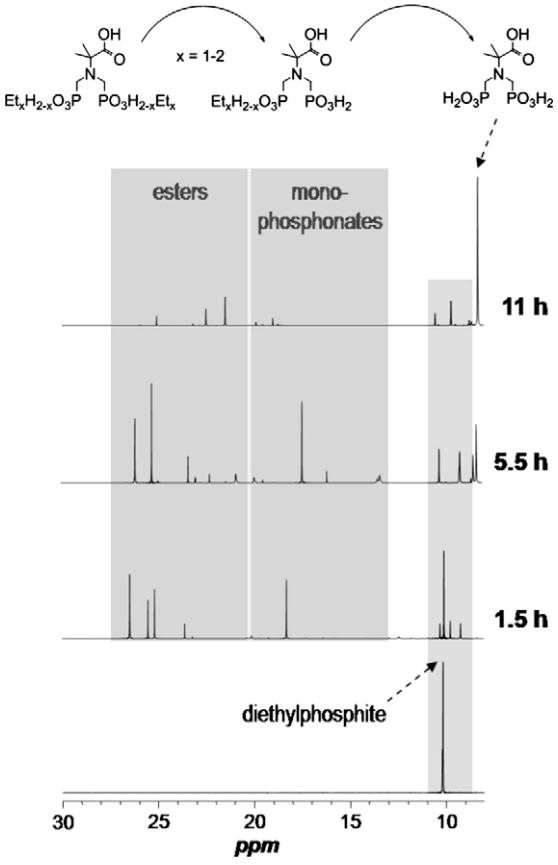


Figure 1. Time-course of the reaction progress of 2-amino-2-methylpropionic acid, diethylphosphite and formaldehyde followed by $^{31}P\{^{1}H\}$ NMR.

acids having C_α–H bonds which form the respective diethylphosphonic esters. A relative ability of phosphonic esters towards hydrolysis is mostly determined by the relative energies of the P–OEt bonds breaking in the ester and P–OH bonds forming the acid. The calculated (at DFT level) vertical P–OEt bond energy (E_{P-OEt}) in phosphonic esters decreases along the row **1d′** > **1a′** > **1c′** > **1b′** (243.9, 236.6, 232.6 and 231.0 kcal/mol, respectively) whereas the P–OH bond energy (E_{P-OH}) in acids **1a–d** varies insignificantly (245.7, 248.3, 246.9 and 246.6 kcal/mol, correspondingly). The difference $E_{P-OEt} - E_{P-OH}$ is only −2.7 kcal/mol for glycine derivatives but reaches (−9.1)–(−15.9) kcal/mol for other derivatives, which correlates well with experimental observations.

3.2. Crystal structure analysis

The results of X-ray diffraction studies of **1a–c** are shown in Figure 2. Selected bond distances (Å) and bond angles (deg) are quoted in the legend to Figure 2. All three compounds crystallized as racemates in the monoclinic space group $P2_1/n$ with one molecule of the corresponding *N*,*N*-bis(phosphonomethyl)amino acid in zwitterionic form and one molecule of water in the asymmetric unit. The non-equivalence of two phosphonate groups (one of which is singly deprotonated) and the protonation of the central *N*1 atom result in chirality of the latter. The angle N–C_α–COOH in **1a–c** is significantly smaller than that in glycine-derived bis(phosphonate) **2** (Table 2)[47] and the ideal tetrahedral angle because of the presence of sterically more demanding substituents at the C_α atom. The P–CH_2 and CH_2–N bond lengths are largely unaffected by the introduced substituents at C_α, while the N–C_α bonds of **1a–c** are slightly longer than in **2**.

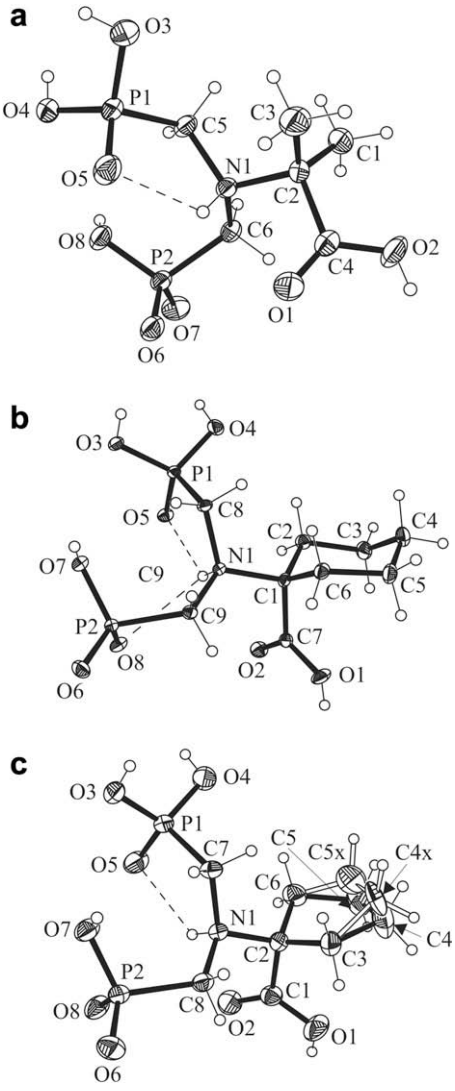


Figure 2. The structures of the zwitter ionic forms of the molecules in **1a–c** with thermal ellipsoids drawn at 50% probability level showing the intramolecular hydrogen bonding interactions N1–H···O5 [N1–H 0.91, H···O5 2.235, N1···O5 2.857 Å, N1–H···O5 125.13°] (**1a**), bifurcated N1–H···O5 and N1–H···O8 [N1–H 0.93, H···O5 2.223, N1···O5 2.865 Å, N1–H···O5 125.46°; H···O8 2.567, N1···O8 3.091 Å, N1–H···O8 116.09°] (**1b**), and N1–H···O5 [N1–H 0.91, H···O5 2.277, N1···O5 2.889 Å, N1–H···O5 124.26°] (**1c**), and disorder in the cyclopentane ring of **1c**. Selected bond distances (Å) and torsion angles (deg): C4–O1 1.2048(18), C4–O2 1.3067(19), P1–O3 1.5295(12), P1–O4 1.5483(12), P1–O5 1.4952(11), P2–O6 1.5133(11), P2–O7 1.4915(12), P2–O8 1.5579(11), N1–C2–C4–O1 –39.76(19), O5–P1–C5–N1 –4.01(13)° (**1a**), C7–O1 1.3083(19), C7–O2 1.2102(19), P1–O3 1.5529(11), P1–O4 1.5336(11), P1–O5 1.4931(11), P2–O6 1.4876(11), P2–O7 1.5640(11), P2–O8 1.5129(11), N1–C1–C7–O2 55.31(16), O5–P1–C8–N1 7.02(11)° (**1b**), C1–O1 1.3034(14), C1–O2 1.2016(16), P1–O3 1.5475(9), P1–O4 1.5278(9), P1–O5 1.4902(9), P2–O6 1.4879(9), P2–O7 1.5575(8), P2–O8 1.5093(9), O2–C1–C2–N1 40.99(14), O5–P1–C7–N1 11.39(9)° (**1c**).

3.3. Effects of *N*,*N*-bis(phosphonomethyl)amino acids on adipogenic and osteogenic differentiation of mesenchymal stem cells isolated from human adipose tissue (hMADS cells)

The effects of **1a–c** and sodium alendronate (also known as FOSAMAX®) were investigated on the differentiation of hMADS cells. The control experiment shows that without addition of compounds adipocytes (lipid droplets in red) and osteoblasts (black dots) were generated (Fig. 3). The addition of compounds **1a–c** did not modify the adipocyte differentiation, but induced the inhibition of osteoblast formation at 10^{-7} M and 3×10^{-7} M as

Table 2
Selected bond lengths (Å) and angles (°) for **1a–c** and the glycine derivative **2**

	1a	1b	1c	Glycine, 2[a]
C_α–COOH	1.542(2)	1.5454(19)	1.5371(15)	1.506(4)
N–C_α	1.5451(19)	1.5448(17)	1.5288(13)	1.493(4)
CH_2–N	1.5150(18)	1.5208(17)	1.5126(12)	1.503(4)
	1.5119(19)	1.5195(17)	1.5162(13)	1.513(4)
P–CH_2	1.8219(15)	1.8205(15)	1.8184(11)	1.830(3)
	1.8243(15)	1.8226(15)	1.8222(11)	1.827(3)
N–C_α–COOH	103.53(11)	103.82(11)	104.79(8)	111.0(3)
CH_2–N–C_α	113.27(11)	113.34(10)	112.74(8)	112.9(2)
	111.86(11)	112.59(10)	110.27(8)	111.6(2)
P–CH_2–N	112.55(9)	112.57(10)	113.13(7)	112.3(2)
	114.66(9)	112.00(9)	115.27(7)	116.7(2)

[a] From Ref. 48.

revealed by the absence of Von Kossa staining, and importantly, no cytotoxic effect was detectable. In contrast, alendronate elicited a cytotoxic effect detectable at 10^{-7} M and more pronounced at 3×10^{-7} M.

Monitoring the GPDH activity revealed no effect of **1a–c** on adipogenesis (Fig. 4), but the ALP activity revealed inhibition of osteogenesis. In the case of alendronate no GPDH and ALP activity was observed at 3×10^{-7} M and this result confirmed the cytotoxic effect of this compound, as also reported on rat osteoblasts.[48]

As a positive effect of bisphosphonates on differentiation of osteoblasts has been reported only when used at low concentration, a series of experiments was conducted at concentrations from 10^{-7} M to 10^{-10} M. Both the toxic effect of alendronate and the inhibitory effect of the compounds were lost when cells were maintained in the presence of 10^{-8} M or lower concentrations (Fig. 5).

4. Conclusions

Amino acids were reacted with diethylphosphite and formaldehyde to give *N*,*N*-bis(phosphonomethyl)amino acids in a one step/one pot procedure, including the cleavage of the ester bonds. This behavior was explained by DFT calculations revealing lower P–OEt bond strength than for compounds having C_α–H bonds. Furthermore, when C_α–H bond-containing amino acids (with the exception of glycine) were reacted to the corresponding *N*,*N*-bis(phosphonomethyl)amino acids, a by-product was formed, which is explained by a weakend C_α–N bond. The synthesized compounds were characterized by spectroscopic methods and elemental analysis and their 3D structures were established by X-ray

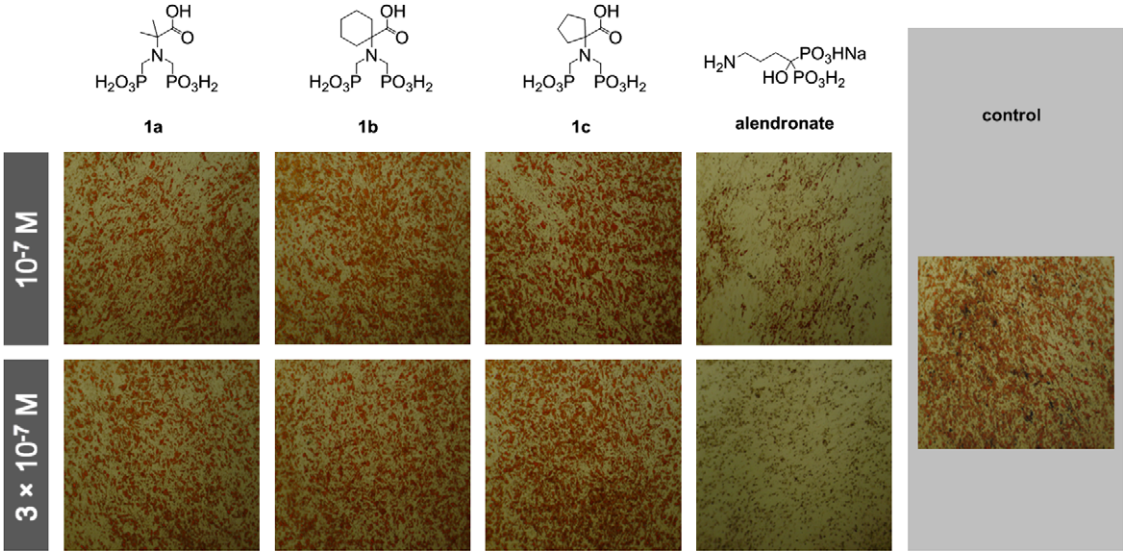


Figure 3. Effects of **1a–c** and the positive control sodium alendronate on the differentiation of hMADS cells and untreated cells as control.

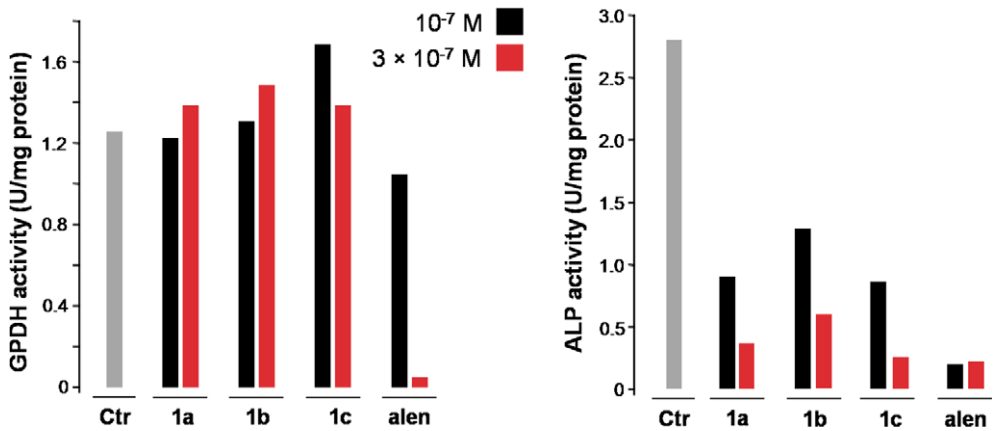


Figure 4. Influence of **1a–c** on the GPDH and the ALP activity.

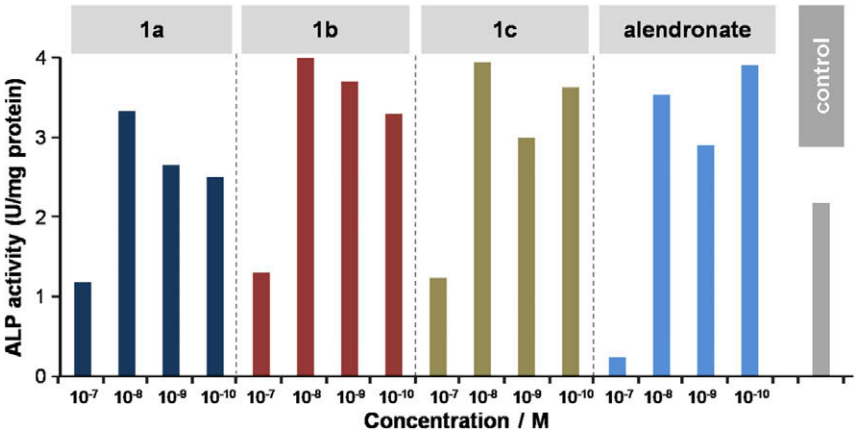


Figure 5. Concentration dependent influence of **1a–c** on the ALP activity.

diffraction analysis. Biological studies on the effects of *N*,*N*-bis(phosphonomethyl)amino acids on the adipogenic and the osteogenic differentiation of mesenchymal stem cells isolated from human adipose tissue (hMADS cells) revealed that, in contrast to alendronate, **1a–c** are not toxic even when applied at high concentrations but they do not affect the adipogenic differentiation.

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