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Original article

PEG-SO_3H catalyzed synthesis and cytotoxicity of α-aminophosphonates

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ABSTRACT

One pot three-component PEG-SO_3H catalyzed reaction of 4-(Pyridin-4-yl)benzaldehyde and triethyl phosphite with various primary amines afforded α-aminophosphonates with high yields by the Kabachnik–Field's reaction. These new structurally diversified set of α-aminophosphonates (**4a**–**j**) were evaluated for their anti-tumor activity on human chronic myeloid leukemia cells (**K 562**), human colon carcinoma cells (**Colo 205**) along with non-cancerous human embryonic kidney cells (**HEK 293**). They showed moderate activity on both cancerous cells and non-cancerous cells.

1. Introduction

α-Aminophosphonates are an important class of bioactive molecules. Their use as peptide mimics [1], enzyme inhibitors [2], antibiotics [3] and anti HIV [4], anti cancer [5] and thrombotic [6] agents has proved their vast potentiality both as direct drugs and as drug precursors for several disease manifestations. Thus, their significant role in life processes prompted the development of many elegant synthetic approaches for them.

The chiral Brønsted acid catalyzed synthesis of α-aminophosphonates had achieved through enantioselective hydrophosphonylation of imines, which is having the synthetic importance [7]. Similarly the Lewis acid promoted nucleophilic phosphite addition to imines is one of the most convenient methods to obtain them [8–13]. The major drawback of reported methodologies is deactivation of the lewis acid catalyst by water released during the course of this reaction. Even the reported catalysts such as $In(OTf)_3$ [14], $H_3PW_{12}O_{40}$ [15] and $Na_2CaP_2O_7$ [16] used for the conversion of carbonyl compounds into α-aminophosphonates could not improve the product yields. Similarly many other reported methods were also not satisfactory since they required toxic chemicals as reagents, complicated experimental set-up, long reaction times and complicated workup procedures. In the recent past, use of soluble polymer supported catalyst for the synthesis of α-aminophosphonates gained attention due to the use of homogeneous reaction conditions [17–22]. However this procedure also could not afford the pure products in satisfactory yields. Our continuous study to synthesize the target specific less toxic and effective bioactive organophosphorus compounds resulted in the development of an elegant one-pot method for the synthesis of α-aminophosphonates using poly ethylene glycol sulfonic acid (PEG-SO_3H) [23,24] as an efficient recyclable catalyst under mild conditions.

In recent, some α-aminophosphonates were reported as antiviral drugs containing a pyridazine [25], amide moieties [26] and anti-tumor drugs containing a chiral α-amino carboxamide [27], benzthiazole moieties [28]. In this connection we had also reported various biologically active organophosphorus compounds of diverse classes [29–31] including α-aminophosphonates [32–35] and some are identified as the potential anti cancer agents [36,37]. Despite reasonable progress achieved in cancer chemotherapy, high toxicity and low specificity of the current medications

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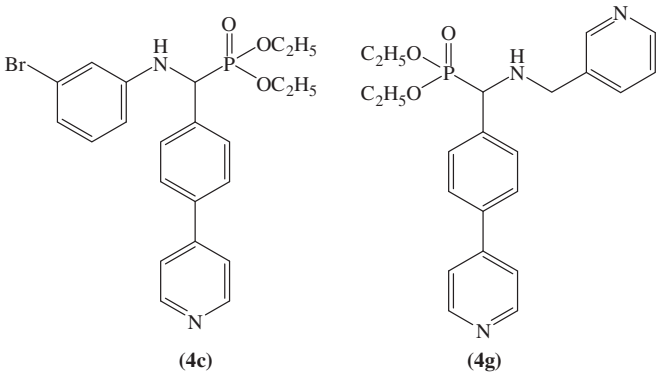


Fig. 1. Cytotoxic α-aminophosphonates **4c** and **4g**.

are driving search for the development of more effective, less toxic cancer drugs. In pursuit of this goal we continued our research and accomplished synthesis of a series of new α-aminophosphonates (**4a**–**j**) and studied their anti-tumor activity. We are successful in identifying the newly synthesized compounds **4c** & **4g** as the potential cancer cytotoxic compounds (Fig. 1). Further, this group of compounds with an aminophosphonate pharmacophore may even have broad range of bioactivity against many other bacterial and viral diseases. Our survey revealed that no such work has been reported on these compounds.

2. Results and discussion

2.1. Chemistry

The three-component reaction of 4-(Pyridin-4-yl)benzaldehyde (**1**) and triethyl phosphite (**3**) with various aryl/heteroaryl substituted primary amines (**2**) led to the formation of diethyl (aryl/heteroaryl amino)(4-(pyridine-4-yl)phenyl)methyl phosphonates (**4a**–**j**) (Scheme 1). In the first step, the corresponding imine intermediate (**2^{I}a**–**j**) was formed from the aldehyde (**1**) and amines (**2a**–**j**) and in the second step subsequent addition of Phosphite (**3**) to the C=N bond of the imine (**2^{I}a**–**j**) occurs to from the corresponding α-aminophosphonates (**4a**–**j**). However, this one-pot Kabachnik–Fields reaction is not straightforward, because water formed during imine formation in the first step hydrolyzes the imine intermediate (**2^{I}a**–**j**) and thus retards the forwarded process of the reaction. This drawback is prevented or at least minimized by the use of PEG-SO_3H catalyst which traps the released water and subsequently drive the reaction forward to the second step facilitating phosphite (**3**) addition to imine (**2^{I}a**–**j**) to form α-aminophosphonates.

Several members of structurally diversified set of α-aminophosphonates (**4a**–**j**) were designed with various aryl/heteroaryl primary amine moieties substituted with different electron withdrawing and donating groups at the α-carbon atom of **4a**–**j**. The main aim in the design and synthesis of the title compounds is to study the variations in cytotoxicity as a function of the chemical structure (Fig. 2).

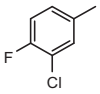
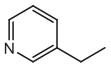
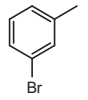
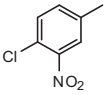
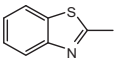
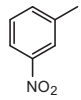
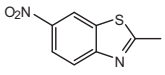
Entry	R	Entry	R
4a		4f	
4b		4g	
4c		4h	
4d		4i	
4e		4j	

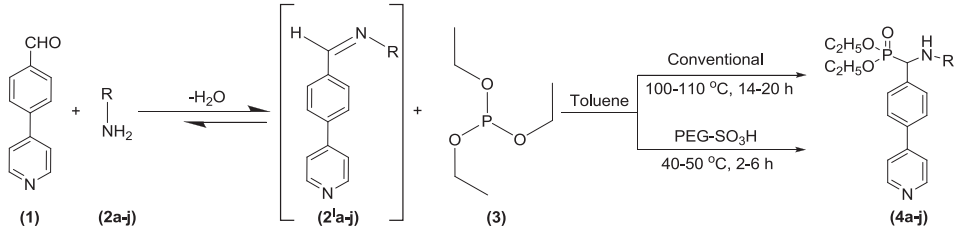
Fig. 2. Structure of diethyl (aryl/heteroaryl amino)(4-(pyridine-4-yl)phenyl)methyl phosphonates (**4a**–**j**).

To establish optimization experimental conditions for the preparation of **4a**–**j**, a typical reaction with aldehyde (**1**), aniline (**2**) and phosphite (**3**), was carried out both by conventional as well as by PEG-SO_3H catalyzed condition in various solvents. In conventional preparation in refluxing toluene even after 14–20 h of reaction, only unsatisfactory product yield (70%) was observed. When the same reaction was performed in the presence of PEG-SO_3H as catalyst at 40–50 °C for the duration of 2 h 96% yield of the product was obtained. Similarly the PEG-SO_3H catalyzed synthesis of **4a** was carried out in various solvents like dichloromethane, tetrahydrofuran, ethanol and toluene at various concentrations of PEG-SO_3H catalyst with 1.0, 0.5, 0.1 M ratios (Table 1). It was found that toluene as a solvent with 0.1 M quantity of the PEG-SO_3H catalyst are ideal for the synthesis of compound **4a**.

The same reaction for the synthesis of compound **4a** with various catalysts in toluene under different temperature conditions was recorded and the analysis is presented in Table 2 as an effective optimization for the selection of catalyst.

The advantage of PEG-SO_3H as a catalyst is that it is water stable, non-corrosive, environmentally benign, recyclable and works efficiently with a wide variety of aryl/heteroaryl substrates. The comparative experimental data on the preparation of all the title compounds with conventional and PEG-SO_3H catalyzed pathways in toluene are given in Table 3.

The proposed structures for the synthesized compounds **4a**–**j** were confirmed by IR, ^{1}H, ^{13}C, ^{31}P and mass spectrometry and elemental analyses. The IR spectra of **4a**–**j** showed the expected absorption bands at 3350–3330 and 1255–1233 cm^{-1} for the NH and



Scheme 1. Synthesis of Diethyl (aryl/heteroaryl amino)(4-(pyridine-4-yl)phenyl)methyl phosphonates (**4a**–**j**).

Table 1
Optimization of catalyst at different concentrations with various solvents for the preparation of **4a**.

Entry	Solvent	Yield (%)[b]		
		1.0[a]	0.5[a]	0.1[a]
1	Dichloromethane	60.0	45.0	20.0
2	Tetrahydrofuran	65.0	55.0	40.0
3	Ethanol	70.0	60.0	55.0
4	Toluene	95.0	93.0	95.0

[a] Moles of PEG-SO_3H.
[b] Yields were given as an average value from two independent consecutive determinations.

P═O stretching vibrations [38]. In the ^1H NMR spectra, the compounds **4a**–**j** gave triplet at δ 5.78–5.02 due to its vicinal coupling with the proton at the α-carbon and in some compounds the NH proton appeared as a broad singlet at δ 5.67–5.04. The doublet of doublet signal at δ 4.98–4.60 is for P–CH proton, the multiplet at δ 4.19–3.66 is for $POCH_2$ proton [39]. In the ^{13}C NMR the doublet at δ 68.6–52.8 is ascribed for the α-carbon. The other carbons gave chemical shifts in their expected region. ^{31}P chemical shifts were observed as singlet at δ 26.45–23.50 for all the compounds **4a**–**j**.

2.2. Pharmacology

The cytotoxicity of synthesized compounds **4a**–**j** was evaluated *in vitro* against human chronic myeloid leukemia cells (**K 562**) and human colon carcinoma cells (**Colo 205**) after 24 h exposure and their IC_{50} values were determined from a graph of cell viability measured over a range of concentrations between 1 and 100 μM. Each data point was the average of two determinations that in all cases differed by 8–10% or less. These results are summarized in Table 4. Initially, the IC_{50} was determined at a broad range of concentrations specifically 1, 10 & 100 μM and then at a narrow range of concentrations specifically 2, 4, 8, 10, 16, 32 & 64 μM of the title compounds against the cell lines. For this data, a line graph was plotted between concentrations (*X*-axis) versus % inhibition (*Y*-axis) and then an intersection drawn at 50% inhibition on *Y*-axis and then correlated to the concentration value on *X*-axis. Finally that concentration value is considered as IC_{50} in μM and these results are summarized in Table 4.

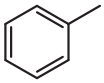
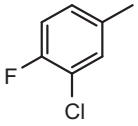
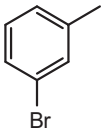
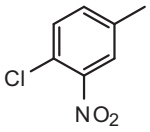
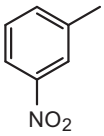
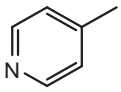
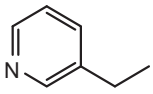
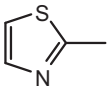
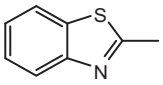
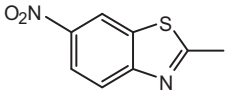
From the data it is revealed that all compounds exhibited different range of significant cytotoxic activities varying from 35 μM to 5 μM due to structural differences. As evident from the cytotoxicity data compounds **4c** and **4g** with 3-bromo aniline and 3-aminopyridine moieties at the α-carbon atom showed highest activity against the **K 562** cell lines with (IC_{50} 10.0 and 10.0 μM respectively). Similarly the same compounds **4c** and **4g** exhibit high cytotxicity against **Colo 205** cell lines with IC_{50} 5.0 and 8.0 μM respectively. The 4-methylamino pyridine derivative (**4f**) also has very low IC_{50} data. Their bioactivity data is presented in the graphical representation also (Fig. 3).

Table 2
Optimization of catalyst at different temperatures with various catalysts for the preparation of **4a**.[a]

Entry	Catalyst	Time (h)	Yield (%)[b]	
			rt	50 °C
1	Bismuth chloride	6	–	92
2	Ferric chloride	2	20	92
3	Silica sulfuric acid	5	87	87
4	Silica supported Tantalum chloride	22	92	92
5	Triphenyl phosphine	12	–	86
6	β-Cyclodextrin	24	64	61
7	Sulfamic acid	6	38	42
8	*p*-Toulenesulfonic acid	1.5	59	59
9	Cellulose Sulfuric acid	1.5	60	91
10	PEG-Sulfuric acid	2	89	98

[a] Reaction conditions: All reactions were carried in toluene with 1 mmol of catalyst.
[b] Isolated yield.

Table 3
Assessment of the synthesis of compounds **4a**–**j** with conventional and PEG-SO_3H catalyzed methods.

Entry	R	Reaction time in h (yield in %)		Melting point (°C)
		Conventional method	PEG-SO_3H Catalyzed method	
4a		14.0 (**75.0**)	2.0 (**96.0**)	155–157
4b		14.5 (**80.0**)	2.5 (**93.0**)	144–146
4c		15.5 (**85.0**)	3.5 (**94.0**)	163–165
4d		14.0 (**79.0**)	2.5 (**91.0**)	175–177
4e		15.5 (**82.0**)	3.0 (**95.0**)	153–155
4f		15.5 (**70.0**)	3.5 (**91.0**)	148–150
4g		17.0 (**76.0**)	4.0 (**87.0**)	150–152
4h		17.5 (**72.0**)	4.5 (**89.0**)	159–161
4i		18.0 (**70.0**)	5.0 (**82.0**)	156–158
4j		20.0 (**71.0**)	6.0 (**85.0**)	170–172

The Percentage inhibition of growth in Human chronic myeloid leukemia cells (**K 562**), Human colon carcinoma cells (**Colo 205**) and Human embryonic kidney cells (**HEK 293**) following treatment with by the compounds **4a**–**j** were measured at 1 μM and 10 μM concentrations which were the mean values determined from two

Table 4
In vitro cytotoxic activity of title compounds (**4a**–**j**) against **K 562** and Colo 205 cell lines.[a]

Entry	IC_{50} (μM)[b]	
	K 562	**Colo 205**
4a	>100.0	18.0
4b	22.0	10.0
4c	10.0	5.0
4d	19.0	12.0
4e	20.0	14.0
4f	32.0	10.0
4g	10.0	8.0
4h	24.0	10.0
4i	35.0	15.0
4j	31.0	10.0

[a] IC_{50} values were determined from growth inhibition curves (1–100 μM).
[b] The reported values are the average of two determinations that in all cases differed by 8–10 % or less.

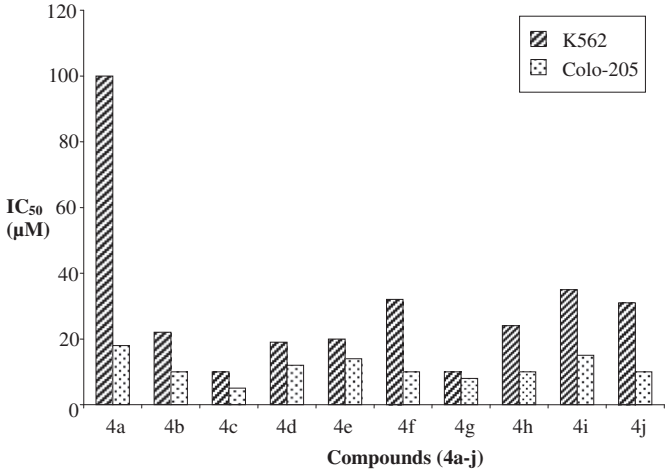


Fig. 3. IC_{50} values of the title compounds (**4a**–**j**) against **K 562** and **Colo 205**.

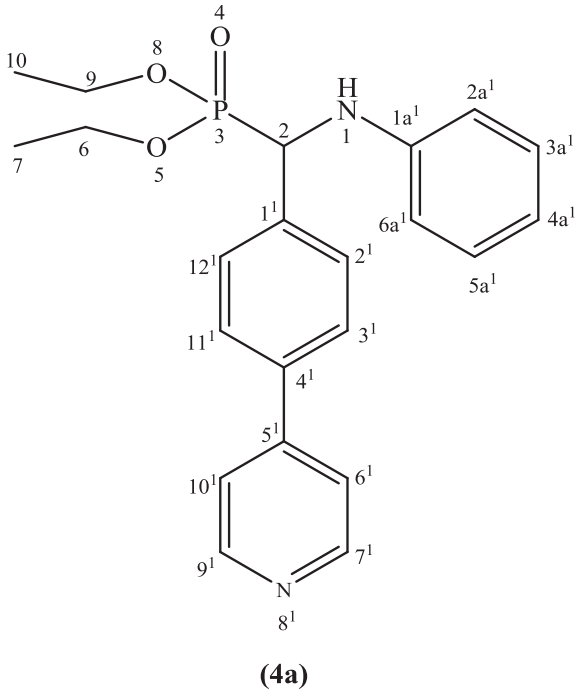
independent experiments with duplicates in each other and the reported values are the average of two determinations that in all cases differed by 7–10% or less.

It is worthwhile to note that all these compounds have no significant cytotoxicity on non-cancerous **HEK 293** cells. This study thus discovered a new family of diethyl (substituted amino) (4-(pyridine-4-yl)phenyl)methyl phosphonates (**4a**–**j**) that have significant target specific cytotoxicity on some cancer cells (Table 5).

Table 5
Percent inhibition of growth of in three cancer cell lines caused by **4a**–**j** 1 μM and 10 μM concentration.

Entry	% of inhibition[b]					
	K 562		**Colo 205**		**HEK293**[a]	
	1.0 μM	10.0 μM	1.0 μM	10.0 μM	1.0 μM	10.0 μM
4a	0.0	0.0	18.1	31.0	0.0	0.0
4b	26.0	26.2	44.5	47.0	0.2	1.2
4c	41.7	53.8	31.3	71.6	1.0	2.3
4d	14.3	24.9	35.0	45.1	1.2	3.4
4e	0.0	25.8	39.5	41.1	0.0	1.2
4f	0.0	15.7	14.0	53.4	0.5	1.9
4g	26.0	48.4	27.6	60.1	3.1	3.6
4h	0.0	24.0	37.1	47.9	1.0	2.5
4i	0.0	15.7	36.6	41.0	2.5	2.5
4j	0.9	18.9	34.4	48.1	0.0	0.3

[a] **HEK 293** is taken as non-cancerous cell line.
[b] The reported values are the average of two determinations that in all cases differed by 7–10% or less.



(4a)

Fig. 4. Diethyl (phenyl amino)(4-(pyridine-4-yl)phenyl)methyl phosphonate.

3. Conclusion

In conclusion, this work describes an efficient one-pot synthesis of α-aminophosphonates in excellent yields by the reaction of aldehyde, amine and tri ethyl phosphite in the presence of PEG-SO_3H as catalyst. The advantages of this method are mild reaction conditions, use of eco-friendly reusable catalyst, easy workup and high product yields. Being an effective and green procedure, this may be the method of choice for commercial production of α-aminophosphonates. Further two of these compounds, **4c** and **4g** exhibited good cytotoxicity on K562 and **Colo 205** cancerous cells.

The structure activity relationship studies analyzed for the title compounds were strengthened the results obtained for the compounds **4c**, **4g** as promising cytotoxic agents. The relation for this had established by the designing itself by the presence of 4-(Pyridin-4-yl)phenyl group as a fixed bioactive moiety in all the title compounds. In addition to this incorporation of active 3-bromophenyl and pyridine-3-methylene electron withdrawing groups at nitrogen atom of the α-aminophosphonate which is linked to phosphorus atom through the bioactive α-carbon. These two compounds **4c** & **4g** were also supported by the significant properties like $\log^P$ (5.72 & 4.55), polaraizability and hydrophobic nature (41.80 & 42.16), total energy (92.94–133.76 & 96.25–138.15) and also sterically feasible ones with molecular weights (475.31 & 411.43) which satisfy the requirements of a potential drug and possible to have precise interaction with the biological systems also. Finally it is concluded that the results provided a foundation for the design and development of some more structurally diversified α-aminophosphonates as potential cytotoxic agents.

4. Experimental

4.1. General

Chemicals were procured from Sigma–Aldrich and used as such without further purification. All solvents used for the spectroscopic

and other physical studies were reagent grade and were further purified by literature methods [40]. Diethyl phosphite was purified by vacuum distillation. All solvents were freshly distilled prior to use. Melting points were determined using a calibrated thermometer by Guna Digital Melting Point apparatus. They were expressed in degrees centigrade (°C) and are uncorrected. IR spectra of samples were recorded as potassium bromide on a Bruker Vector 21 FT–IR spectrophotometer. Absorptions were reported in wave numbers (cm^{-1}). ^1H, ^{13}C and ^{31}P NMR spectra were recorded as solutions in DMSO-d_6 on a Bruker AMX 500 MHz spectrometer operating at 400 MHz for ^1H, 100 MHz for ^{13}C and 161.9 MHz for ^{31}P NMR. The ^1H and ^{13}C chemical shifts were expressed in parts per million (ppm) with reference to tetramethylsilane (TMS) as an internal standard and ^{31}P chemical shifts to 85% H_3PO_4 as an external standard. LCMS mass spectra were recorded on a Jeol SX 102 DA/600 Mass spectrometer. Elemental analysis was performed on Thermo Finnigan Insturment.

4.2. Synthesis

4.2.1. Synthesis of Diethyl (phenyl amino)(4-(pyridine-4-yl)phenyl) methyl phosphonate (**4a**)

In a 50 mL round bottom flask a mixture of 4-(Pyridin-4-yl) benzaldehyde (**1**) (0.0025 mol, 0.46 g), aniline (**2**) (0.0025 mol, 0.23 mL) and triethyl phosphite (**3**) (0.0030 mol, 0.52 mL) were taken in toluene (10 mL) as solvent and stirred for 15 min in the presence of PEG-SO_3H (0.1 mol) for a duration of 2 h at 40–50 °C. The reaction progress was monitored with Thin Layer Chromatography by using ethyl acetate, hexane mixture (1:9) as mobile phase. After completion of the reaction the reaction mixture was allowed to settle for 15 min the catalyst was separated by filtration washed with toluene and dried, for its reuse. The solvent from filtrate was removed in rotaevaporator. The crude compound obtained was purified by column chromatography using neutral silica gel as an absorbent, ethyl acetate, hexane mixture (3:7) as an eluant. Finally the collected product is recrystallized from hot ethanol to get the pure diethyl(phenylamino) (4-(pyridine-4-yl)phenyl)methyl phosphonate **4a** (Yield 96%; mp: 155–157 °C) (Fig. 4). The same procedure is followed for the synthesis of the remaining title compounds **4b–j**.

4.2.2. Preparation of PEG-SO_3H

Chlorosulfonic acid (10 mmol) was added to a solution of PEG-6000 (1 mmol) in CH_2Cl_2 (10 ml) at °C and the resulting solution was stirred at room temperature overnight. Then, the solution was concentrated under reduced presence and ether (25 ml) was added to it. The resulting precipitate was filtered and washed with ether (10 ml) three times to afford PEG-SO_3H as a gummy solid [41,42]. After its first use, it was again successfully reused for three times.

4.3. Spectral data of the synthesized compounds (**4a–j**)

4.3.1. Diethyl (phenyl amino)(4-(pyridine-4-yl)phenyl) methylphosphonate (**4a**)

Yield: 96%. mp: 155–157 °C. IR (KBr) cm^{-1}: 3330 (N–H), 1235 (P=O). ^1H NMR (400 MHz, DMSO-d_6) δ: 8.62 (d, 2H, J = 8.0 Hz, Ar–H), 7.78–7.35 (m, 6H, Ar–H), 7.25 (s, 1H, Ar–H), 6.95–6.75 (m, 3H, Ar–H), 6.45 (d, 1H, J = 8.3 Hz, Ar–H), 5.22 (t, 1H, J = 8.3 Hz, NH), 4.69 (dd, 1H, J = 24.9, 7.55 Hz, CHP), 4.17–4.09 (m, 2H, OCH_2), 4.01–3.92 (m, 1H, OCH_2), 3.78–3.70 (m, 1H, OCH_2), 1.37 (t, 3H, J = 6.7 Hz, CH_3), 1.11 (t, 3H, J = 6.79 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 149.9 (C-7′ & C-9′), 147.4 (C-1a'), 147.4 (C-5′), 137.6 (C-1′), 136.6 (C-4′), 130.4 (C-3a' & C-5a'), 128.4 (C-2′ & C-12′), 127.2 (C-3′ & C-11′), 123.0 (C-6′ & C-10′), 121.3 (C-4a'), 116.5 (C-6a'), 112.2 (C-2a'), 68.5 (d, J = 150.5 Hz, C-2), 63.3 (d, J = 6.1 Hz, –O–$\underline{C}H_2$–CH_3), 16.2 (d, J = 5.8 Hz, –O–CH_2–$\underline{C}H_3$). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 24.73. LCMS, (m/z): 397 $[M+1]^+$. Anal. Calcd for $C_{22}H_{25}N_2O_3P$ (%): C, 66.66; H, 6.36; N, 7.07. Found (%): 66.42; H, 6.21; N, 6.96.

4.3.2. Diethyl (3-chloro-4-fluorophenylamino)(4-(pyridine-4-yl) phenyl)methylphosphonate (**4b**)

Yield : 93%. mp: 144–146 °C. IR (KBr) cm^{-1}: 3350 (N–H), 1255 (P=O). ^1H NMR (400 MHz, DMSO-d_6) δ: 8.58 (d, 2H, J = 8.1, Ar–H), 7.92–7.45 (m, 8H, Ar–H), 6.91–6.80 (m, 1H, Ar–H), 5.67 (br s, 1H, NH), 4.90 (dd, 1H, J = 22.8, 8.5 Hz, CHP), 4.17–4.07 (m, 2H, OCH_2), 3.99–3.87 (m, 2H, OCH_2), 1.29 (t, 3H, J = 6.9 Hz, CH_3), 1.16 (t, 3H, J = 6.9 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 149.9 (C-7′ & C-9′), 147.7 (C-3a'), 147.4 (C-1a'), 147.3 (C-5′), 137.6 (C-1′), 136.6 (C-4′), 130.4 (C-5a'), 128.4 (C-2′ & C-12′), 127.2 (C-3′ & C-11′), 123.0 (C-6′ & C-10′), 121.3 (C-4a'), 116.5 (C-6a'), 112.2 (C-2a'), 63.3 (d, J = 7.2 Hz, –O–$\underline{C}H_2$–CH_3), 56.3 (d, J = 150.5 Hz, C-2), 16.1 (d, J = 5.9 Hz, –O–CH_2–$\underline{C}H_3$). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 24.56. LCMS, (m/z): 450 $[M+2]^+$, 449 $[M+1]^+$. Anal. Calcd for $C_{22}H_{23}ClFN_2O_3P$ (%): C, 58.87; H, 5.16; N, 6.24. Found (%): C, 58.68; H, 5.02; N, 6.17.

4.3.3. Diethyl (3-bromophenylamino)(4-(pyridine-4-yl)phenyl) methylphosphonate (**4c**)

Yield : 94%. mp: 163–165 °C. IR (KBr) cm^{-1}: 3202 (N–H), 1247 (P=O). ^1H NMR (400 MHz, DMSO-d_6) δ: 8.66 (d, 2H, J = 8.1 Hz, Ar–H), 7.84–7.50 (m, 6H, Ar–H), 6.94 (d, 1H, J = 8.0 Hz, Ar–H), 6.82 (d, 1H, J = 7.9 Hz, Ar–H), 6.78–6.49 (m, 2H, Ar–H), 5.02 (t, 1H, J = 8.2 Hz, NH), 4.79 (dd, 1H, J = 24.5, 9.1 Hz, CHP), 4.14–4.03 (m, 2H, OCH_2), 3.99–3.85 (m, 1H, OCH_2), 3.76–3.66 (m, 1H, OCH_2), 1.32 (t, 3H, J = 7.05 Hz, CH_3), 1.16 (t, 3H, J = 7.06 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 149.9 (C-7′ & C-9′), 147.7 (C-3a′), 147.4 (C-1a′), 147.3 (C-5′), 137.6 (C-1′), 136.6 (C-4′), 130.4 (C-5a'), 128.4 (C-2′ & C-12′), 127.2 (C-3′ & C-11′), 123.0 (C-6′ & C-10′), 121.3 (C-4a'), 116.5 (C-3a'), 112.2 (C-2a'), 63.3 (d, J = 6.1 Hz, –O–$\underline{C}H_2$–CH_3), 56.2 (d, J = 150.5 Hz, C-2), 16.2 (d, J = 5.9 Hz, –O–CH_2–$\underline{C}H_3$). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 23.68. LCMS, (m/z): 476 $[M+2]^+$, 475 $[M+1]^+$. Anal. Calcd for $C_{22}H_{24}BrN_2O_3P$ (%): C, 55.59; H, 5.09; N, 5.89. Found (%): C, 54.97; H, 5.03; N, 5.82.

4.3.4. Diethyl (4-chloro-3-nitrophenylamino)(4-(pyridine-4-yl) phenyl)methylphosphonate (**4d**)

Yield : 91%. mp: 175–177 °C. IR (KBr): 3339 (N-H), 1238 (P=O). ^1H NMR (400 MHz, DMSO-d_6) δ: 8.63 (d, 2H, J = 8.0 Hz, Ar–H), 7.80–6.33 (m, 9H, Ar-H), 5.04 (br s, 1H, NH), 4.60 (dd, 1H, J = 22.8, 7.8 Hz, CHP), 4.12–4.10 (m, 2H, O–CH_2), 4.03–3.99 (m, 1H, OCH_2), 3.60 (m, 1H, OCH_2), 1.41 (t, 3H, J = 7.03 Hz, CH_3), 1.18 (t, 3H, J = 7.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 149.9 (C-7′ & C-9′), 147.7 (C-3a'), 147.4 (C-1a'), 147.3 (C-5′), 137.6 (C-1′), 136.6 (C-4′), 130.4 (C-5a'), 128.4 (C-2′ & C-12′), 127.2 (C-3′ & C-11′), 123.0 (C-6′ & C-10′), 121.3 (C-4a'), 116.5 (C-6a'), 112.2 (C-2a'), 63.3 (d, J = 6.9 Hz, –O–$\underline{C}H_2$–CH_3), 55.8 (d, J = 152.2 Hz, C-2), 16.2 (d, J = 6.8 Hz, –O–CH_2–$\underline{C}H_3$). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 26.45. LCMS, (m/z): 477 $[M+2]^+$, 476 $[M+1]^+$. Anal. Calcd for $C_{22}H_{23}ClN_3O_5P$ (%): C, 55.53; H, 4.87; N, 8.83. Found (%): 55.35; H, 4.65; N, 8.68.

4.3.5. Diethyl (3-nitrophenylamino)(4-(pyridine-4-yl)phenyl) methylphosphonate (**4e**)

Yield : 95%. mp: 153–155 °C. IR (KBr) cm^{-1}: 3340 (N–H), 1237 (P=O). ^1H NMR (400 MHz, DMSO-d_6) δ: 8.65 (d, 2H, J = 5.7 Hz, Ar–H), 7.64–7.55 (m, 4H, Ar–H), 7.50 (d, 2H, J = 5.9 Hz, Ar–H), 6.90–6.43 (m, 4H, Ar–H), 5.10 (br s, 1H, NH), 4.70 (dd, 1H, J = 21.8, 9.6 Hz, CHP), 4.18–4.13 (m, 2H, OCH_2), 4.01–3.98 (m, 1H, OCH_2),

3.80 (m, 1H, OCH_2), 1.31 (t, 3H, J = 7.03 Hz, CH_3), 1.16 (3H, t, J = 7.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 149.9 (C-7′ & C-9′), 147.7 (C-6a'), 147.4 (C-1a'), 147.3 (C-5′), 137.6 (C-1′), 136.6 (C-4′), 130.4 (C-5a'), 128.4 (C-2′ & C-12′), 127.2 (C-3′ & C-11′), 123.0 (C-6′ & C-10′), 121.3 (C-4a'), 116.5 (C-3a'), 112.2 (C-2a'), 63.3 (d, J = 6.3 Hz, −O−CH_2−CH_3), 56.7 (d, J = 151.5 Hz C-2), 16.2 (d, J = 6.9 Hz, −O−CH_2−CH_3). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 25.30. LCMS, (m/z): 442 $[M+1]^+$. Anal. Calcd for $C_{22}H_{24}N_3O_5P$ (%): C, 59.86; H, 5.48; N, 9.52. Found (%): C, 59.66; H, 5.23; N, 9.39.

4.3.6. Diethyl (pyridine-4-ylamino)(4-(pyridine-4-yl)phenyl) methylphosphonate(**4f**)

Yield : 91%. mp: 148–150 °C. IR (KBr) cm^{-1}: 3340 (N–H), 1245 (P═O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.68 (d, 2H, J = 8.1 Hz, Ar–H), 8.51 (d, 2H, J = 8.2 Hz, Ar–H), 7.68–7.12 (m, 8H, Ar–H), 5.78 (t, 1H, J = 8.1 Hz, NH), 4.94 (dd, 1H, J = 23.9, 8.5 Hz, CHP), 4.14–3.65 (m, 4H, OCH_2), 1.68 (t, 3H, J = 7.1 Hz, CH_3), 1.29 (t, 3H, J = 7.1 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 149.9 (C-7′ & C-9′), 148.4 (C-6a' & 5a'), 147.4 (C-1a'), 147.4 (C-5′), 137.6 (C-1′), 136.6 (C-4′), 128.4 (C-2′ & C-12′), 127.2 (C-3′ & C-11′), 123.0 (C-6′ & C-10′), 121.3 (C-4a'), 112.2 (C-2a'), 63.3 (d, J = 5.8 Hz, −O−CH_2−CH_3), 56.8 (d, J = 151.6 Hz, C-2), 16.2 (d, J = 6.4 Hz, −O−CH_2−CH_3). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 23.50. LCMS, (m/z): $[M+1]^+$ 398. Anal. Calcd for $C_{21}H_{24}N_3O_3P$ (%): C, 63.57; H, 6.09; N, 10.57. Found (%): C, 62.87; H, 6.02; N, 10.45.

4.3.7. Diethyl(pyridine-4-ylmethylamino)(4-(pyridine-4-yl)phenyl) methylphosphonate (**4g**)

Yield : 87%. mp: 150–152 °C. IR (KBr) cm^{-1}: 3330 (N–H), 1233 (P═O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.66 (d, 2H, J = 8.0 Hz, Ar–H), 7.67–7.43 (m, 6H, Ar–H), 7.26 (d, 2H, J = 7.1 Hz, Ar–H), 6.91 (d, 2H, J = 6.96 Hz, Ar–H), 5.34 (t, 1H, J = 8.0 Hz, NH), 4.85 (dd, 1H, J = 23.1, 8.6 Hz, CHP), 4.72–4.68 (m, 2H, OCH_2), 4.19–4.14 (m, 2H, CH_2), 4.05–3.99 (m, 1H, OCH_2), 3.80–3.75 (m, 1H, OCH_2), 1.17 (t, 3H, J = 7.04 Hz, CH_3), 1.31(t, 3H, J = 7.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 149.5 (C-7′ & C-9′), 147.4 (C-5′), 146.6 (C-3a'), 145.1 (C-5a'), 140.6 (C-4′), 134.7 (C-2a'), 135.4 (C-7a'), 132.6 (C-1′), 127.3 (C-2′ & C-12′), 127.1 (C-3′ & C-11′), 122.3 (C-6a'), 120.8 (C-6′ & C-10′), 63.2 (d, J = 5.8 Hz, −O−CH_2−CH_3), 53.2 (C-1a'), 52.8 (d, J = 151.6 Hz, C-2), 16.3 (d, J = 6.4 Hz, −O−CH_2−CH_3). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 24.89. LCMS, (m/z): 412 $[M+1]^+$. Anal. Calcd for $C_{22}H_{26}N_3O_3P$ (%): C, 64.22; H, 6.37; N, 10.21. Found (%): C, 63.51; H, 6.29; N, 10.09.

4.3.8. Diethyl(thiazol-2-ylamino)(4-(pyridine-4-yl)phenyl) methylphosphonate (**4h**)

Yield : 89%. mp: 159–161 °C. IR (KBr) cm^{-1}: 3346 (N–H), 1242 (P═O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.60 (d, 2H, J = 8.1 Hz, Ar–H), 8.26 (s, 1H, Ar–H), 7.78–7.54 (m, 5H, Ar–H), 7.41 (d, 1H, J = 6.5 Hz, Ar–H), 6.79 (d, 1H, J = 6.5, Ar–H), 5.58 (br s, 1H, NH), 4.96 (dd, 1H, J = 29.0, 13.0 Hz, CHP), 4.04–3.92 (m, 2H, OCH_2), 3.52–3.41 (m, 2H, OCH_2), 1.31 (t, 3H, J = 6.9 Hz, CH_3), 1.19 (t, 3H, J = 6.9 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.8 (C-1a'), 149.7 (C-7′ & C-9′), 147.3 (C-5′), 139.5 (C-4′), 136.8 (C-4a'), 136.4 (C-1′), 127.4 (C-2′ & C-12′), 127.1 (C-3′ & C-11′), 120.8 (C-6′ & C-10′), 112.2 (C-3a'), 68.6 (d, J = 151.6 Hz, C-2), 62.8 (d, J = 5.8 Hz, −O−CH_2−CH_3), 16.4 (d, J = 6.4 Hz, −O−CH_2−CH_3). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 25.63. LCMS, (m/z): 404 $[M+1]^+$. Anal. Calcd for $C_{19}H_{22}N_3O_3PS$ (%): C, 56.57; H, 5.50; N, 10.42. Found (%): C, 55.94; H, 5.44; N, 10.31.

4.3.9. Diethyl(benzo[d]thiazol-2-ylamino)(4-(pyridine-4-yl)phenyl) methyl phosphonate (**4i**)

Yield: 82%. mp: 156–158 °C; IR (KBr) cm^{-1}: 3337 (N–H), 1235 (P═O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.64 (d, 2H, J = 8.2 Hz, Ar–H), 8.20–7.15 (10H, m, Ar–H), 5.75 (t, 1H, J = 8.1, NH), 4.90 (dd, 1H, J = 23.8, 8.3 Hz, CHP), 4.16–3.64(m, 4H, OCH_2), 1.63 (t, 3H, J = 7.0 Hz, CH_3), 1.33 (t, 3H, J = 7.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.3 (C-1a'), 152.9 (C-8a'), 149.4 (C-7′ & C-9′), 147.1 (C-5′), 136.5 (C-1′), 139.8 (C-4′), 129.9 (C-3a'), 127.3 (C-2′ & C-12′), 127.0 (C-3′ & C-11′), 125.6 (C-6a'), 124.7 (C-5a'), 121.1 (C-4a'), 120.5 (C-6′ & C-10′), 119.6 (C-7a'), 62.5 (d, J = 5.8 Hz, −O−CH_2−CH_3), 56.7 (d, J = 151.6 Hz, C-2), 16.3 (d, J = 6.4 Hz, −O−CH_2−CH_3). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 23.58. LCMS, (m/z): 454 $[M+1]^+$. Anal. Calcd for $C_{23}H_{24}N_3O_3PS$ (%): C, 60.92; H, 5.33; N, 9.27. Found (%): C, 60.24; H, 5.27; N, 9.16.

4.3.10. Diethyl(6-nitrobenzo[d]thiazol-2-ylamino)(4-(pyridine-4-yl)phenyl)methyl phosphonate (**4j**)

Yield : 85%. mp: 170–172 °C. IR (KBr) cm^{-1}: 3341 (N–H), 1239 (P═O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.62 (d, 2H, J = 8.2 Hz, Ar–H), 8.35–7.18 (m, 9H, Ar–H), 5.78 (t, 1H, J = 8.0 Hz, NH), 4.98 (dd, 1H, J = 22.9, 8.1 Hz, CHP), 4.10–3.66 (m, 4H, OCH_2), 1.60 (t, 3H, J = 7.0 Hz, CH_3), 1.35 (t, 3H, J = 7.0 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 173.9 (C-1a′), 159.6 (C-8a′), 149.7 (C-7′ & C-9′), 147.3 (C-5′), 144.7 (C-5a′), 140.2 (C-4′), 136.7 (C-1′), 130.8 (C-3a′), 127.7 (C-2′ & C-12′), 127.4 (C-3′ & C-11′), 121.6 (C-6a′), 119.7 (C-4a′), 120.2 (C-6′ & C-10′), 117.7 (C-7a'), 62.4 (d, J = 5.8 Hz, −O−CH_2−CH_3), 55.6 (d, J = 151.6 Hz, C-2), 16.1 (d, J = 6.4 Hz, −O−CH_2−CH_3). ^{31}P NMR (161.9 MHz, DMSO-d_6) δ: 24.62. LCMS, (m/z): 499 $[M+1]^+$. Anal. Calcd for $C_{23}H_{23}N_4O_5PS$ (%): C, 55.42; H, 4.65; N, 11.24. Found (%): C, 54.81; H, 4.59; N, 11.12.

4.4. Biological assay

4.4.1. Cell lines and culture conditions

The title compounds **4a**–**j** were evaluated for cytotoxic activity against human chronic myeloid leukemia cells, **K 562**, human colon carcinoma cells, **Colo 205** and human embryonic kidney cells, **HEK 293**. They were procured from National Center for Cell Sciences, Pune, India. All cells were grown in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mm-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were subcultured twice each week, seeding at a density of about 2 × 10^3 cells/mL. Before the analysis of the compounds, cells were washed with PBS and fresh medium was added. For final analysis, exponentially growing cells were collected and resuspended in fresh culture medium with 10% FBS.

4.4.2. MTT assay

Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann [43]. All the cells (5 × 10^3 cells/well) were seeded to 96-well culture plate and cultured with or without compounds at 1 μM and 10 μM concentration for 24 h in a final volume of 200 μL. After treatment, the medium was removed and 20 μL of MTT (5 mg/mL in PBS) was added to the fresh medium. After 2 h incubation at 37 °C, 100 μL of DMSO was added to each well and plates were agitated for 1 min. The colored precipitate formed was dissolved in 100 μL of DMSO and the absorbance was read at 570 nm on a multi-well plate reader (Victor3, Perkin Elmer). The Percent inhibition of proliferation of the title compounds were calculated with respect to the control. The concentration that inhibited the cell growth by 50% (IC_{50}) was determined from cell survival plots and the detailed procedure for the determination of IC_{50} values was followed by the graphical method as explained here. The IC_{50} was preliminarily determined at a broad range of concentrations viz., 1, 10 & 100 μM and then at a narrow range of concentrations viz., 2, 4, 8, 10, 16, 32 & 64 μM of the title compounds against the cell lines. For this data, a line graph was plotted between concentrations (X-axis) versus %

inhibition (*Y*-axis) and then an intersection drawn at 50% inhibition on *Y*-axis and then correlated to the concentration value on *X*-axis. Finally that concentration value is considered as IC_{50} in μM and the same procedure is applied for the evaluation of MTT assay of all the compounds.

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