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ORIGINAL PAPER

Nano ZnO catalyzed green synthesis and cytotoxic assay of pyridinyl and pyrimidinyl bisphosphonates

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Abstract A straight forward approach ensuring the anchoring of bisphosphonate moiety onto various pyridinyl and pyrimidinyl amines is described by the three-component reaction of an amine, diethyl phosphite, and triethyl orthoformate in the presence of catalytic amount of nano ZnO as an environmentally benign and heterogeneous catalyst under solvent-free microwave irradiation conditions. All the synthesized compounds are evaluated for anti-cell proliferation activity against human breast (MCF-7), prostate (DU-145), osteosarcoma (MG-63), fibrosarcoma (HT-1080), multiple myeloma (RPMI-8226) cancer cell lines using sulforhodamine-B (SRB) assay method, and adriamycin as a reference drug. All the title compounds showed promising cytotoxic activity on the five cell lines. One compound was two times more active than adriamycin on all the five cancer cell lines.

Graphical abstract

Keywords Bisphosphonates · Nano ZnO · Solvent-free · Eco-friendly · Anticancer activity · SRB assay

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Introduction

Heteroaromatic compounds those bearing nitrogen as a heteroatom draw an enormous interest due to their wide-range spectrum of biological and pharmaceutical applications [1–4]. The pyridine and pyrimidine moieties are common core heterocyclic motifs present in some well-known components of human organisms [5–8]. Pyridine derivatives are an important class of aza-heterocycle occurred in many natural products and active pharmaceuticals, including vitamin B_3 (niacin), vitamin B_6 (pyridoxine), nicotine, isoniazid, and other nitrogen-containing plant products [9]. Pyrimidines are very important biologically active heterocycles that represent the most abundant fellows of the diazine family with cytosine, uracil, and thymine being constituents of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). In addition to this, pyrimidine moieties also occur in many natural products such as vitamin B_1 (thiamine) and many pharmaceutical compounds, such as barbituric acid and the HIV drug zidovudine [10].

Aminomethylene bisphosphonates are well established in the management of cancer-induced skeletal disorders [11]. Recent research studies suggest that nitrogen-containing bisphosphonates (N-BPs) stimulate the apoptosis of tumor cells as well as osteoclasts in bone metastatic sites [12]. They are potent inhibitors of osteoclast-mediated bone resorption and play a vital role in the supportive care of patients with bone metastases [13]. Several N-BPs such as clodronate, pamidronate, and zoledronic acid develop the clinical outcome for patients with multiple myeloma [14] and metastatic breast cancer [15]. Recently, zoledronic acid was found to increase disease-free survival and overall survival in some adjuvant breast cancer settings and prolonged survival in patients with several advanced cancers [16]. Besides the bone resorption and antitumor activity of

bisphosphonates, they are also showing antioxidant [17], antimicrobial [18], antitrypanosomal [19, 20], and herbicidal [21] activities. Along with their biological importance, aminomethylene bisphosphonates are also known for their metal-chelating ability. The uranyl (UO_2^{2+})-binding properties of these compounds were listed [22] to have excellent association constants.

The common synthetic routes to aminomethylene bisphosphonates (N-BPs) involve acid-catalyzed reactions of nitriles with phosphorous acid or phosphites, Beckmann rearrangement of oximes in the presence of phosphites [23], and reductive amination of carbonyl derivatives with aminomethyl diphosphonate [24]. However, the aforementioned procedures have one or more drawbacks such as long reaction times, require stoichiometric quantities of toxic catalysts, give product yields poor, and generate vast amounts of wastage [25].

It is evident from the recent literature that nanochemistry is an up growing research area due to their unique properties. These materials exhibit better catalytic activity compared to their bulk-sized counterparts [26–28]. The usage of nanomaterial provides advantages of high atom efficiency, simplified isolation of product, easy recovery, and recyclability of the catalysts. Among the nanoparticles, zinc oxide (nano ZnO) has received considerable attention due to its low toxicity, cost effectiveness, air and water compatibility, ease of handling, good reactivity, recyclability, experimental simplicity, remarkable ability to suppress side reactions in acid-sensitive substrates, environment friendly, and many other potential applications in diverse fields [29–35].

In recent days, microwave-assisted organic chemistry has growing at a rapid rate, because this technique provides an effective and alternative energy source for carrying out the chemical reactions and processes [36]. Thus, application of microwave energy to promote organic reactions is of increasing attention and offers numerous advantages over the conventional techniques [37]. Microwave reactions are eco-friendly and find valuable applications in organic synthesis [37–40], peptide synthesis [41], nanotechnology [42], polymer chemistry [43], and biochemical processes [44, 45]. In this study, we accomplished the synthesis and in vitro anticancer activity studies of tetraethyl (pyridinyl/pyrimidinyl amino)methylene bisphosphonates (TPAM-BPs) using nano ZnO as environmentally benign, reusable, heterogeneous acid catalyst under solvent-free and microwave irradiation conditions.

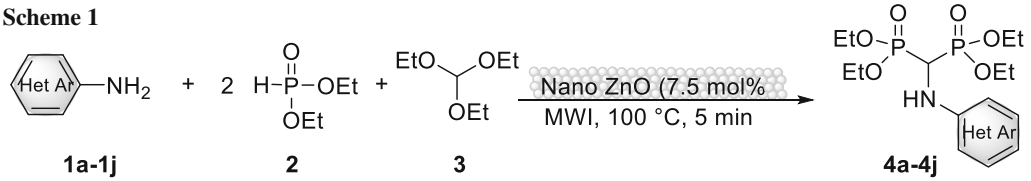
Results and discussion

A series of aminomethylene bisphosphonates **4a–4j** were synthesized via tri-component one-pot neat reaction of various pyridinyl and pyrimidinyl amines (**1a–1j**), diethyl phosphite (**2**), and triethyl orthoformate (**3**) using nano ZnO as an efficient catalyst under solvent-free and microwave irradiation conditions (Scheme 1). The products were obtained in high yields after simple work-up procedure.

In order to determine the best experimental conditions, we accomplished a model reaction by taking 3-aminopyridine (**1a**; 1 mmol), diethyl phosphite (**2**; 2.2 mmol), and triethyl orthoformate (**3**; 1 mmol). Initially, we have run the reaction under solvent-free conditions by using different catalysts such as $FeCl_3$, $ZnCl_2$, I_2, $CuCl_2$, $AlCl_3$, p-TSA, $BF_3{\cdot}SiO_2$, and Amberlyst 15 along with nano ZnO at 100 °C, and the results are listed in Table 1. Among all these catalysts, it was found that nano ZnO showed better catalytic activity. Most excitingly, when nano ZnO was used in 7.5 mol%, the reaction proceeded very smoothly and gave the product **4a** in 93% yield (Table 1, entry 10).

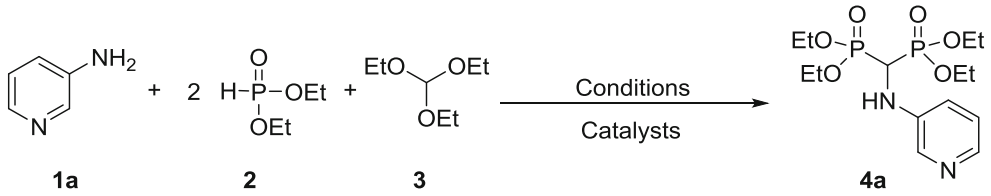
Further, to determine the exact requirement of catalyst for the reaction, we investigated the model reactions at different concentrations of catalyst such as 1, 2.5, 5, 7.5, and 10 mol% were loaded and yields were 35, 60, 75, 93, and 92%, respectively (Fig. 1). This indicates that 7.5 mol% of nano ZnO is sufficient for the best result by considering the reaction time and yield of product. Further, there is no development in the product yield even though the amount of catalyst is amplified. Thus, we generalized the procedure that 7.5 mol% of nano ZnO is sufficient for the synthesis of aminomethylene bisphosphonates under neat conditions. The catalytic activity of the recycled nano ZnO was also examined. Nano ZnO could be reused five times for the reaction without noticeable loss of activity (Fig. 2). When the effect of microwave irradiation power was studied on this reaction, use of high watts microwave irradiation not only enhanced the reaction rate but also avoided the side reactions.

Scheme 1



1a-1j **2** **3** **4a-4j**

Table 1 Influence of the catalyst on the synthesis of tetraethyl [(pyridin-3-ylamino)methylene]bis(phosphonate) (**4a**)



Entry	Catalyst/mol%	Solvent	Time/min	Yield[a]/%
1	$FeCl_3$ (10)	Solvent-free	5	30
2	$ZnCl_2$ (10)	Solvent-free	5	45
3	I_2 (10)	Solvent-free	5	40
4	$CuCl_2$ (10)	Solvent-free	5	55
5	$AlCl_3$ (10)	Solvent-free	5	50
6	p-TSA (10)	Solvent-free	5	55
7	$BF_3.SiO_2$ (10)	Solvent-free	5	60
8	Amberlyst 15 (10)	Solvent-free	5	70
9	Nano ZnO (10)	Solvent-free	5	92
10	Nano ZnO (7.5)	Solvent-free	5	93
11	Nano ZnO (5)	Solvent-free	5	75
12	Nano ZnO (2.5)	Solvent-free	5	60
13	Nano ZnO (1)	Solvent-free	5	35

Reaction of 3-aminopyridine (1 mmol), diethyl phosphite (2.2 mmol), and triethyl orthoformate (1 mmol) using various catalysts along with nano ZnO under neat conditions at 100 °C

[a] Isolated yield

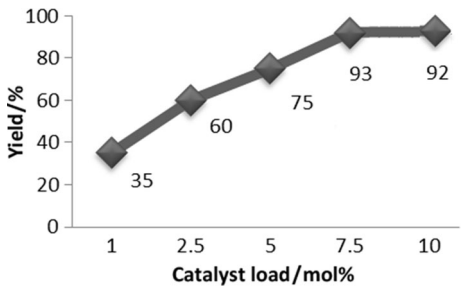


Fig. 1 Optimization of catalyst concentration

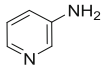
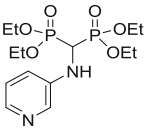
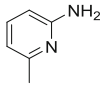
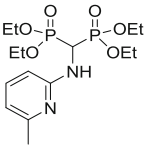
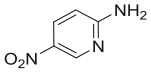
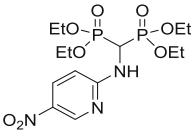
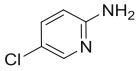
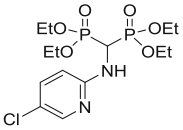
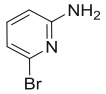
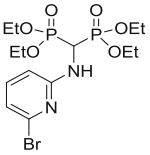
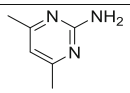
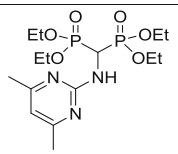
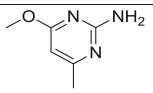
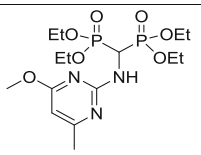
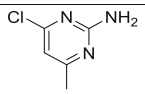
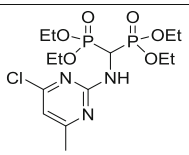
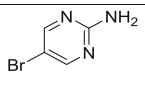
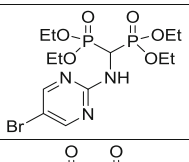
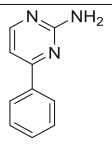
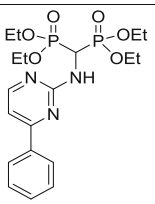
We investigated the scope and limitations of the catalyst for the reaction using various substituted pyridinyl/pyrimidinyl amines **1a**–**1j**, diethyl phosphite (**2**), and triethyl orthoformate (**3**), as shown in Table 2. The experimental results indicated that this catalyst was efficient for all the substituted pyridinyl/pyrimidinyl amines. From the results, we found that the 7.5 mol% of the catalyst nano ZnO, MWI at 100 °C for 5 min without solvent are the optimum conditions for the formation of title compounds **4a**–**4j** in good to excellent yields. High yields of the product, less reaction time, low catalyst loading, and reusability of the catalyst (up to five cycles), and microwave irradiation conditions are the advantages of this method.

Reusability of the catalyst

The recyclability of the nano ZnO catalyst was checked for the model reaction (**4a**). After each run, dichloromethane (2×15 cm^3) was added to the reaction mixture and filtered. The catalyst residue was washed with chloroform, dried, and reused for next run. The yields obtained are in 93, 91, 90, 88, and 86% yields over five cycles (Fig. 2). The results indicate that the catalyst can be reused for five times without loss of its activity.

Formation of the title compounds **4a**–**4j** in the presence of nano ZnO (Scheme 2) involves the mechanism of the three-component condensation [47, 48], which is similar to the Kabachnik–Fields reaction in which a carbonyl component is replaced by triethyl orthoformate of corresponding electrophilic character. Imines are commonly suggested as intermediates of the Kabachnik–Fields reaction. The first step of the condensation is the interaction of orthoformate with the nano ZnO surface that generated the more electrophilic carbon center followed by the nucleophilic attack of amine to give imine-type intermediate **I**. The more electrophilic carbon center generated by catalytic surface was attacked by the nucleophilic amine. In this step, nano ZnO as strong Lewis acid enhances the electrophilicity of the active carbon of the

Table 2 Synthesis of aminomethylene bisphosphonates **4a–4j** on nano ZnO catalyst by MWI via Scheme 1

Entry	**1a–1j**	**4a–4j**	Time/min	Yield[a]/%	M.p./°C
1			5	93	154–156
2			5	91	142–144 [46]
3			5	90	147–149
4			5	92	185–187
5			5	91	133–135
6			5	90	156–158
7			5	92	166–168
8			5	93	175–177
9			5	93	140–142
10			5	91	149–151

Reaction conditions: various pyridinyl/pyrimidinyl amines, diethyl phosphonate, and triethyl orthoformate on 7.5 mol% nano ZnO catalyst at 100 °C

[a] Isolated yields

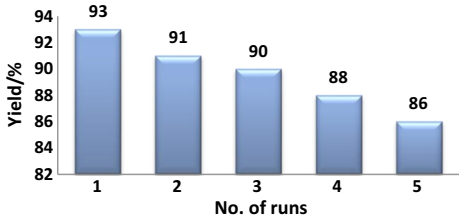


Fig. 2 Reusability of the catalyst

orthoformate. In the next steps, the nucleophilic addition of two moles diethyl phosphite to the C=N bond of the imines results in phosphonates **II** and **III**, respectively. Then, the elimination of an ethanol may lead to the formation of bisphosphonates **IV**.

The compounds **4a**–**4j** were characterized by physical and spectral (IR, NMR, and mass) data. They showed strong IR absorption bands in the regions of 3296–3214, 1264–1224, 1024–1009, and 760–720 cm^{-1} for –NH, –P=O, –P–O–C, and P–C aliphatic stretching frequencies, respectively. In ^{1}H NMR spectra, the singlet at $\delta = 6.86$–5.82 ppm confirmed the –NH protons. The chemical shifts in the region of 8.95–6.48 ppm are due to aromatic protons and the multiplet at 5.64–4.85 ppm corresponds to HC–P proton. The multiplet in the region of 4.18–4.04 and multiplet at 1.38–1.14 ppm are due to the O–CH_2–CH_3 and O–CH_2–CH_3. In ^{13}C NMR spectra, the chemical shifts in the region of 184.5–101.3 ppm are assigned to carbons of aromatic ring; the signals in the region of 64.6–58.4, 52.6–43.4, and 16.4–16.1 ppm confirmed the –O–CH_2–CH_3, HC–P, and –O–CH_2–CH_3 carbons. The ^{31}P NMR chemical shifts of the title compounds appeared in the range of 16.2–24.4 ppm.

In vitro cytotoxic activity

In order to investigate the effectiveness of the in vitro cell cytotoxic properties of synthesized aminomethylene bisphosphonates, they were subjected to the well-known sulforhodamine-B (SRB) cytotoxic assay [49] against human breast (MCF-7), prostate (DU-145), osteosarcoma (MG-63), fibrosarcoma (HT-1080), and multiple myeloma (RPMI-8226) cancer cell lines. All the data were expressed as IC_{50} values (the dose which causes 50% inhibition of viable cells) of the tested compounds. The results are illustrated in Table 3. Compounds **4i** and **4j** have the most active and broad spectrum activities on the five cancer cell lines which are more active than that of standard drug adriamycin. Compound **4j** was two times more active than that of adriamycin in all the five cell lines. While compounds **4d**, **4e**, **4f**, **4g**, and **4h** showed moderate cytotoxic

Scheme 2

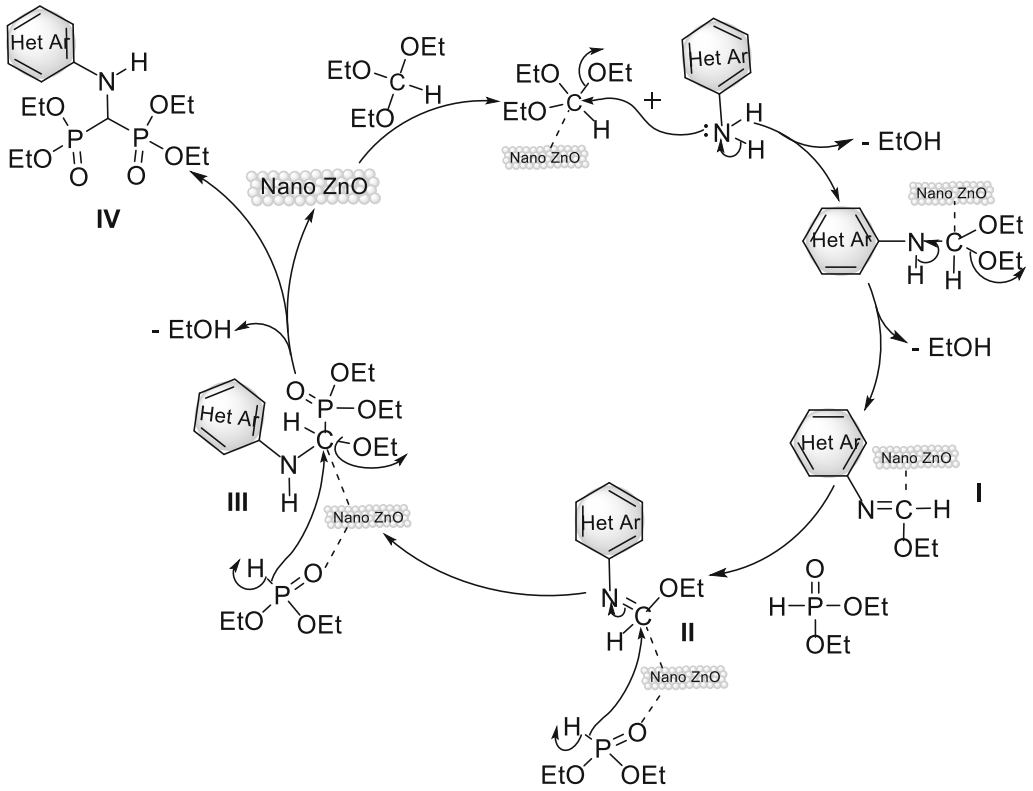


Table 3 In vitro anticancer activity of human breast (MCF-7), prostate (DU-145), osteosarcoma (MG-63), fibrosarcoma (HT-1080), and multiple myeloma (RPMI-8226) cancer cell lines

Samples	MCF-7	DU-145	MG-63	HT-1080	RPMI-8226
IC_{50}/nM ± SD					
4a	47.53 ± 0.006	48.08 ± 0.017	49.63 ± 0.003	50.51 ± 0.005	54.05 ± 0.007
4b	38.63 ± 0.019	36.34 ± 0.025	42.69 ± 0.177	41.63 ± 0.019	48.08 ± 0.025
4c	30.97 ± 0.019	32.03 ± 0.025	37.62 ± 0.177	33.52 ± 0.019	35.04 ± 0.025
4d	23.76 ± 0.055	29.87 ± 0.008	27.54 ± 0.015	21.36 ± 0.023	28. 61 ± 0.003
4e	18.78 ± 0.055	19.67 ± 0.008	17.74 ± 0.015	20.42 ± 0.023	21.85 ± 0.003
4f	16.92 ± 0.026	17.89 ± 0.035	15.31 ± 0.006	19.48 ± 0.004	20.78 ± 0.015
4g	14.94 ± 0.026	15.64 ± 0.035	12.34 ± 0.006	14.63 ± 0.004	13.81 ± 0.015
4h	12.87 ± 0.005	11.64 ± 0.037	8.97 ± 0.013	10.83 ± 0.024	9.88 ± 0.016
4i	8.79 ± 0.006	7.81 ± 0.017	7.23 ± 0.093	8.76 ± 0.026	7.25 ± 0.005
4j	6.15 ± 0.006	5.48 ± 0.017	4.32 ± 0.093	5.47 ± 0.026	4.63 ± 0.005
Adriamycin	12.42 ± 0.0015	11.57 ± 0.002	8.79 ± 0.004	10.23 ± 0.015	9.76 ± 0.002

activity, the compounds **4b**, **4c**, and **4a** could not show effective cytotoxic activity on all the five cell lines.

In the previous literature [50], adriamycin was used as a standard drug against various cancer cell lines such as colon (SW620), breast (MCF7), cervix (HeLa), and liver (HEPG2), and the values were 35.8, 43.8, 57.2, and 46.6, respectively. Likewise, in another report [51], for colon (SW480), lung carcinoma cells (A549), hepatic carcinoma cells (HepG2), and cervical cancer cells (HeLa), the obtained standard values are 10.9, 13.5, 11.5, and 12.5, respectively. Comparatively the same drug as standard in another report [52] acted against breast (MDA-MB453, MCF7), lung (NCI-H522, NCI-H23), and kidney (HEK-2933T) cancer cell lines and the values are 23.98, 19.01, 25.92, 26.76, and 70.23, respectively. While our compounds when treated for various cancer lines such as human breast (MCF-7), prostate (DU-145), osteosarcoma (MG-63), fibrosarcoma (HT-1080), multiple myeloma (RPMI-8226), using adriamycin as a standard drug, the obtained values are 12.42, 11.57, 8.79, 10.23, and 9.76, respectively. When our compounds **4a**–**4j** are treated with the aforementioned cancer cell lines, all the compounds showed moderate to good activity against all the five cancer call lines, but compound **4j** showed (6.15, 5.48, 4.32, 5.47, and 4.63) twice more activity than that of adriamycin in all the five cell lines.

Structure–activity relationship (SAR)

Structure–activity relationship studies showed that the anticancer activity exhibited by the newly synthesized aminomethylene bisphosphonates depended on two series: one is pyridine series (**4a**–**4e**) and another one is pyrimidine series (**4f**–**4j**). In general, the pyrimidine series exhibited better antitumor activity than pyridine series. Among the pyrimidine series, **4j** and **4i** exhibited highest tumor activity when compared to adriamycin. This might be due to the presence of the benzene ring and bromine attached to pyrimidine ring, respectively.

Conclusions

In conclusion, nano ZnO was found to be an efficient catalyst for the synthesis of aminomethylene bisphosphonates **4a**–**4j** in good to excellent yields. The notable advantages of the present synthetic protocol are operational simplicity, eco-friendly, catalyst reusability, simple work-up, and high yields. Among the synthesized compounds, **4i** and **4j** showed potent cytotoxic activity against various cell lines than that of the standard drug Adriamycin. Interestingly, compound **4j** is twice more active than the standard drug. We believe that this method serves a practical alternative to existing methods for the synthesis of aminomethylene bisphosphonates.

Experimental

All the solvents and chemicals were procured from Merck and Sigma-Aldrich and were used without further purification. Microwave irradiation was carried out in microwave oven, catalyst system (CATA-4R). Melting points of the compounds were determined in open capillary tube on Guna melting pointing apparatus and are uncorrected. Infrared spectra were recorded on Bruker ALPHA interferometer instrument. ^{1}H, ^{13}C, and ^{31}P NMR were recorded on Bruker 400 MHz instrument. Mass spectra were recorded on ESI–MS model in positive mode and elemental analysis was carried out in FLASH EA Thermo

Finnigan 1112 instrument. The cancer cell lines for anti-cancer activity were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured with RPMI 1640 medium (GIBCO BRL, Grand Island, NY, USA).

General procedure for the synthesis of aminomethylene bisphosphonates 4a–4j

Various pyridinyl/pyrimidinyl amines (**1a**–**1j**; 1 mmol), diethyl phosphite (**2**; 2.2 mmol), triethyl orthoformate (**3**; 1 mmol), and 7.5 mol% of nano ZnO were taken in a flat-bottomed flask and irradiated with microwave irradiation using Catalyst System (CATA-4R) at 400 W. The progress of the reaction was monitored by TLC (3:2; *n*-hexane:ethyl acetate) for every 1 min. The reaction was completed in 5 min. After completion of reaction, the mixture was dissolved in 10 cm^3 of DCM and filtered to remove the catalyst as residue. The organic layer was washed with water (2 × 5 cm^3) and the water layer was discarded. The combined organic mixture was washed with 5 cm^3 brine solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure; the solid obtained was washed with cold water, air-dried, and recrystallized from ethanol to get the pure compounds.

Tetraethyl [(pyridin-3-ylamino)methylene]bis(phosphonate) (**4a**, $C_{14}H_{26}N_2O_6P_2$)
Yield 93% (white solid); m.p.: 154–156 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.21 (1H, s, Ar–H), 8.10 (1H, d, J = 8 Hz, Ar–H), 7.27 (1H, d, J = 8 Hz, Ar–H), 6.97 (1H, d, J = 8 Hz, Ar–H), 5.87 (1H, s, NH), 5.48–5.40 (1H, m, P–CH), 4.12–4.06 (8H, m, PO$\underline{CH_2}$$CH_3$), 1.22–1.18 (12H, m, POCH$_2$$\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 146.33, 142.48, 138.98, 126.34, 121.31, 64.04 (d, $^1J_{P–C}$ = 11 Hz, PO$\underline{C}$H$_2$CH$_3$) 44.87 (d, $^2J_{P–C}$ = 36 Hz, P–C–H), 16.35 (d, $^3J_{P–C}$ = 28 Hz, POCH$_2$$\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 21.16 ppm; IR (neat): ν = 3217 (NH), 1260 (P=O), 1017 (P–O–C), 760 (P–C aliphatic) cm^{-1}; ESI–MS: m/z (%) = 381 (48, $[M + 1]^+$), 380 (35, $[M]^+$), 355 (22), 346 (23), 152 (54), 91 (16), 90 (2), 77 (64).

Tetraethyl [(6-methylpyridin-2-ylamino)methylene]-bis(phosphonate) (**4b**)
Yield 91% (white solid); physical data were found to agree with those described in Ref. [46].

Tetraethyl [(5-nitropyridin-2-ylamino)methylene]bis(phosphonate) (**4c**, $C_{14}H_{25}N_3O_8P_2$)
Yield 90% (brown solid); m.p.: 147–149 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.93 (1H, s, Ar–H), 8.37 (1H, d, J = 8 Hz, Ar–H), 6.92 (1H, d, J = 4 Hz, Ar–H), 5.93 (1H, s, NH), 5.48–5.42 (1H, m, P–CH), 4.11–4.06 (8H, m, PO$\underline{CH_2}$$CH_3$), 1.17–1.20 (12H, m, POCH$_2$$\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 161.90, 148.82, 141.42, 136.57, 133.51, 107.76, 64.32 (d, $^1J_{P–C}$ = 32 Hz, PO$\underline{C}$H$_2$-CH$_3$), 54.87 (d, $^2J_{P–C}$ = 36 Hz, P–C–H), 16.35 (d, $^3J_{P–C}$ = 28 Hz, POCH$_2$$\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 8.26 ppm; IR (neat): ν = 3236 (NH), 1215 (P=O), 1018 (P–O–C), 723 (P–C aliphatic) cm^{-1}; ESI–MS: m/z (%) = 426 (78, $[M + 1]^+$), 425 (35, $[M]^+$), 395 (43), 326 (22), 252 (51), 141 (18), 110 (8), 87 (34).

Tetraethyl [(5-chloropyridin-2-ylamino)methylene]bis-(phosphonate) (**4d**, $C_{14}H_{25}ClN_2O_6P_2$)
Yield 91% (white solid); m.p.: 185–187 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.31 (1H, s, Ar–H), 7.67 (1H, d, J = 8 Hz, Ar–H), 6.74 (1H, d, J = 4 Hz, Ar–H), 5.86 (1H, s, NH), 5.32–5.24 (1H, m, P–CH), 4.14–4.08 (8H, m, PO$\underline{CH_2}$$CH_3$), 1.18–1.22 (12H, m, POCH$_2$$\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 153.10, 146.62, 137.93, 126.48, 133.51, 117.76, 114.92, 64.06 (d, $^1J_{P–C}$ = 32 Hz, PO$\underline{C}$H$_2$CH$_3$), 52.66 (d, $^2J_{P–C}$ = 36 Hz, P–C–H), 16.32 (d, $^3J_{P–C}$ = 28 Hz, POCH$_2$$\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 18.58 ppm; IR (neat): ν = 3248 (NH), 1247 (P=O), 1010 (P–O–C), 720 (P–C aliphatic) cm^{-1}; ESI–MS: m/z (%) = 415 (84, $[M + 1]^+$), 414 (65, $[M]^+$), 395 (28), 366 (32), 315 (50), 291 (16), 262 (6), 97 (64).

Tetraethyl [(6-bromopyridin-2-ylamino)methylene]bis-(phosphonate) (**4e**, $C_{14}H_{25}BrN_2O_6P_2$)
Yield 88% (orange solid); m.p.: 133–135 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.58 (1H, t, ^2J = 16 Hz, Ar–H), 7.07 (1H, d, J = 8 Hz, Ar–H), 6.78 (1H, d, J = 8 Hz, Ar–H), 5.83 (1H, s, NH), 5.56–5.48 (1H, m, P–CH), 4.12–4.07 (8H, m, PO$\underline{CH_2}$$CH_3$), 1.22–1.12 (12H, m, POCH$_2$$\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.15, 143.40, 138.44, 117.6, 127.71, 116.01, 113.09, 106.07, 64.38 (d, $^1J_{P–C}$ = 163.4 Hz, PO$\underline{C}$H$_2$CH$_3$), 54.86 (d, $^2J_{P–C}$ = 36 Hz, P–C–H), 16.38 (d, $^3J_{P–C}$ = 28 Hz, POCH$_2$$\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 21.20 ppm; IR (neat): ν = 3273 (NH), 1256 (P=O), 1021 (P–O–C), 735 (P–C aliphatic) cm^{-1}; ESI–MS: m/z (%) = 460 (95, $[M + 1]^+$), 459 (75, $[M]^+$), 455 (22), 446 (63), 252 (34), 191 (26), 98 (2), 72 (44).

Tetraethyl [(4,6-dimethylpyrimidin-2-ylamino)methylene]-bis(phosphonate) (**4f**, $C_{15}H_{29}N_3O_6P_2$)
Yield 92% (white solid); m.p.: 156–158 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.74 (1H, s, Ar–H), 5.78 (1H, s, NH), 5.14–4.96 (1H, m, PCH), 4.11–4.06. (8H, m, PO$\underline{CH_2}$$CH_3$), 2.58 (6H, s, 2 Ar-$CH_3$), 1.22–1.14 (12H, m, POCH$_2$$\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 164.89, 157.16, 115.49, 110.3, 107.2, 64.06 (d, $^2J_{P–C}$ = 7.6 Hz, PO$\underline{C}$H$_2$CH$_3$), 53.80 (d, $^1J_{P–C}$ = 163.4 Hz, P–C–H), 24.75 (Ar-CH_3), 16.35 (d,

$^{3}J_{P-C}$ = 7.0 Hz, $POCH_2\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 20.85 ppm; IR (neat): ν = 3248 (NH), 1245 (P=O), 1018 (P–O–C), 733 (P–C aliphatic) cm^{-1}; ESI–MS: *m/z* (%) = 410 (84, $[M + 1]^+$), 409 (65, $[M]^+$), 385 (42), 346 (42), 252 (34), 168 (16), 90 (16), 67 (24).

Tetraethyl [(4-methoxy-6-methylpyrimidin-2-ylamino)-methylene]bis(phosphonate) (**4g**, $C_{15}H_{29}N_3O_7P_2$)
Yield 87% (pale yellow solid); m.p.: 166–168 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.12 (1H, s, Ar–H), 5.92 (1H, s, NH), 5.18–5.06 (1H, m, PCH), 4.14–4.09 (8H, m, $PO\underline{CH_2}CH_3$), 2.54 (3H,s, Ar–O-CH_3), 1.48 (3H, s, Ar-CH_3), 1.20–1.14 (12H, m, $POCH_2\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.33, 166.51, 159.67, 110.69, 64.08 (d, $^{2}J_{P-C}$ = 7.0 Hz, $PO\underline{CH_2}CH_3$), 53.75 (Ar–O–CH_3), 51.76 (d, $^{1}J_{P-C}$ = 163.4 Hz, P–C–H), 24.86 (Ar–CH_3), 16.35 (d, $^{3}J_{P-C}$ = 7.0 Hz, $POCH_2\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 17.95; IR (neat): ν = 3267 (NH), 1234 (P=O), 1024 (P–O–C), 742 (P–C aliphatic) cm^{-1}; ESI–MS: *m/z* (%) = 426 (68, $[M + 1]^+$), 425 (42, $[M]^+$), 385 (24), 346 (28), 242 (54), 176 (26), 98 (34), 64(16).

Tetraethyl [(4-chloro-6-methylpyrimidin-2-ylamino)-methylene]bis(phosphonate) (**4h**, $C_{14}H_{26}ClN_3O_6P_2$)
Yield 89% (orange solid); m.p.: 175–177 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.84 (1H, s, Ar–H), 5.74 (1H, s, NH), 5.14–5.02 (1H, m, PCH), 4.13–4.08 (8H, m, $POCH_2CH_3$), 2.57 (3H, s, Ar-CH_3), 1.22–1.16 (12H, m, $POCH_2\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.88, 162.58, 161.46, 112.31, 63.98 (d, $^{2}J_{P-C}$ = 7.5 Hz, $PO\underline{CH_2}CH_3$), 52.78 (d, $^{1}J_{P-C}$ = 18 Hz, P–C–H), 24.76 (Ar-CH_3), 16.35 (d, $^{3}J_{P-C}$ = 7.0 Hz, $POCH_2\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 22.63 ppm; IR (neat): ν = 3276 (NH), 1247 (P=O), 1016 (P–O–C), 728 (P–C aliphatic) cm^{-1}; ESI–MS: *m/z* (%) = 430 (79, $[M + 1]^+$), 429 (65, $[M]^+$), 375 (28), 346 (32), 252 (45), 178 (16), 96 (21), 76 (46).

Tetraethyl [(5-bromopyrimidin-2-ylamino)methylene]-bis(phosphonate) (**4i**, $C_{13}H_{24}BrN_3O_6P_2$)
Yield 92% (white solid); m.p.: 140–142 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.65 (2H, s, Ar–H), 6.25 (1H, s, NH), 5.20–5.08 (1H, m, PCH), 4.15–4.07 (8H, m, $PO\underline{CH_2}CH_3$), 1.22–1.16 (12H, m, $POCH_2\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.82, 156.3, 107.41, 64.36 (d, $^{2}J_{P-C}$ = 7.0 Hz, $PO\underline{CH_2}CH_3$), 52.78 (d, $^{1}J_{P-C}$ = 163.4 Hz, P–C–H), 16.36 (d, $^{3}J_{P-C}$ = 7.5 Hz, $POCH_2\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 21.50; IR (neat): ν = 3248 (NH), 1245 (P=O), 1018 (P–O–C), 733 (P–C aliphatic) cm^{-1}; ESI–MS: *m/z* (%) = 460 (82, $[M + 1]^+$), 459 (68, $[M]^+$), 425 (22), 386 (24), 256 (64), 190 (26), 145 (14), 82 (63).

Tetraethyl [(4-phenylpyrimidin-2-ylamino)-methylene]bis(phosphonate) (**4j**, $C_{19}H_{29}N_3O_6P_2$)
Yield 90% (yellow solid); m.p.: 149–151 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.14 (1H, d, *J* = 4.0 Hz, Ar–H), 7.66 (2H, d, *J* = 4.0 Hz, Ar–H), 7.46 (2H, d, *J* = 4.0 Hz, Ar–H), 7.38 (1H, t, ^{2}J = 8.0 Hz, Ar–H), 6.88 (1H, d, *J* = 4.0 Hz, Ar–H), 6.18 (1H, s, NH), 5.28–5.16 (1H, m, PCH), 4.15–4.10 (8H, m, $PO\underline{CH_2}CH_3$), 1.24–1.16 (12H, m, $POCH_2\underline{CH_3}$) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 159.93, 159.70, 159.14, 137.54, 130.44, 128.92, 128.11, 111.10, 62.38 (d, $^{2}J_{P-C}$ = 7.0 Hz, $PO\underline{CH_2}CH_3$), 52.56 (d, $^{1}J_{P-C}$ = 18.5 Hz, P–C–H), 16.39 (d, $^{3}J_{P-C}$ = 7.5 Hz, $POCH_2\underline{CH_3}$) ppm; ^{31}P NMR (161.7 MHz, $CDCl_3$): δ = 20.88 ppm; IR (neat): ν = 3296 (NH), 1263 (P=O), 1019 (P–O–C), 724 (P–C aliphatic) cm^{-1}; ESI–MS: *m/z* (%) = 458 (84, $[M + 1]^+$), 457 (45, $[M]^+$), 411 (28), 346 (23), 277 (54), 162 (16), 98 (12), 84 (24).

In vitro cytotoxicity assay

All the synthesized compounds **4a**–**4j** were tested for their cytotoxic activity using sulforhodamine-B (SRB) assay method [49]. MCF-7, DU-145, MG-63, HT-1080 and RPMI-8226 cancer cell lines were seeded for 24 h in a 96-well microtiter plates at a concentration of 1000–2000 cells/well and then the cells were incubated for 48 h with several concentrations of 100–6.25 μmol/cm^3 (each repeated thrice). After treatment of 10% trichloroacetic acid, 150 mm^3/well was added for 1 h at 4 °C, washed with distilled water for 3 times. Wells were stained with 0.4% (w/v) sulforhodamine-B 50 mm^3/well for 10–30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. The dye was solubilized with 100 mm^3 of 10 mM Tris-base solution. The optical density (OD) of each well was measured spectrophotometrically with an ELISA microplate reader at 545 nm. To achieve the survival curve, the percent of surviving cells was calculated and plotted against different concentrations of the tested compounds. The IC_{50} values were calculated using sigmoidal concentration–response curve fitting models (Sigma plot software).

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