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Original article

Synthesis and bio-activity evaluation of tetraphenyl(phenylamino) methylene bisphosphonates as antioxidant agents and as potent inhibitors of osteoclasts *in vitro*

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ABSTRACT

A new series of tetraphenyl bisphosphonates have been elegantly synthesized by one-pot method and were characterized by elemental analysis, FTIR, ^{1}H, ^{13}C, ^{31}P NMR, mass spectra and evaluated for their *in vitro* antibone resorptive activity by inhibiting growth of osteoclasts. Two bisphosphonates **3g** and **3f** showed marked inhibition ratio (8 μM and 10 μM) and emerged as lead compounds. All compounds were tested for their antioxidant (DPPH scavenging, reducing power and inhibition of lipid peroxidation). They exhibited potent *in vitro* antioxidant activity dose-dependently.

1. Introduction

Osteoporosis is characterized by extensive bone resorption leading to decreased bone mass and increased fragility and susceptibility to fractures. At the cellular level, osteoporosis is disturbance in the bone micro architecture, which typically develops in women after menopause, due to decrease in serum estrogen levels [1]. The clinical manifestations of osteoporosis occur most often as fractures in the spine, femoral neck and at the wrist. Its prevention and treatment is therefore of a paramount importance. The pronounced selective activity of bisphosphonates for bone tissues rather than others is the basis for their value in clinical practice. Their preferential uptake and adsorption by mineral surfaces in bone bring them into close contact with osteoclasts. Many studies have shown that bisphosphonates affect osteoclasts mediated bone resorption in a variety of ways, including effects on osteoclasts recruitment, differentiation, resorptive activity, and may also induce apoptosis. Bisphosphonates could also inhibit bone resorption by preventing osteoclasts formation, in addition to affecting mature osteoclasts *in vitro*. Bisphosphonates inhibit dose-dependently the formation of osteoclasts-like cells in long-term human bone marrow cultures [2]. In organ cultures also some bisphosphonates can inhibit the generation of mature osteoclasts, possibly by preventing the fusion of osteoclasts precursors [3,4].

Nitrogen-containing bisphosphonates are the mainstay of anti-resorptive therapy for osteoporosis [5,6]. They inhibit bone resorption by reducing osteoclastic activity and induce osteoclasts apoptosis [7]. Over the past two decades, several bisphosphonate derivatives have been developed and used in the treatment of bone skeletal disorders such as Pagets disease, myeloma and bone metastases and reduce bone fracture risks associated with post-menopausal osteoporosis in women [8,9]. Nitrogen-containing bisphosphonates are also reported to inhibit farnesyl diphosphate synthase, a key enzyme in the mevalonate pathway, and decrease prenylation of essential GTP-binding proteins.

The latest third generation bisphoshonates having nitrogen moiety as one of the side chains are usually preferred over non-nitrogen side chain preparations because of their high potency [10].

In human rheumatoid arthritis (RA), osteoclasts are found at sites of tissues invasion and emerge from synovial inflammatory tissue, which provides signals allowing their differentiation from monocytic precursors [11]. Bone loss in experimental models of arthritis resembling human RA depends on osteoclasts formation. Inhibition of osteoclastogenesis is a powerful approach to prevent

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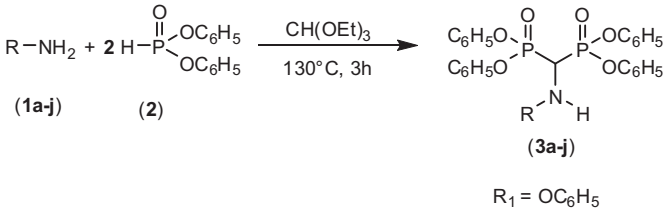
arthritic bone destruction. In addition reactive oxygen species (ROS) have been proved to play a major role in enhancing inflammatory process in rheumatoid arthritis (RA) and also contribute to the destruction of cartilage and bone [12]. In the inflamed bone joints, superoxide, hydrogen peroxide, hydroxyl and hypochlorous acid radicals may be produced by macrophages, neutrophils and chondrocytes and cause hyper-fusion cycle in inflamed joints leading to increased generation of ROS [13,14]. Consequently, antioxidants may have a therapeutic role in RA by suppressing the inflammation. In this study, we accomplished synthesis of tetraphenyl(phenylamino)methylene bisphosphonates (TPM-BPs) and investigated their influence on inhibition of osteoclasts and their antioxidant profile *in vitro*.

2. Chemistry

Tetraphenyl(phenylamino) methylene bisphosphonates (TPM-BPs) **3a**–**j** are synthesized in moderate yields (Scheme 1) by one-pot reaction by reacting an amine with triethyl orthoformate and diphenyl phosphite. In this particular reaction, triethyl orthoformate performs dual role as a reagent and solvent. The reaction conditions in the present protocol are mild and do not require any catalyst to facilitate the reaction. Another important feature of this reaction is the survival of a variety of functional groups such as, chloro, fluoro, methoxy and nitro under these reaction conditions. The reactions were clean and completed within 3 h. All the reactions were monitored by thin layer chromatography using ethylacetate–hexane (4:6) as eluent. Evaporation of the solvent from the reaction mixture under reduced pressure afforded pure products in 55–72% yields which are obviously dependent on the position and nature of the substituents on the amine **1**. The key step in the one-pot synthesis of TPM-BPs is the nucleophillic addition of an amine to triethyl orthoformate followed by addition of a phosphite to the resulting imine [15].



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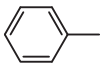
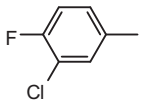
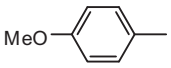
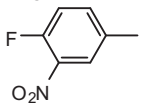
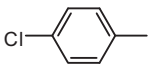
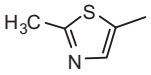
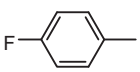
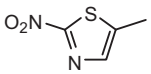
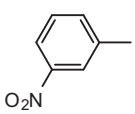
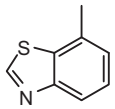
3a		3f	
3b		3g	
3c		3h	
3d		3i	
3e		3j	

Scheme 1. Synthesis of tetraphenyl(phenylamino) methylene bisphosphonates **3a**–**j**.

IR absorptions for **3a**–**j** appeared for P=O in the region 1232–1224 cm^{-1} confirms the presence of P=O group. The stretching frequencies of N–H and P–$C_{aliphatic}$ groups are observed respectively at 3436–3312 cm^{-1} and 764–752 cm^{-1} [16]. The aromatic protons showed multiplets in the region of δ 6.10–8.36. The P–C–H exhibited multiplet at δ 5.52–6.90 due to its coupling with phosphorus [16]. The –NH signal was observed at δ 5.10–6.31.The carbon chemical shifts for P–C–H, P–OC_6H_5 in the title compounds were observed in their expected regions. Their ^{31}P NMR chemical shifts were observed at δ 24.26–28.04 for **3a**–**j** [17]. Chemical ionization mass spectra of **3a**, **3b**, and **3e** gave molecular ions and diagnostic daughter ion peaks at their respective expected *m/z* values.

3. Pharmacology

The antioxidant activity was evaluated by Diphenyl picryl hydrazyl method (DPPH), Reducing power and Lipid peroxidation (LPO) methods and their inhibitory concentration (IC_{50}) varied according to substitution on the phenyl ring and Vitamin C was measured as standard for antioxidant activity. To the best of our knowledge a systematic antioxidant profile of TPM-BPs has not been studied. Anti-bone resorption activity, in terms of inhibitory ratios of osteoclasts were measured by MTT assay.

4. Results and discussion

According to our predicted results, the TMP-BPs **3a**–**j** have the ability to scavenge the DPPH radical by donating one electron. In the lipid peroxidation process iron plays an important key role in complex formation via Fenton's reagent. Since BPs are known for their affinity for divalent cations, the high reactive hydroxyl radicals formed in the inflamed joints are responsible to cause lipid peroxidation and tissue damage. Since phosphorus atom has affinity towards oxygen, it can easily bind and scavenge ROS and has ability to scavenge effectively. Our synthesized compounds **3a**–**j**, containing phosphorus, oxygen, nitrogen are expected to be more active due to the presence of hetero atoms containing non-bonded electron pairs that serve as binding sites in the bio-matrix. **3e**, **3g** and **3h** displayed appreciable antioxidant activity. **3g** showed the highest activity because both fluorine and $-NO_2$ substituents which affect the electron and hydrogen donating capacities appears to be useful in inducing antioxidant activity. Since fluorine shows highly negative inductive effect and $-NO_2$ is highly electron withdrawing moiety, thereby electron density around P=O moiety decreases and increases affinity towards oxygen derived free radicals and mobilizes ROS to be scavenged out of living system. Besides, the presence of chlorine on aryl ring in **3c** and **3f** can also strongly polarize that parent molecule and seems to have profound effect on biological properties of title compounds. This points to the fact electron withdrawing substituent in **3a**–**j** appears to prevent to some extent oxidative metabolic pathways in the living cells.

4.1. DPPH radical scavenging activity

The scavenging activity of TMP-BPs against DPPH radical was performed in accordance with Choi et al. [18]. 85 μM of DPPH was added to a medium containing different TMP-BPs concentrations. The medium was incubated for 30 min at room temperature. The decrease in absorbance was measured at 518 nm. Ascorbic acid was used as standard reference to record maximal decrease in DPPH radical absorbance. The values are expressed in percentage of inhibition of DPPH radical absorbance with those of the standard

control values without the title compounds (Fig. 1) (ascorbic acid maximal inhibition was considered 100% of inhibition).

$$\text{DPPH Scavenged (\%)} = \frac{(A_{\text{cont}} - A_{\text{test}})}{A_{\text{cont}}} \times 100$$

In the case of TMP-BPs **3a**–**j**, fluoro-nitro substituted compound **3g** showed the highest DPPH radical scavenging activity with IC_{50} at 30.1 μg/mL when compared with other compounds. The remaining compounds exhibited DPPH radical scavenging activity in the following order: **3f** (IC_{50} 32.9 μg/mL), **3e** (IC_{50} 39.6 μg/mL), **3b** (IC_{50} 40.7 μg/mL), **3c** (IC_{50} 41.5 μg/mL), **3j** (IC_{50} 47.9 μg/mL), **3d** (IC_{50} 48.2 μg/mL), **3i** (IC_{50} 48.6 μg/mL), **3h** (IC_{50} 58.4 μg/mL), **3a** (IC_{50} 80.6 μg/mL) and when compared with ascorbic acid (IC_{50} 32.2 μg/mL).

4.2. Reducing power assay

The reducing power of synthesized compounds **3a**–**j** was determined according to the method of Oyaizu [19]. The compounds having 50–100 μM were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide and incubated at 50 °C for 20 min. To this mixture 2.5 mL of 10% trichloroacetic acid (TCA) was added and the mixture was centrifuged at 3000 rpm for 20 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride and the UV absorbance was measured at 700 nm using a spectrophotometer. Increases of absorbance of the reaction mixture indicate higher reducing power. Mean values from three independent samples were calculated for each compound and standard deviations were less than 5%.

In the case of TMP-BPs (Fig. 2) **3a**–**j**, derivatives **3e** showed the highest reducing power with IC_{50} of 2.09 μg/mL when compared with other compounds. The remaining compounds exhibited reducing power activity in the following order: **3f** (IC_{50} 2.14 μg/mL), **3g** (IC_{50} 2.27 μg/mL), **3j** (IC_{50} 2.44 μg/mL), **3c** (IC_{50} 2.71 μg/mL), **3b** (IC_{50} 2.73 μg/mL), **3d** (IC_{50} 2.86 μg/mL), **3h** (IC_{50} 3.01 μg/mL), **3a** (IC_{50} 3.12 μg/mL), **3i** (IC_{50} 3.54 μg/mL) and when compared with ascorbic acid (IC_{50} 2.51 μg/mL).

4.3. Lipid peroxidation assay

Lipid peroxidation was induced by Fe^{2+} ascorbate complex system in rat liver cells and was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al. [20]. Experiments *in vitro* lipid peroxidation were carried out to clarify the mode of the protective effect of the tetraphenyl bisphosphonates against oxidative stress-induced cell damage. The inhibition of lipid peroxidation has been used as a model to elucidate antioxidant activity.

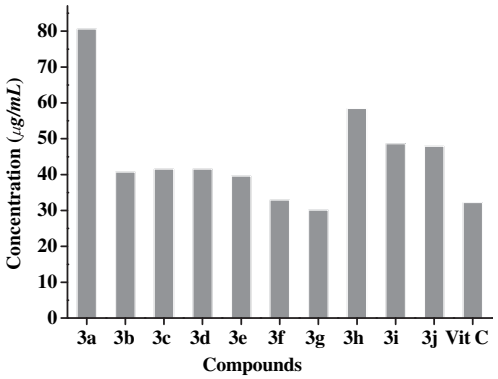


Fig. 1. DPPH radical scavenging activity of compounds **3a**–**j**.

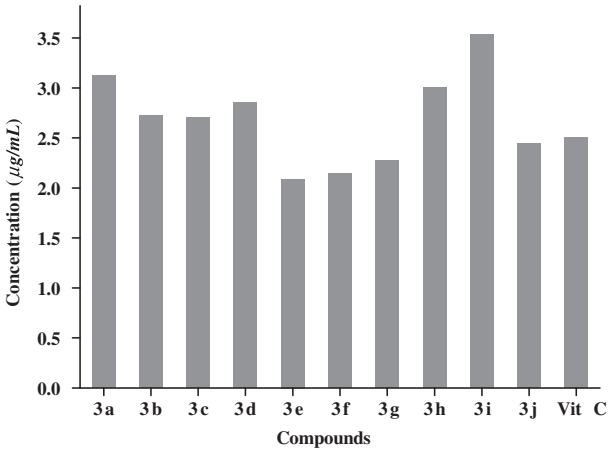


Fig. 2. Reducing power activity of compounds **3a**–**j**.

According to the results obtained, **3h** (IC_{50} 75.2 μg/mL) (Fig. 3) significantly inhibited the ferric ion plus ascorbic acid in rat liver cells. The remaining compounds exhibited hydroxyl radical scavenging activity in the following order: **3g** (IC_{50} 78.5 μg/mL), **3f** (IC_{50} 80.6 μg/mL), **3j** (IC_{50} 84.9 μg/mL), **3e** (IC_{50} 85.7 μg/mL), **3d** (IC_{50} 86.2 μg/mL), **3c** (IC_{50} 87.5 μg/mL), **3b** (IC_{50} 89.8 μg/mL), **3a** (IC_{50} 96.0 μg/mL), **3i** (IC_{50} 97.9 μg/mL) and when compared with ascorbic acid (IC_{50} 81.4 μg/mL).

4.4. Bone anti-resorptive activity – inhibition of osteoclasts by MTT assay

Reduced osteoblasts survival along with increased osteoclasts functions are the two key cellular mechanisms observed in osteoporosis. Osteoclasts are responsible for dissolving both the mineral and organic bone matrix [21] and represent terminally differentiated cells expressing a unique polarized morphology with specialized membrane areas and several membrane and cytoplasmic markers, such as tartrate resistant acid phosphatase (TRAP) [22]. The effects of TMP-BPs **3a**–**j** and alendronate (one of the most potent bone resorption inhibitor) as reference compound on bone resorption were examined against osteoclasts on the basis of inhibitory ratio of osteoclasts. The results are presented in Table 1. Their inhibitory activity was related to the nature of the substituents such as fluoro, chloro, nitro and methoxy groups present in the aryl ring. Osteoclasts treated with 10, 50, 100 and 250 μM of title compounds **3a**–**j** were harvested after 24, 48 and 72 h and were

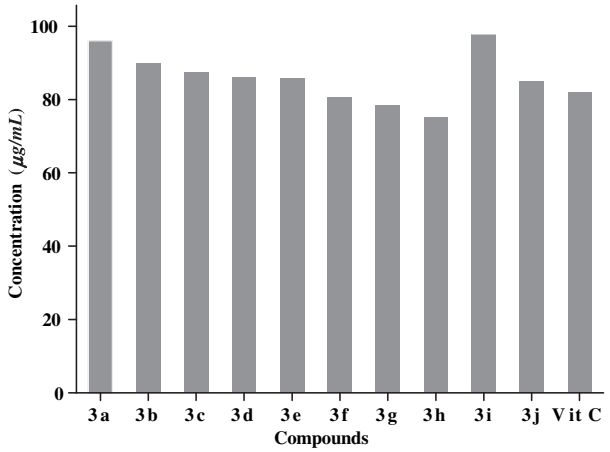


Fig. 3. Lipid peroxidation of compounds **3a**–**j**.

Table 1
Osteoclast inhibitory activity of **3a**–**j** by MTT assay.

Compounds	Osteoclast inhibitory ratio mean ± SD[a]		
	24 h	48	72 h
3a	201.6 ± 1.97	168.2 ± 1.35	104.4 ± 1.05
3b	138.7 ± 1.85	102.6 ± 1.06	67.7 ± 0.68
3c	191.4 ± 0.73	140.3 ± 0.98	71.8 ± 0.59
3d	126.1 ± 1.44	89.1 ± 0.76	56.4 ± 0.50
3e	49.6 ± 1.42	28.8 ± 1.08	14.6 ± 0.54
3f	32.7 ± 0.76	21.6 ± 0.66	10.4 ± 0.97
3g	31.2 ± 1.22	19.1 ± 0.95	8.1 ± 0.58
3h	88.4 ± 0.49	64.2 ± 0.76	48.8 ± 0.74
3i	106.6 ± 1.20	87.7 ± 0.51	51.6 ± 0.80
3j	125.9 ± 1.62	95.9 ± 1.06	54.5 ± 0.63
Alen[b]	52.7 ± 1.55	29.6 ± 1.32	20.4 ± 1.29

[a] The inhibitory ratio (%) of TPM-BPs μ of **3a-j** were analyzed by MTT assay after 24, 48 and 72 h.
[b] Alendronate (standard reference).

subjected to MTT assay. Inhibitory ratio for **3e** was 49 μM at 24 h, 28 μM at 48 h, and 14 μM at 72 h of treatment. Inhibitory ratio for **3f** was 32 μM at 24 h, 21 μM at 48 h and 10 μM at 72 h of treatment. Inhibitory ratio for **3g** was 31 μM at 24 h, 19 μM at 48 h and 8 μM at 72 h of treatment. Results showed that cell viability was affected upon treatment with compounds **3a**–**j** at 100 and 250 μM, especially after 72 h. Although, it is difficult to explain the observed variations in the activity with respect to substituents in growth inhibition. Compounds **3e**, **3f** and **3g** showed remarkable decrease osteoclastic bone resorption than other compounds and even than the standard alendronate. These results suggest that inhibition of osteoclasts is greatly influenced by nitro and halo substituted, secondary amine moiety at the α-carbon of TPM-BPs. This may be attributed to their interference in the osteoclasts unique polarized morphology and decreases their metabolic rate.

During the process of bone resorption, the sub-cellular space beneath the osteoclast is acidified by the action of vacuolar-type proton pumps in the ruffled border of the osteoclast membrane [23]. The acidic P^H normally favors the dissolution of the bone mineral (hydroxyapatite) and provides optimal conditions for the action of the proteinases secreted by osteoclasts. When the osteoclasts stops resorption and moves away from the resorption lacuna, phagocytes clean up the remains and make room for osteoblasts to begin bone formation in the newly formed resorption cavity [24]. Therefore, there is a need to interrupt the function of osteoclasts. The results demonstrated that the secondary nitrogen atom bearing a sufficiently bulky substituent-bearing halogens and nitro group are effective in inhibiting osteoclasts. Among the potent compounds **3e**, **3g** and **3f** are selected for further studies and are being investigated on survival of osteoblasts and promotion of bone formation.

4.5. Structure-activity relationship and mechanism of action

The P–C–P moiety of bisphosphonate is responsible for the strong affinity and for binding to hydroxyapatite and the methylene carbon allows for a number of structural variations with the help of substituents R (2°-amine) and $R_1(-OC_6H_5)$. The ability of the bisphosphonates to bind to hydroxyapatite crystals and to prevent both crystal growth and dissolution was enhanced when the R side chain is a secondary amine with aromatic ring having substituent with electronwithdrawing groups such as halogens and nitro.

5. Conclusion

An effective and simple method for synthesis of novel tetraphenyl(phenylamino) bisphosphonates **3a**–**3j** is reported. Their structures were characterized by physical and analytical data. In the MTT assay, tetraphenyl(4-fluoro-3-nitro phenylamino) methylene diphosphonate is highly effective in inhibiting osteoclasts cells. Its activity is much higher than that of standard bone anti-resorption drug. These results encourage further *in vivo* studies and explore their possible therapeutic applications. This is the first report on the anti-bone resorptive/anti-oxidant activity of TMP-BPs.

6. Experimental protocols

6.1. Chemistry

Melting points were recorded on Buchi R-535 apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer FTIR 240-c spectrophotometer using KBr optics. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on AMX 400 MHz NMR spectrometer operating at 400 MHz for ^1H NMR, 100 MHz for ^{13}C and 161.89 MHz for ^{31}P NMR. NMR data recorded in DMSO were referenced to TMS (^1H and ^{13}C) and 85% H_3PO_4 (^{31}P). Mass spectra were recorded on a Finnigan MAT 1020/Micro-Mass Q-T of micro AMPS MAX 10/6A, Hz 60/50 system fitted with a built-in inlet system. Elemental analyses were performed using Perkin Elmer 2400 instrument at the Central Drug Research Institute (CDRI), Lucknow, India.

Synthesis of tetraphenyl(phenylamino) methylene diphosphonate (**3a**): A mixture of aniline **1a** (0.45 g, 0.005 mol), triethyl orthoformate (30 mL) and 2 mol of diphenyl phosphite (1.91 g, 0.01 mol) was stirred at 130 °C for 2.5 h with continuous removal of ethanol formed. The reaction was monitored by TLC on silica gel using petroleum ether and ethyl acetate (1:2 v/v). After cooling, the volatiles were removed *in vacuo*. The residue was chromatographed on silica gel using $CHCl_3$-MeOH (9:1). The other compounds **3b**–**j** were prepared employing the above procedure.

6.1.1. Tetraphenyl(phenylamino) methylene diphosphonate (*3a*)

Yield 66%, mp 145–147 °C; IR (KBr): 3436,1225, 758 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.81–8.20 (m, 25 H, Ar-H), 6.40–6.75 (m, 1H, CHP), 6.15 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 52.4, 114.8, 116.7, 127.6, 139.8 (C-1–C-7); 126.6, 127.03, 129.7, 135.2 (C-1′–C-6′ and C-1″–C-6″); 126.7, 127.7, 129.8, 135.37 (C-1a′–C-6a′ and C-1a″–C-6a″). ^{31}P NMR (DMSO-d_6): δ 27.52. CIMS: (*m*/*z*) 572[MH]$^+$. Elemental analysis: Calcd. $C_{31}H_{27}NO_6P_2$: C, 65.15; H, 4.76; N, 2.45. Found C, 65.04; H, 4.71; N, 2.40

6.1.2. Tetraphenyl(4-methoxyphenyl amino) methylene diphosphonate (*3b*)

Yield 62%, mp 160–162 °C; IR (KBr): 3318, 1228, 757 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 7.10–8.22 (m, 24 H, Ar-H), 6.45–6.85 (m, 1H, CHP), 6.14 (s, 1H, NH). ^{13}C NMR(DMSO-d_6): 53.2, 115.2, 117.8, 129.4, 132.9, 140.2, 142.3 (C-1–C-7); 126.6, 127.7, 129.7, 135.2 (C-1′–C-6′ and C-1″–C-6″); 126.7, 127.3, 129.8, 135.3 (C-1a′–C-6a′ and C-1a″–C-6a″). ^{31}P NMR (DMSO-d_6): δ 27.04. CI (*m*/*z*): 602[MH]$^+$. Elemental analysis: Calcd. $C_{32}H_{29}NO_7P_2$: C, 63.89; H, 4.86; N, 2.33. Found C, 63.77; H, 4.81; N, 2.27.

6.1.3. Tetraphenyl(4-chlorophenylamino) methylene diphosphonate (*3c*)

Yield 61%, mp 154–156 °C; IR (KBr): 3316, 1224, 752 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.46–8.21 (m, 24 H, Ar-H), 6.10–6.32 (m, 1H, CHP), 5.85 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 53.4, 116.2, 118.1, 128.4, 131.1, 139.2, 141.3 (C-1–C-7); 126.6, 127.7, 129.3, 136.3 (C-1′–C-6′ and C-1″–C-6″); 126.8, 127.8, 129.7, 136.4 (C-1a′–C-6a′and C-1a″–C-6a″). ^{31}P NMR (DMSO-d_6): δ 27.24. Elemental analysis: Calcd. $C_{31}H_{26}ClNO_6P_2$: C, 61.45; H, 4.32; N, 2.31. Found C, 61.30; H, 4.26; N, 2.26.

*6.1.4. Tetraphenyl(4-fluoro phenylamino) methylene diphosphonate (**3d**)*

Yield 64%, mp 162–164 °C; IR (KBr): 3342, 1226, 764 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.24–8.24 (m, 24 H, Ar-H), 5.82–5.94 (m, 1H, CHP), 5.72 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 53.3, 117.2, 119.2, 129.2, 130.2, 138.2, 141.1 (C-1–C-7); 125.7, 126.4, 128.3, 136.2 (C-1′–C-6′ and C-1″–C-6″); 125.9, 126.8, 128.7, 136.4 (C-1a′–C-6a′ and C-1a″–C-6a″). ^{31}P NMR (DMSO-d_6): δ 25.24. Elemental analysis Calcd. $C_{31}H_{26}FNO_6P_2$: C, 63.16; H, 4.45; N, 2.38. Found C, 63.04; H, 4.40; N, 2.32.

*6.1.5. Tetraphenyl(3-nitro phenylamino) methylene diphosphonate (**3e**)*

Yield 72%, mp 152–154 °C; IR (KBr): 3320, 1230, 755 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 7.21–8.22 (m, 24 H, Ar-H), 6.88–6.90 (m, 1H, CHP), 6.31 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 52.1, 112.7, 113.8, 118.0, 132.9, 139.4, 142.3 (C-1–C-7); 125.7, 126.9, 128.7, 134.2 (C-1′–C-6′ and C-1″–C-6″); 126.4, 127.9, 129.4, 134.3 (C-1a′–C-6a′ and C-1a″–C-6a″). ^{31}P NMR (DMSO-d_6): δ 28.04. CIMS (*m*/*z*): 617 $[MH]^+$. Elemental analysis: Calcd. $C_{31}H_{26}N_2O_8P_2$: C, 60.39; H, 4.25; N, 4.54. Found C, 60.28; H, 4.21; N, 4.49.

*6.1.6. Tetraphenyl(3-chloro-4-fluoro phenylamino) methylene diphosphonate (**3f**)*

Yield 65%, mp 156–158 °C; IR (KBr): 3312, 1231, 762 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.21–8.34 (m, 23 H, Ar-H), 5.80–5.92 (m, 1H, CHP), 5.64 (s, 1H, NH). ^{31}P NMR (DMSO-d_6): δ 26.72. Elemental analysis: Calcd. $C_{31}H_{25}ClFNO_6P_2$: C, 59.68; H, 4.04; N, 2.24. Found C, 59.56; H, 3.98; N, 2.19.

*6.1.7. Tetraphenyl(4-fluoro-3-nitro phenylamino) methylene diphosphonate (**3g**)*

Yield 66%, mp 152–154 °C; IR (KBr): 3316, 1228, 764 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.20–8.36 (m, 23 H, Ar-H), 5.62–5.78 (m, 1H, CHP), 5.34 (s, 1H, NH). ^{31}P NMR (DMSO-d_6): δ 26.24. Elemental analysis: Calcd. $C_{31}H_{25}FN_2O_8P_2$: C, 58.68; H, 3.97; N, 4.42. Found C, 58.55; H, 3.91; N, 4.35.

*6.1.8. Tetraphenyl(2-methyl thiazol-5-yl amino) methylene diphosphonate (**3h**)*

Yield 57%, mp 168–170 °C; IR (KBr): 3342, 1232, 756 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.15–8.10 (m, 23 H, Ar-H), 5.54–5.63 (m, 1H, CHP), 5.12 (s, 1H, NH). ^{31}P NMR (DMSO-d_6): δ 26.12. Elemental analysis: Calcd. $C_{29}H_{26}N_2O_6P_2S$: C, 58.78; H, 4.42; N, 4.73. Found C, 57.55; H, 4.39; N, 4.68.

*6.1.9. Tetraphenyl(2-nitro thiazol-5-yl amino) methylene diphosphonate (**3i**)*

Yield 59%, mp 142–144 °C; IR (KBr): 3338, 1228, 759 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.10–8.10 (m, 23 H, Ar-H), 5.64–5.75 (m, 1H, CHP), 5.10 (s, 1H, NH). ^{31}P NMR (DMSO-d_6): δ 24.28. Elemental analysis: Calcd. $C_{28}H_{23}N_3O_8P_2S$: C, 53.94; H, 3.72; N, 6.74. Found C, 53.84; H, 3.67; N, 6.68.

*6.1.10. Tetraphenyl(benzo [d] thiazol-7-yl amino) methylene diphosphonate (**3j**)*

Yield 55%, mp 158–160 °C; IR (KBr): 3324, 1226, 754 cm^{-1}; ^1H NMR (DMSO-d_6; 400 MHz): δ 6.10–8.24 (m, 26 H, Ar-H), 5.52–5.62 (m, 1H, CHP), 5.15 (s, 1H, NH). ^{31}P NMR (DMSO-d_6): δ 24.26. Elemental analysis: Calcd. $C_{32}H_{26}N_2O_6P_2S$: C, 61.15; H, 4.17; N, 4.46. Found C, 61.02; H, 4.12; N, 4.40.

6.2. Isolation and culture of osteoclasts

Osteoclasts from 1- or 2-day-old Sprague–Dawley rat pup long bones were scraped mechanically into DMEM (Dulbecco's modified Eagle's medium) buffered with 20 mM Hepes and containing 0.84 g of sodium bicarbonate/liter, 2 mM L-glutamine, 100 IU of penicillin/ml, 100 μg of streptomycin/ml and 7–10% heat-inactivated fetal calf serum (FCS). The cells were allowed to attach to sonicated bovine cortical bone slices for 30 min. After the attachment period, non-attached cells were rinsed away, and the bone slices with the remaining cells were transferred into 24-well plates containing fresh medium with appropriate substances to be tested. Cells were cultured at 37 °C (5% CO_2, 95% air) for up to 48 h. Medium was changed every 3 days. Cell viability was measured using an MTT Cell Proliferation Assay [25]. Experiments were carried out in triplicates and absorbance readings were taken at 492 nm.

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