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Preliminary communication

Synthesis and anticancer activity of new class of bisphosphonates/phosphanamidates

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Abstract

Novel bis-heterocyclic bisphosphonates/phosphonamidates were synthesized utilizing the Pudovick reaction. The employment of Nb_2O_5 as catalyst was found to increase the yields and purity of the bisbenzoxazaphosphine derivatives (**13a–h**). Their anticancer activity studies *in vitro*, on three human tumor cell lines NCI-H460 (lung large cell), MCF-7 (breast adenocarcinoma), and SF-268 (central nervous system glioblastoma), showed that bis-[3-(3-chloro-4-fluorophenyl)-2-oxo-3,4-dihydro-2H-2λ^5-benzo[*e*][1,3,2]oxazaphosphinin-2-yl]arylmethanes (**13a–h**) and [(4-chlorophenyl)-(hydroxyamidophosphinoyl)-methyl]phosphonic acid (**14**) exhibited significant anticancer activity.

Keywords: Cytotoxicity; Pudovick reaction; Bisphosphonates; Bisphosphanamidates; Adenocarcinoma; Glioblastoma

1. Introduction

Simple bisphosphonates (BPs) were primarily used in the early days as antiscaling, anticorrosive and metal complexing agents [1]. However, it was only in 1960s that their potential for the treatment of various bone diseases was realized after Fleisch and co-workers discovered that bisphosphonates impair the formation and dissolution of calcium phosphate crystals *in vitro* [2–4].

Alendronate and olpadronate have good bone antiresorptive potency. Zolendronic acid with heteroaromatic substituent at methylene carbon is both efficacious and safe for the treatment of tumor-induced hypercalcemia, Paget's disease of bone, osteolytic metastases and post-menopausal osteoporosis [5]. Oxy-diphenylbutylidene and pyrozoline containing bisphosphonate esters are both anti-inflammatory and antiarthritic agents [6,7]. Several nitrogen containing hydroxyl bisphosphonates with nitrogen separated by two carbon atoms from the bisphosphonate carbon back-bone are potential antiparasitic agents [8]. BPs are gaining increased attention as anticancer drugs following reports that elodronate exhibited anti-metastatic activity decreasing tumor burden in the bone [9]. Even simple carbonyl BPs inhibit HIV-I replication *in vitro* [10]. Another use is in nuclear medicine as ligands for radio metals as bone seeking diagnostic and therapeutic agents [11]. More importantly, being orally active, BPs could be orally administered in the patients. Currently, this area of research is very active and new discoveries on the chemistry and pharmacology of bisphosphonates may lead to the development of new commercial drugs.

Considering the pharmaceutical potential of this class of compounds, in this work, we describe the preparation of several new bisphosphonates and bisphosphanamidates. The cytotoxicities of 17 new products were screened against three tumor cell lines.

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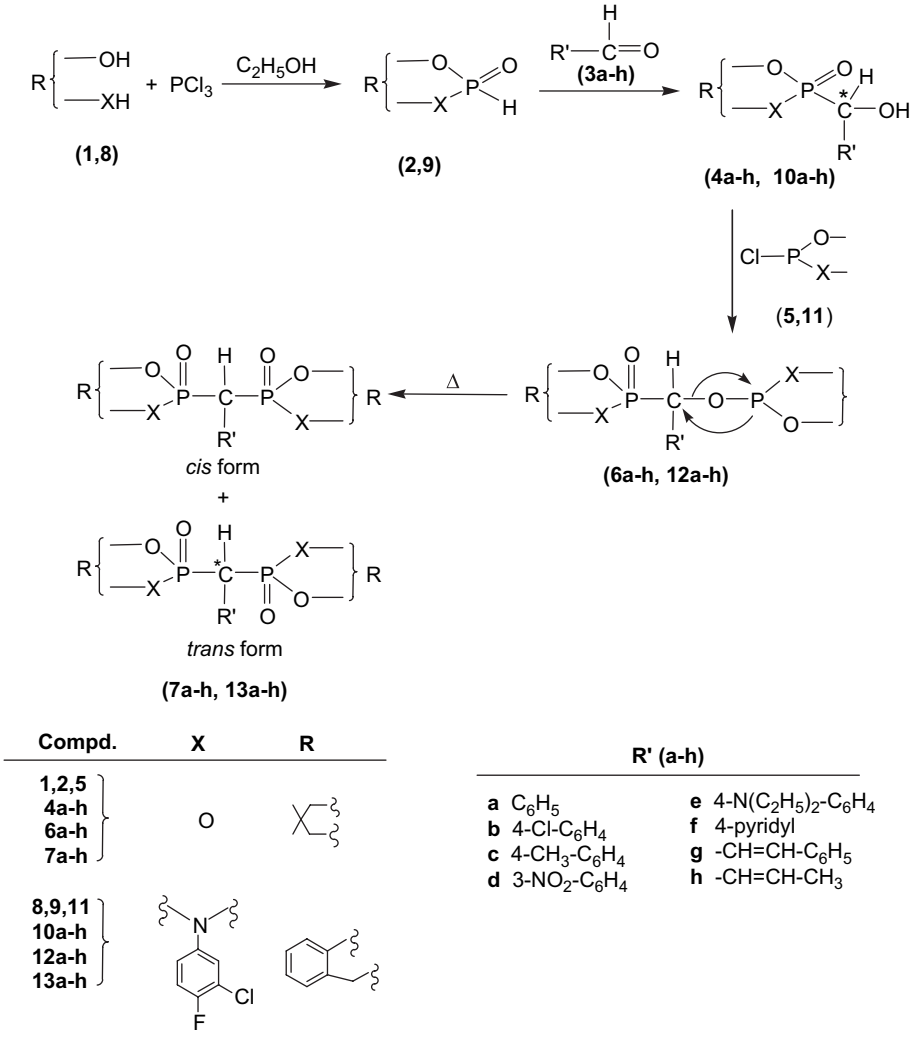
2. Results and discussion

2.1. Chemistry

Synthesis of novel bis-(5,5-dimethyl-2-oxo-2λ^5-[1,3,2]dioxaphosphinan-2-yl)-arene/alkene substituted methanes (**7a–h**) and bis-[3-(3-chloro-4-fluorophenyl)-2-oxo-3,4-dihydro-2*H*-2λ^5-benzo[*e*][1,3,2]oxaphosphonin-2-yl]arene/alkene substituted methanes (**13a–h**) (Scheme 1) was accomplished by Pudovick reaction. Addition of an equimolar amount of 2,2-dimethyl-1,3-propanediol (**1**)/2-(3-chloro-4-fluorophenylamino)methylphenol (**8**) and ethanol to PCl_3 or vice-versa afforded the cyclic hydrogen phosphite (**2**)/phosphoramidate (**9**) corresponding to the starting materials **1** and **8**, respectively. This is a better procedure for the preparation of compounds **2** and **9** when compared to the method that involves the reaction of **1** and **8** directly with PCl_3 followed by addition of water. In the latter procedure the acid formed catalyzes the reverse reaction resulting in the recovery of the starting material [12]. Aldehydes (**3a–h**) were added to **2** and **9** and corresponding substituted 3-(5,5-dimethyl-1,3-propanediol-2-oxa-2λ^5-[1,3,2]dioxaphosphinan-2-yl methanols and 3-(3-chloro-4-fluorophenyl)-2-oxo-3,4-dihydro-2*H*-2λ^5-benzo[*e*][1,3,2]oxazaphosphin-2-yl methanols (**4a–h**, **10a–h**) were formed. The addition reactions between aldehydes (**3a–h**) and cyclic hydrogen phosphoramidate (**9**) were facilitated by the presence of catalytic amount of Nb_2O_5. Besides that, the yields for compounds **10a–h** were improved. The dioxaphosphinon-2-yl methanol derivatives **4a–h** were obtained in good yields even without addition of this catalyst. Further, the reaction times required for the formation of compounds **4a–h** and **10a–h** depended on the nature of aldehydes. Reaction of **4a–h** and **10a–h** with $P_{(III)}$ monochloride of **1** and **8** (compounds **5** and **11**) yielded the respective intermediates BPs (**6a–h**) and bisphosphonamidates (**12a–h**). These compounds, in turn, underwent rearrangement under stirring and refluxing conditions to afford bis-(5,5-dimethyl-2-oxo-2λ^5-[1,3,2]dioxaphosphinan-2-yl)methane derivatives (**7a–h**) and bis-[3-chloro-4-fluorophenyl-2-oxo-3,4-dihydro-2*H*-2λ^5-benzo[*e*][1,3,2]-oxazaphosphin-2-yl]methane derivatives (**13a–h**) as *cis* and *trans* isomers. Restricted rotation around the P_α–C–P_β bonds contributes for the rigidity of the compounds. Hydrolysis of one member (**13b**) also afforded *cis* and *trans* isomers of 4-chlorophenyl-4-hydroxyamino



Compd.	X	R
1,2,5 4a-h 6a-h 7a-h	O	
8,9,11 10a-h 12a-h 13a-h		

R' (a-h)	
a C_6H_5	**e** 4-N$(C_2H_5)_2$-C_6H_4
b 4-Cl-C_6H_4	**f** 4-pyridyl
c 4-CH_3-C_6H_4	**g** -CH=CH-C_6H_5
d 3-NO_2-C_6H_4	**h** -CH=CH-CH_3

Scheme 1.

phosphinoyl methyl phosphonic acid (**14**) (Scheme 2). This points to the fact that **13b** exists as a mixture of *cis* and *trans* forms. Attempts to separate them by fractional crystallization and column chromatography were unsuccessful.

The structures of all the compounds were confirmed by elemental analysis, infrared, ^{31}P, ^{13}C and ^{1}H NMR spectroscopic data and mass spectrometry. Two distinct ^{31}P chemical shifts were observed for all the members of **7** (δ 23.81–12.65 and −4.27 to 15.52), **13** (δ 23.93–4.40 and −3.40 to 15.15) and **14** (δ −19.03, −39.36). These data confirm that each one of the products of **7a–h** and **13a–h** is a mixture of *cis* and *trans* isomers. Only one ^{31}P chemical shift is expected for the two P_α and P_β atoms of *cis* and *trans* isomers because both of them are present in the identical environment due to their bonding to similar groups.

The six carbon atoms of the two dioxaphosphinane units (C_4 to C_6 and C_4' to C_6') in both the *cis* and *trans* isomers in **7a–h** gave only 3 carbon-13 signals and the 26 carbons of the two *N*-phenyl-benzooxazaphosphine moieties in **13a–h** gave only 13 carbon-13 signals in their ^{13}C NMR spectra suggesting their symmetrical disposition around bis-phosphoryl methane (P_α–C–P_β) systems. P–C coupling is observed only up to two bond distance in **7a–h**. But similar P–C coupling is not discernible in **13a–h**.

The aromatic hydrogen atoms of **7a–h**, **13a–h** and **14** showed multiplets at δ 8.49–6.03, 8.95–6.42 and 7.68–7.34, respectively. The C4 and C4′ methylene hydrogens of **13a–h** resonated as two multiplets at δ 4.96–3.94 indicating their non-equivalence and coupling with phosphorus of the benzoxazaphosphorin-2-oxide system [13]. Compound **14** gave two low intensity deshielded signals at δ 10.12 and 9.89 for the OH and NH_2 hydrogen atoms, respectively [14,15]. Absence of signals for C-4 and 4′ methylene and aromatic hydrogens [13] of the starting compound **13b** supports formation of **14** from **13b** after hydrolysis. A significant point is the appearance of two signals for the hydrogen of the bisphosphoryl methane carbon (P_α–C–P_β), one corresponding to the hydrogen of the *cis*-form and the other to that of the *trans* isomer of **7a–h** and **13a–h**. Presence of M^+ for **7a–h** and **14** and M^+, M^{2+}, M^{4+} for **13a–h** at the respective *m/z* values with expected percent ratio for chlorine isotope peaks further confirm their proposed structures.

2.2. Anticancer activity

A comparative study on the inhibitory effect of bisphosphonamidates (**13a–h**) and non-nitrogen bisphosphonates (**7a–h**) in the growth of three human tumor cell lines NCl-H460 (lung), MCF-7 (breast) and SF-268 (central nervous system) was made. None of the oxygen bisphosphonates (**7a–h**) exhibited antiproliferative activity against the investigated tumor cell lines while bisphosphonamidate derivatives (**13a–h**) inhibited the proliferation of the three tumor cell lines investigated. From this series, compound **13a** was the most active one with an average IC_{50} value of 3.54 μM while the acyclic bishydroxyamino-4-chlorophenyl methyl phosphonic acid (**14**) was the least active compound with an IC_{50} value of 79 μM (Table 1, Fig. 1).

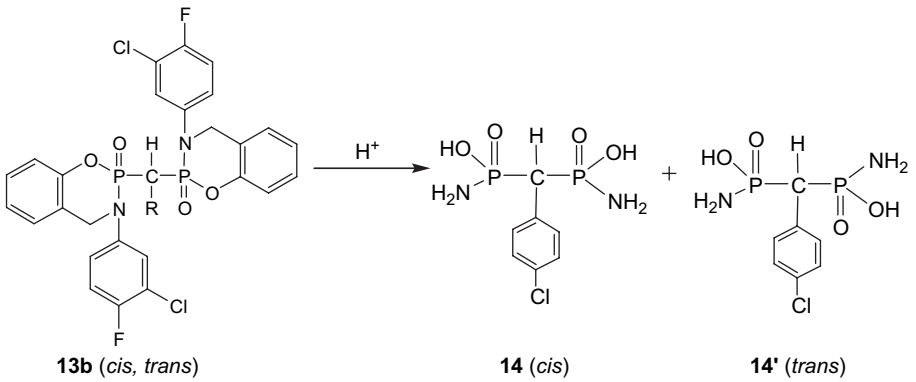
3. Conclusions

Novel aryl and alkenyl substituted BPs and biscyclophosphonamidates were synthesized by Pudovick reaction. The use of Nb_2O_5 as catalyst facilitated the formation and increased the yields of biscyclophosphonamidates (**13a–h**). Compounds **13a–h** inhibited the proliferation of three human tumor cell lines (NCI-H460, MCF-7 and SF-268) with IC_{50} values ranging from 3.54 to 13.8 μM while the acyclic *N*,*N*′-((4-chlorophenyl)methylene)diphosphonamidic acid (**14**) was less active with an IC_{50} value of 79 μM. Simple oxygen cyclobisphosphonates (**7a–h**) have no activity against these tumor cell lines.

4. Experimental section

4.1. Synthesis

All experiments were conducted under nitrogen atmosphere and anhydrous conditions. Solvents used were dried and distilled before use [16]. Progress of the reactions was monitored by thin-layer chromatography using glass plates coated



13b (*cis, trans*) **14** (*cis*) **14'** (*trans*)

Scheme 2.

Table 1
IC_{50} values for tumor cell growth inhibition of compounds **13a–h**, **14**, and **7a–h**

Compound	NCI-H460 (μM)	MCF-7 (μM)	SF-268 (μM)	Mean (μM)
13a	3.77	4.05	2.80	3.54
13e	5.69	5.71	3.85	5.08
13h	8.22	11.3	8.50	9.34
13c	9.00	12.7	7.69	9.80
13f	12.3	11.5	11.7	11.8
13b	10.9	14.3	11.2	12.1
13g	11.8	13.1	11.6	12.2
13d	13.0	17.6	10.7	13.8
14	75.2	71.3	90.4	79.0
7a–h	>1000	>1000	>1000	–

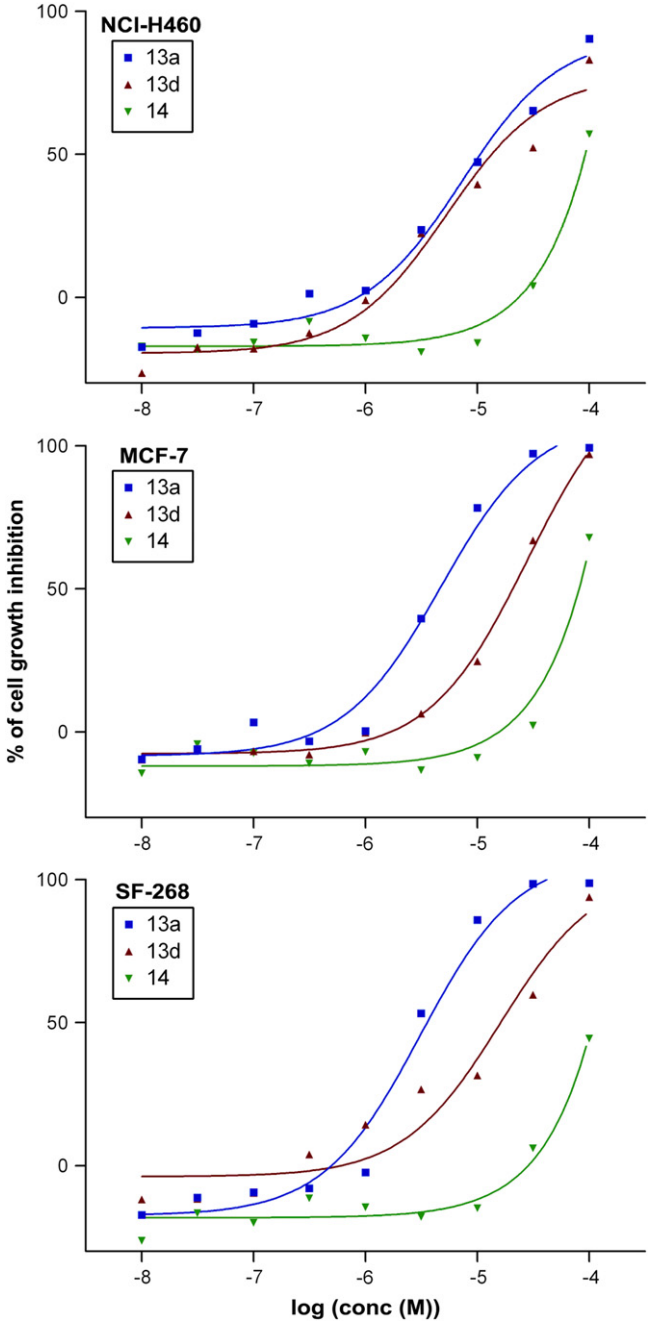


Fig. 1. Dose–response curves of compounds **13a**, **13d** and **14**.

with silica gel 60 FG254 in hexane–ethyl acetate (3:2 v/v) and iodine as visualizing agent. Solvents from the reaction mixtures were removed under reduced pressure in a rotatory evaporator. Crude residues obtained were purified by recrystallization or trituration from appropriate solvent. Melting points were determined in open capillary tubes on Melt-temp apparatus and were uncorrected. Microanalyses were performed at the Environmental Engineering Laboratory, S.V. University, Tirupati-517 502, India. IR spectra were recorded on a Jasco FT/IR-5300 spectrometer employing KBr pellet. ^{31}P, ^{13}C and ^{1}H NMR spectra were recorded on Varian Mercury 300 (300, 75.46 and 121.5 MHz, respectively) in acetone-d_6 using TMS as internal standard for ^{1}H and ^{13}C and 85% H_3PO_4 for ^{31}P chemical shifts. The coupling constants were given in hertz. Mass spectra were recorded on Jeol 5 × 102 DA/600 mass spectrometer using argon/xenon (6 kV, 10 mA) as the fast atom bombardment (FAB) gas.

2,2-Dimethyl-1,3-propanediol (**1**) was procured from Aldrich (Milwaukee, WI, USA). 2-(3-Chloro-4-fluorophenylamino)methylphenol (**8**) was prepared according to the procedure described in the literature [13]. Cyclic hydrogen phosphate (**2**) was prepared according to the reported procedure [17,18].

*4.1.1. Typical procedure for the synthesis of **7a–h***

Aldehyde (**3a–h**, 0.01 mol) and triethylamine (0.005 mol), dissolved in benzene (20 mL) were added to the stirred solution of **2** in benzene (20 mL) at 5–8 °C. After stirring the reaction mixture for 4 h, the solid formed was removed by filtration to yield 3-(5,5-dimethyl-1,3-propanediol-2-oxo-2λ^5-[1,3,2]dioxaphosphinane-2-yl) substituted aryl/alkenyl-methane derivatives (**4a–h**). To the cold and stirred solution of **4a–h** and triethylamine (0.01 mol) taken in a new reaction flask, 2-chloro-5,5-dimethyl-[1,3,2]dioxaphosphinane (**5**, 0.01 mol) in benzene (10 mL) was added dropwise. Temperature was slowly raised to 50 °C and stirring was continued for 6 h at this temperature. Finally, the reaction mixture was refluxed for another 4 h. After this time, triethylamine hydrochloride formed was separated by filtration. The filtrate was concentrated under reduced pressure to yield a residue that was recrystallised from ethanol to afford the required product (**7a–h**).

*4.1.1.1. Bis-(5,5-dimethyl-2-oxo-2λ^5-[1,3,2]-dioxaphosphinan-2-yl)phenylmethane (**7a**).* Yield: 80%; mp 102–103 °C; IR (KBr) ν_{max} 1271 (P=O), 793 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 15.42, −8.44; ^{1}H NMR (acetone-d_6) δ 7.67–7.24 (m, 5H, ArH), 6.01, 5.92 (d, J_{PH} = 12.9, 18.7 Hz, 1H, H–*C–), 4.07–3.74 (m, 8H, 4 × $-CH_2-$), 1.20, 0.91 (2s, 6H, 2 × CH_3) and 1.19, 0.90 (2s, 6H, 2 × CH_3); ^{13}C NMR (acetone-d_6) δ 78.41 and 78.17 (d, $^{2}J_{CP(4)}$ = 6.7 and 6.8 Hz, C-4 and C-4′), 46.15 (C-5 and C-5′), 77.93 and 76.67 (d, $^{2}J_{CP(6)}$ = 7.1 and 6.5 Hz, C-6), 22.03, 21.35, 21.23 and 21.16 (4 × CH_3), 33.36, 31.92 (*C), 138.53 (C-1″), 129.25 (C-2″ and C-6″), 128.64 (C-3″ and C-5″), 127.88 (C-4″); FABMS *m/z* (%): 389 (16) [M$^+$], 369 (24), 331 (21), 283 (40), 154 (11), 149 (27), 102 (100).

Anal. Calcd for ($C_{17}H_{26}O_6P_2$) (%): C, 52.55; H, 6.75. Found: C, 52.61; H, 6.78.

*4.1.1.2. Bis-(5,5-dimethyl-2-oxo-2λ⁵-[1,3,2]-dioxaphosphinan-2-yl)-4-chlorophenylmethane (**7b**).* Yield: 78%; mp 215–217 °C; IR (KBr) ν_{max} 1227 (P═O), 763 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 14.66, −5.26; ^1H NMR (acetone-d_6) δ 8.05–7.53 (m, 4H, ArH), 4.35, 4.10 [(d, J_{PH} = 10.5, 14.7 Hz), 1H, *H*–*C–], 3.47–3.28 (m, 8H, 4 × –CH_2–), 1.43, 0.98 (2s, 6H, 2 × CH_3) and 1.42, 0.96 (2s, 6H, 2 × CH_3); ^{13}C NMR (acetone-d_6) δ 78.41 and 78.17 (d, $^2J_{CP(4)}$ = 6.7 and 6.8 Hz, C-4 and C-4′), 46.15 (C-5 and C-5′), 77.93 and 76.67 (d, $^2J_{CP(6)}$ = 7.1 and 6.5 Hz, C-6 and C-6′), 21.24, 21.17, 20.79 and 20.28 (4 × CH_3), 32.41, 31.92 (*C), 129.65 (C-1″), 128.83 (C-2″ and C-6″), 131.52 (C-3″ and C-5″), 138.83 (C-4″); FABMS *m/z* (%): 422 (13) [M + 2], 420 (34) [$M^{+\bullet}$], 392 (32), 350 (59), 322 (35), 273 (54), 232 (40), 167 (27), 149 (29), 102 (100). Anal. Calcd for ($C_{17}H_{25}ClO_6P_2$) (%): C, 48.27; H, 5.96. Found: C, 48.35; H, 6.01.

*4.1.1.3. Bis-(5,5-dimethyl-2-oxo-2λ⁵-[1,3,2]-dioxaphosphinan-2-yl)-4-methylphenylmethane (**7c**).* Yield: 85%; mp 152–154 °C; IR (KBr) ν_{max} 1237 (P═O), 765 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 20.14, 15.52; ^1H NMR (acetone-d_6) δ 7.51–7.12 (m, 4H, ArH), 4.71, 4.60 [(d, J_{PH} = 9.7, 11.4) 1H, *H*–*C], 4.43–4.37 (m, 4H, 2 × –CH_2–) and 4.18–3.98 (m, 4H, 2 × –CH_2–), 2.94 (m, 3H, Ar-CH_3), 1.35, 0.99 (2s, 6H, 2 × CH_3) and 1.3, 0.98 (2s, 6H, 2 × CH_3); ^{13}C NMR (acetone-d_6) δ 79.32 and 78.29 ($^2J_{CP(4)}$ = 8.9 and 7.7 Hz, C-4 and C-4′), 45.75 (C-5 and C-5′), 75.89 and 76.24 ($^2J_{CP(6)}$ = 8.1 and 7.6 Hz, C-6 and C-6′), 21.07, 20.23, 20.17 and 20.07 (4 × CH_3), 34.36, 33.17 (*C), 143.27 (C-1″), 129.40 (C-2″ and C-6″), 131.15 (C-3″ and C-5″), 139.15 (C-4″), 21.8 (Ar-*C*H_3). Anal. Calcd for ($C_{18}H_{28}O_6P_2$) (%): C, 53.70; H, 7.02. Found: C, 53.75; H, 7.07.

*4.1.1.4. Bis-(5,5-dimethyl-2-oxo-2λ⁵-[1,3,2]-dioxaphosphinan-2-yl)-3-nitrophenylmethane (**7d**).* Yield: 77%; mp 178–179 °C; IR (KBr) ν_{max} 1251 (P═O), 753 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 18.50, −5.55; ^1H NMR (acetone-d_6) δ 8.35–7.47 (m, 4H, ArH), 5.25, 4.94 [(d, J_{PH} = 18.7, 11.8 Hz), 1H, *H*–*C–], 4.08–3.07 (m, 8H, 4 × –CH_2–), 1.29, 0.98 (2 s, 6H, 2 × CH_3) and 1.17, 0.86 (2 s, 6H, 2 × CH_3); ^{13}C NMR (acetone-d_6) δ 80.02 and 79.29 (d, $^2J_{CP(4)}$ = 9.9 and 8.5 Hz, C-4 and C-4′); 46.67 (C-5 and C-5′), 76.89 and 75.94 ($^2J_{CP(6)}$ = 7.8 and 9.3 Hz, C-6 and C-6′), 22.17, 21.12, 21.04 and 21.01 (4 × CH_3), 35.36, 33.29 (*C), 148.45 (C-1″), 123.02 (C-2″); 118.45 (C-3″), 123.31 (C-4″), 129.38 (C-5″), 139.95 (C-6″); FABMS *m/z* (%): 437 (16) [$M^+ + 4H^+$], 433 (9) [M^+], 391 (18), 302 (89), 289 (18), 284 (8), 154 (100), 136 (59). Anal. Calcd for ($C_{16}H_{25}NO_6P_2$) (%): C, 47.09; H, 5.81. Found: C, 47.14; H, 5.92.

*4.1.1.5. Bis-(5,5-dimethyl-2-oxo-2λ⁵-[1,3,2]-dioxaphosphinan-2-yl)-4-N,N-dimethyl phenylmethane (**7e**).* Yield: 77%; mp 178–179 °C; IR (KBr) ν_{max} 1261 (P═O), 753 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 23.81, −5.68; ^1H NMR (acetone-d_6) δ 8.05–7.53 (m, 4H, ArH), 4.35, 4.10 [(d, J = 10.5, 14.7 Hz), 1H, *H*–*C–], 3.47–3.28 (m, 8H, 4 × –CH_2–), 2.59–1.53 (m, 10H, N–(C_2H_5)$_2$), 1.43, 0.98 (2s, 6H, 2 × CH_3) and 1.42, 0.96 (2s, 6H, 2 × CH_3); ^{13}C NMR (acetone-d_6) δ 79.24 and 78.17 ($^2J_{CP(4)}$ = 5.7 and 6.9 Hz, C-4 and C-4′), 46.15 (C-5 and C-5′), 78.93 and 77.68 ($^2J_{CP(6)}$ = 8.1 and 7.5 Hz, C-6 and C-6′), 23.63, 22.45, 22.33 and 22.20 (4 × CH_3), 34.46, 34.19 (*C), 136.31 (C-1″), 128.17 (C-2″ and C-6″), 115.73 (C-‴ and C-5″), 153.97 (C-4″), 46.22 (N–(*C*H_2–)$_2$), 13.81 (2 × -*C*H_3). Anal. Calcd for ($C_{21}H_{35}NO_6P_2$) (%): C, 54.90; H, 7.68. Found: C, 54.97; H, 7.69.

*4.1.1.6. Bis-(5,5-dimethyl-2-oxo-2λ⁵-[1,3,2]-dioxaphosphinan-2-yl)-pyridylmethane (**7f**).* Yield: 72%; mp 165–166 °C; IR (KBr) ν_{max} 1243 (P═O), 712 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 15.62, −8.14; ^1H NMR (acetone-d_6) δ 8.49–7.16 (m, 4H, ArH), 4.61, 4.23 [(d, J_{PH} = 16.8, 17.5 Hz), 1H, H–*C–], 3.69–3.08 (m, 8H, 4 × –CH_2–), 0.77, 0.68 (2s, 6H, 2 × CH_3) and 0.69, 0.67 (2s, 6H, 2 × CH_3); ^{13}C NMR ($CDCl_3$): δ 79.24 and 78.17 ($^2J_{CP(4)}$ = 5.7 and 6.9 Hz, C-4 and C-4′), 46.15 (C-5 and C-5′), 78.93 and 77.68 ($^2J_{CP(6)}$ = 8.1 and 7.5 Hz, C-6 and C-6′), 23.08, 22.85, 22.38 and 22.07 (4 × CH_3), 34.46, 34.19 (*C), 137.32 (C-1″), 128.47 (C-2″ and C-″), 149.53 (C-3″ and C-5″). Anal. Calcd for ($C_{17}H_{25}NO_8P_2$) (%): C, 49.36; H, 6.47. Found: C, 49.43; H, 6.51.

*4.1.1.7. Bis-(5,5-dimethyl-2-oxo-2λ⁵-[1,3,2]-dioxaphosphinan-2-yl)-styrylmethane (**7g**).* Yield: 73%; mp 167–168 °C; IR (KBr) ν_{max} 1237 (P═O), 742 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 12.65, −4.27; ^1H NMR (acetone-d_6) δ 7.82–6.03 (m, 5H, Ar–H), 4.20, 3.80 [(d, J_{PH} = 5.7, 6.1 Hz) 1H, H–*C–], 3.47–3.25 (m, 8H, 4 × –CH_2–), 1.02, 0.94 (2s, 6H, 2 × CH_3) and 1.00, 0.91 (2s, 6H, 2 × CH_3). The signals for the vinyl unit ^{a}CH═^{b}CH– are not distinguishable due to their merging with aromatic hydrogen signals; ^{13}C NMR ($CDCl_3$) δ 81.79 and 79.27 ($^2J_{CP(4)}$ = 9.7 and 8.9 Hz, C-4 and C-4′), 47.25 (C-5 and C-5′), 78.93 and 77.68 ($^2J_{CP(6)}$ = 8.1 and 7.5 Hz, C-6 and C-6′), 22.15, 21.97, 21.92 and 21.86 (4 × CH_3), 35.54 and 35.19 (J = 7.9 and 6.3 Hz, *C), 129.17 (^{a}C), 151.87 (^{b}C), 139.12 (C-1″), 127.92 (C-2″ and C-6″), 131.24 (C-3″ and C-5″), 130.31 (C-4″). Anal. Calcd for ($C_{19}H_{28}O_6P_2$) (%): C, 55.05; H, 6.81. Found: C, 55.13; H, 6.87.

*4.1.1.8. Bis-(5,5-dimethyl-2-oxo-2λ⁵-[1,3,2]-dioxaphosphinan-2-yl)-1-propenylmethane (**7h**).* Yield: 68%; mp 161–163 °C; IR (KBr) ν_{max} 1239 (P═O), 757 cm^{-1} (P–*$C_{aliphatic}$). ^{31}P NMR ($CDCl_3$) δ 22.44, 13.43; ^1H NMR (acetone-d_6) δ 6.99 (1H, –^{b}C*H*), 5.82 (1H, ^{a}C*H*–), 4.21, 3.80 [(d, J_{PH} = 5.9, 6.4 Hz) 1H, H–*C–], 3.47–3.25 (m, 8H, 4 × –CH_2–), 1.78 (3H, ^{c}CH_3–), 1.09, 0.98 (2s, 6H, 2 × CH_3) and 1.08, 0.89 (2s, 6H, 2 × CH_3); ^{13}C NMR (acetone-d_6) δ 79.24 and 78.17 ($^2J_{CP(4)}$ = 5.7 and 6.9 Hz, C-4 and C-4′), 46.15 (C-5 and C-5′), 78.93 and 77.68 ($^2J_{CP(6)}$ = 8.1 and 7.5 Hz, C-6 and

C-6′), 23.73, 22.23, 22.06 and 22.26 (4 × CH_3), 34.46, 34.19 (*C), 117.29 (aC), 137.81 (bC), 21.09 (cC). Anal. Calcd for (%) ($C_{14}H_{26}O_6P_2$): C, 47.10; H, 7.44. Found: C, 57.75; H, 7.52.

*4.1.2. General procedure for the synthesis of bis-[3-chloro-4-fluorophenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]-oxazaphosphanine-2-yl]aryl/alkenyl substituted methanes (**13a–h**)*

Phosphorus trichloride (0.2 mol) in benzene (10 mL) was added dropwise to a stirred solution containing 2-(3-chloro-4-fluorophenylamino)methylphenol (**8**) (0.2 mol) [13] and ethanol (0.2 mol) in benzene (20 mL) at 5–10 °C. The resultant reaction mixture was stirred for 30 min at room temperature and at 50–55 °C for another 3 h. It was then degassed for 30 min under reduced pressure to afford 3-(3-chloro-4-fluorophenyl)3,4-dihydrobenzo[*e*][1,3,2]oxazaphosphinine-2-oxide (**9**) [17], which was not isolated. To this crude product (**4**), in a round bottom flask, kept at 5–8 °C, was added benzene (25 mL), followed by benzaldehyde (**3a–h**) (0.01 mol), triethylamine (0.05 mol) and catalytic quantity of Nb_2O_5 in benzene (20 mL). The temperature of the reaction mixture was slowly allowed to rise to 50 °C and was further stirred for 4 h before it was filtered to yield the required product [3-(3-chloro-4-fluorophenyl)-2-oxo-3,4-dihydro-2*H*-2λ⁵-benzo[*e*][1,3,2]oxazaphosphinine-2-yl]phenyl-methanol (**10a–h**).

The filtrate containing **10a–h** was transferred into another round bottom flask and kept under N_2 atmosphere. To this flask, triethylamine (0.01 mol in 10 mL of benzene) followed by 2-chloro-3-(3-chloro-4-fluorophenyl)-3,4-dihydro-2*H*-benzo[*e*][1,3,2]oxazaphosphinine **11** (0.01 mol in 10 mL of benzene) were added. The reaction mixture was stirred at 45 °C for an additional 6 h and then refluxed for another 4 h. The triethylamine hydrochloride formed was separated by filtration and the filtrate was concentrated under reduced pressure to yield the required product as a residue. This residue was washed with water and further recrystallised from petroleum ether.

*4.1.2.1. Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-phenylmethane (**13a**).* Yield: 84%; mp 188–189 °C; IR (KBr) ν_{max} 1227 (P═O), 762 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 23.93, 15.15; ^1H NMR (acetone-d_6) δ 8.21–7.32 (m, 19H, Ar–H), 5.35, 5.20 [(d, J_{PH} = 9.5, 10.8 Hz) 1H, H–*C–], 4.96–4.24 (m, 4H, 2 × $-CH_2-$); ^{13}C NMR (acetone-d_6) δ 51.82 and 51.09 (C-4 and C-4′), 132.33 and 131.67 (C-5 and C-5′), 124.19 and 123.52 (C-6 and C-6′), 128.07 (C-7 and C-7′), 118.39 and 118.19 (C-8 and C-8′), 158.58 (C-9 and C-9′), 126.92 (C-10 and C-10′), 147.42 and 146.91 (C-1a and C1-a′), 118.88 (C-2a and C-2a′), 123.80 (C-3a and C-3a′), 155.06 (C-4a and C-4a′), 117.68 (C-5a and C-5a′), 114.07 (C-6a and C-6a′), 31.42 (*C), 138.11 (C-1″), 130.61 (C-2″), 129.45 (C-3″), 129.91 (C-4″), 129.45 (C-5″), 130.61 (C-6″). Anal. Calcd for ($C_{33}H_{24}N_2O_4P_2F_2Cl_2$) (%): C, 57.97; H, 3.54. Found: C, 57.99; H, 3.59.

*4.1.2.2. Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-4-chlorophenyl-methane (**13b**).* Yield: 83%; mp 193–194 °C; IR (KBr) ν_{max} 1225 (P═O), 759 cm^{-1} (P–$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 15.50, 1.17; ^1H NMR ($CDCl_3$) δ 7.35–6.47 (m, 18H, Ar–H), 5.50, 5.43 [(d, J_{PH} = 4.5, 4.5 Hz) 1H, H–*C–], 4.48–4.18 (m, 4H, 2 × $-CH_2-$); ^{13}C NMR ($CDCl_3$) δ 49.82 and 48.49 (C-4 and C-4′), 131.52 and 131.34 (C-5 and C-5′), 123.42 and 123.19 (C-6 and C-6′), 127.36 and 127.06 (C-7 and C-7′), 117.11 and 117.20 (C-8 and C-8′), 156.22 (C-9 and C-9′), 125.89 and 125.72 (C-10 and C-10′), 143.87 (C-1a and C-1a′), 116.67 and 116.59 (C-2a and C-2a′), 122.33 (C-3a and C-3a′), 153.98 (C-4a and C-4a′), 117.99 and 117.91 (C-5a and C-5a′), 115.07 and 115.01 (C-6a and C-6a′), 32.52 (*C), 139.14 (C-1″), 129.63 (C-2″), 130.14 (C-3″), 132.91 (C-4″), 130.01 (C-5″), 129.63 (C-6″); FABMS *m/z* (%): 722 (8) [M + 6], 720 (13%) (M + 4), 718 (45) [M + 2], 716 (40) [M+], 606 (25), 466 (56), 420 (77), 374 (67), 358 (94), 296 (20), 268 (100), 107 (90). Anal Calcd for ($C_{32}H_{23}N_2O_4P_2$ F_2Cl_3) (%): C, 55.19; H, 3.23. Found: C, 55.25; H, 3.27.

*4.1.2.3. Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-4-methylphenyl-methane (**13c**).* Yield: 87%; mp 195–197 °C; IR (KBr) ν_{max} 1224 (P═O), 758 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 12.02, −8.18; ^1H NMR (acetone-d_6) δ 8.15–7.39 (m, 18H, Ar–H), 5.51, 5.43 [(d, J_{PH} = 8.8, 6.9 Hz) 1H, H–*C–], 4.94–4.54 (m, 4H, 2 × $-CH_2-$), 2.96 (s, 3H, Ar–CH_3); ^{13}C NMR (acetone-d_6) δ 51.34 and 50.82 (C-4 and C-4′), 134.98 and 134.33 (C-5 and C-5′), 124.18 (C-6 and C-6′), 127.96 and 127.21 (C-7 and C-7′), 117.91 and 117.32 (C-8 and C-8′), 157.43 (C-9 and C-9′), 125.93 (C-10 and C-10′), 146.92 and 146.21 (C-1a and C-1a′), 116.86 and 116.22 (C-2a and C-2a′), 122.89 (C-3a and C-3a′), 156.06 (C-4a and C-4a′), 118.98 (C-5a and C-5a′), 114.73 and 114.16 (C-6a and C-6a′), 31.52 (*C–), 144.11 (C-1″), 128.61 (C-2″), 130.54 (C-3″), 140.91 (C-4″), 130.54 (C-5″), 128.69 (C-6″), 22.3 (Ar–CH_3). Anal. Calcd for ($C_{34}H_{26}N_2O_4P_2F_2Cl_2$) (%): C, 58.59; H, 3.72. Found: C, 58.53; H, 3.75.

*4.1.2.4. 4-Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-methyl-3-nitrophenylmethane (**13d**).* Yield: 81%; mp 183–185 °C; IR (KBr) ν_{max} 1237 (P═O), 742 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 16.09, −3.29; ^1H NMR (acetone-d_6) δ 8.95–7.43 (m, 18H, Ar–H), 5.39, 5.22 [(d, J_{PH} = 6.8, 8.1 Hz) 1H, H–*C–], 4.34–3.94 (m, 4H, 2 × $-CH_2-$); ^{13}C NMR (acetone-d_6) δ 52.91 and 51.68 (C-4 and C-4′), 133.83 and 133.01 (C-5 and C-5′), 122.49 and 122.04 (C-6 and C-6′), 127.96 (C-7 and C-7′), 117.90 and 117.08 (C-8 and C-8′), 157.58 and 156.92 (C-9 and C-9′), 124.94 (C-10 and C-10′), 146.98 and 146.48 (C-1a and C-1a′), 116.86 and 116.21 (C-2a and C-2a′), 121.78 (C-3a and C-3a′), 155.16 (C-4a and C-4a′), 118.91 and 118.08 (C-5a and C-5a′), 112.96 and 112.14 (C-6a and C-6a′), 32.47 (*C), 128.11 (C-1″), 122.61 (C-2″), 149.54 (C-3″), 134.84 (C-4″), 128.91 (C-5″), 140.19 (C-6″); FABMS *m/z*

(%): 731 ([M + 4], 10), 729 (26) (M + 2), 727 (32) [M + 2], 699 (45), 593 (37), 544 (35), 497 (27), 449 (20), 431 (16), 358 (78), 279 (59), 252 (92), 208 (12), 107 (100). Anal. Calcd for ($C_{33}H_{23}N_3O_6P_2F_2Cl_2$) (%): C, 54.46; H, 3.23. Found: C, 54.39; H, 3.18.

*4.1.2.5. 4-Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-N-N′-diethyl phenylmethane (**13e**).* Yield: 83%; mp 137–138 °C; IR (KBr) ν_{max} 1228 (P═O), 768 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ −4.47, −16.47; ^1H NMR (acetone-d_6) δ 8.65–6.96 (m, 18H, Ar–H), 5.47, 5.34 [(d, J_{PH} = 6.8, 8.3 Hz) 1H, H–*C–], 4.87–4.34 (m, 4H, 2 × $-CH_2-$), 2.43–1.39 (m, 10H, $N(C_2H_5)_2$); ^{13}C NMR (acetone-d_6) δ 48.23 and 47.92 (C-4 and C-4′), 134.77 and 134.48 (C-5 and C-5′), 124.92 and 124.28 (C-6 and C-6′), 127.86 (C-7 and C-7′), 116.96 (C-8 and C-8′), 158.95 and 158.58 (C-9 and C-9′), 126.12 and 125.82 (C-10 and C-10′), 146.49 and 145.99 (C-1a and C-1a′), 115.96 and 115.11 (C-2a and C-2a′), 122.98 and 122.57 (C-3a and C-3″), 154.86 and 154.23 (C-4a and C-4a′), 118.08 (C-5a and C-5a′), 112.96 and 112.32 (C-6a and C-6a′), 34.74 (*C); 137.11 (C-1″), 128.62 (C-2″), 116.59 (C-3″), 154.91 (C-4″), 116.59 (C-5″), 128.62 (C-6″), 48.19 (N–(CH_2)$_2$–), 12.9 (2 × CH_3). Anal. Calcd for ($C_{37}H_{33}N_3O_4F_2Cl_2$) (%): C, 58.90; H, 4.41. Found: C, 58.94; H, 4.49.

*4.1.2.6. 4-Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-pyridylmethane (**13f**).* Yield: 79%; mp 180–182 °C; IR (KBr) ν_{max} 1226 (P═O), 759 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 14.47, −7.71; ^1H NMR (acetone-d_6) δ 8.93–6.42 (m, 18H, Ar–H), 5.17, 4.96 [(d, J_{PH} 11.8, 9.7 Hz) 1H, *H*–C–], 4.64–4.32 (m, 4H, 2 × $-CH_2-$); ^{13}C NMR (acetone-d_6) δ 49.94 and 48.74 (C-4 and C-4′), 135.13 and 134.33 (C-5 and C-5′), 123.19 and 122.97 (C-6 and C-6′), 127.16 (C-7 and C-7′), 116.98 and 116.31 (C-8 and C-8′), 158.53 and 158.27 (C-9 and C-9′), 124.82 (C-10 and C-10′), 147.75 and 147.42 (C-1a and C-1a′), 115.27 and 115.16 (C-2a and C-2a′), 122.80 (C-3a and C-3a′), 156.16 (C-4a and C-4a′), 117.98 and 117.24 (C-5a and C-5a′), 114.96 and 114.38 (C-6a and C-6a′), 33.75 (*C); 137.14 (C-1″), 128.01 (C-2″), 151.43 (C-3″), 151.43 (C-5″), 128.01 (C-6″). Anal. Calcd for ($C_{33}H_{23}N_3O_4P_2F_2Cl_2$) (%): C, 56.13; H, 3.39. Found: C, 56.22; H, 3.42.

*4.1.2.7. Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-styrylmethane (**13g**).* Yield: 75%; mp 78–80 °C; IR (KBr) ν_{max} 1224 (P═O), 769 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 15.65, −8.16; ^1H NMR (acetone-d_6) δ 7.85–6.67 (m, 19H, Ar–H), 5.92, 5.72 [(d, J_{PH} = 11.4, 10.6 Hz) 1H, H–*C–], 4.84–4.44 (m, 4H, 2 × $-CH_2-$); The signals for the vinyl unit –^{a}CH═^{b}CH– are not distinguishable due to coincidence with the chemical shifts with aromatic hydrogens. ^{13}C NMR (acetone-d_6) δ 50.04 and 49.81 (C-4 and C-4′), 135.17 and 134.23 (C-5 and C-5′), 124.27 and 124.10 (C-6 and C-6′), 126.86 and 126.00 (C-7 and C-7′), 117.84 and 117.09 (C-8 and C-8′), 157.58 and 157.13 (C-9 and C-9′), 124.94 (C-10 and C-10′), 146.67 and 146.47 (C-1a and C-1a′), 116.94 and 116.87 (C-2a and C-2a′), 122.80 (C-3a and C-3a′), 154.86 (C-4a and C-4a′), 116.97 and 116.18 (C-5a and C-5a′), 113.26 and 112.99 (C-6a and C-6a′), 32.42 (*C), 129.52 (^{a}C), 150.75 (^{b}C), 138.17 (C-1″), 128.61 (C-2″), 132.54 (C-3″), 130.93 (C-4″), 132.54 (C-5″), 128.61 (C-6″). Anal. Calcd for ($C_{35}H_{26}N_2O_4P_2F_2Cl_2$) (%): C, 59.23; H, 3.69. Found: C, 59.27; H, 3.74.

*4.1.2.8. Bis-[3-(3-chloro-4-fluoro-phenyl)-2-oxo-3,4-dihydro-2H-2λ⁵-benzo[e][1,3,2]oxazaphosphinine-2-yl]-1-propenylmethane (**13h**).* Yield: 72%; mp 114–116 °C; IR (KBr) ν_{max} 1222 (P═O), 754 cm^{-1} (P–*$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ 9.40, −3.4; ^1H NMR (acetone-d_6) δ 7.35–7.12 (m, 14H, Ar–H), 5.37, 5.28 [(d, J_{PH} = 12.3, 10.8) 1H, H–*C–], 4.64–4.14 (m, 4H, 2 × $-CH_2-$), 1.82 (3H, ^{c}CH_3). The signals for the vinyl unit 2H, ^{a}CH═^{b}CH are not distinguishable due to coincidence with the chemical shifts with aromatic hydrogens. ^{13}C NMR (acetone-d_6) δ 46.34 and 45.82 (C-4 and C-4′), 134.11 and 133.33 (C-5 and C-5′), 124.72 and 124.19 (C-6 and C-6′), 127.32 and 127.16 (C-7 and C-7′), 116.87 and 116.21 (C-8 and C-8′), 158.05 and 157.54 (C-9 and C-9′), 124.93 (C-10 and C-10′), 146.45 and 145.96 (C-1a and C-1a′), 115.87 and 115.12 (C-2a and C-2a′), 122.08 (C-3a and C-3a′), 155.16 (C-4a and C-4a′), 117.32 and 117.28 (C-5a and C-5a′), 113.52 and 113.36 (C-6a and C-6a′), 32.93 (*C), 119.21 (C-1″), 136.61 (C-2″), 20.54 (C-3″). Anal. Calcd for ($C_{30}H_{24}N_2O_4P_3F_2Cl_2$) (%): C, 55.63; H, 3.73: Found: C, 55.71; H, 3.74.

*4.1.3. Preparation of N,N′-((4-chlorophenyl)methylene)diphosphonamidic acid (**14**)*

Compound **13b** (0.01 mol) was dissolved in benzene and cooled to 5–10 °C. To this, HCl (0.04 mol) in dichloromethane was added dropwise and the mixture was stirred for 72 h at room temperature. The solvent was removed, then 20 mL of water was added. The mixture was stirred for 24 h and evaporated to dryness in vacuum. The residue was agitated with dichloromethane (10 mL) for 6 h, the solvent was decanted and the product was suspended in diethyl ether and filtered to provide compound **14** [19,20].

Yield: 73%; mp 211 °C (dec); IR (KBr) ν_{max} 1227 (P═O), 3415 (PNH_2), 2923 ($P_{(V)}OH$), 762 cm^{-1} (P–$C_{aliphatic}$); ^{31}P NMR ($CDCl_3$) δ −19.03, −39.36; ^1H NMR ($CDCl_3$) δ 10.12 (2H, 2 × −OH), 9.89 (4H, 2 × $-NH_2$), 7.68–7.34 (m, 4H Ar–H), 2.63 (s, 1H, H–*C);^{13}C NMR ($CDCl_3$) δ 145.72 (C-1), 129.43 (C-2 and C-6), 128.79 (C-3 and C-5), 137.67 (C-4), 29.93 (*C). Anal. Calcd for ($C_7H_{11}N_2O_4P_2Cl$) (%): C, 29.52; H, 3.89. Found: C, 29.59; H, 3.95.

4.2. Bioassay

4.2.1. Cell growth inhibition assay

The *in vitro* anticancer activity of compounds **7a–h**, **13a–h** and **14** was determined on three human tumor cell

lines: NCI-H460 (lung large cell), MCF-7 (breast adenocarcinoma) and SF-268 (central nervous system glioblastoma) obtained from the National Cancer Institute, Bethesda, Maryland, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 μg/mL of gentamycin at 37 °C, 5% CO_2 atmosphere and 100% humidity. In order to determine the IC_{50} values of each compound, the cells were inoculated at a density of 5000 cells/well into 96-well flat bottom culture plates containing 10 μL of the test compound, previously half-log serial diluted (from 0.316 mM to 0.1 pM) for a final volume of 100 μL. The bisphosphonates were dissolved in DMSO (DMSO carrier had no effect on cell proliferation). After 4 days of incubation under the same conditions, the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay was performed in order to obtain dose–response curves [21] (Fig. 1). GraphPad PRISM® version 4.0 [22] was used to fit the data to a rectangular hyperbolic function:

$$I = \frac{I_{\max} C}{IC_{50} + C}$$

where I is the percent inhibition, $I_{\max} = 100\%$ inhibition, C is the concentration of the inhibitor, and IC_{50} is the concentration for 50% growth inhibition. In order to rank the compounds based on activity and due to the high correlation between the three tumor cell lines, the average of the IC_{50} values for each compound in the three human tumor cell lines was calculated.

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