Investigation of anti-proliferative and antioxidative effects of some bis (α-amino) phosphinic acid derivatives

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Abstract: Aminophosphinic acids which are organophosphorus compounds widely investigated for potential production of antibacterial, antitumor and antiviral materials. In vitro antioxidant, cytotoxic and antimicrobial activities of synthesized novel compounds of 8 different bis(α-amino alkyl)phosphinic acids (4a-h) were investigated on MCF-7 breast adenocarcinoma cell and human umbilical vein endothelial cell (HUVEC) cultures. Malondialdehyde (MDA) levels were evaluated as an indication of lipid peroxidation in cell cultures for antioxidant capacities. In vitro antioxidant activities in cell cultures were determined by evaluating totals of antioxidant, oxidant, thiol levels and activities of paraoxanase, aryl esterase. It was found that 4c compound reduced MDA level significantly while 4a and 4g compounds increased MDA levels significantly compared to control. 4c compound was found most effective in reducing MDA levels by neutralizing reactive oxygen species to prevent cell damage while compounds 4c, 4f and 4h were found presenting adequate activity with other antioxidants. In vitro anti-proliferation was evaluated on MCF-7 and HUVEC cells using XTT to investigate anti-cancer potentials as therapeutics. Compounds 4c, 4e and 4f were exhibited better compared to others. Most compounds were found cytotoxic to both MCF-7 and HUVECs. Antimicrobial and antifungal activities were investigated by disc diffusion and compared to MICs of Gentamycin and Nystatin.

Keywords: anticancer, antimicrobial, bis phosphinic acids, cytotoxicity, oxidative stress.

INTRODUCTION

It is acknowledged that amino acids are main components of various proteins which undertake important structural and physiological roles and which ensure that many systems such as transportation, defense, circulation, excretion and nervous systems working properly during lifespans of organisms. 1-aminophosphinic acid molecules are phosphorus analogues of naturally occurring amino acids, and are selective inhibitors particularly of proteolytic enzymes such as metalloproteinases (Latajka *et al.,* 2008; Cates and Li, 1985; Ye *et al.,* 2008; Sarac *et al.,* 2016). Aminophosphinic acids are widely investigated for developments of antibacterial, antitumor and antiviral materials, and they are becoming increasingly more important as their roles in biological processes are being understood (Gittens *et al.,* 2005; Sanders *et al.,* 2003; Collinsova and Jiracek, 2000). There are various proposals on Aminophosphinic acid ligands and associated complexes due to their unique structures and qualities (Kafarski *et al.,* 1995; Luckman *et al.,* 1998; Katoh *et al.,* 1996; Kabuodin and As-Habei, 2003; Kabuodin *et al.,* 2007; Kabuodin and Jafari, 2008). Aminophosphinic acids may also exist as components of natural compounds (Kukhar and Hudson, 2000). Although it is proved that they are pharmacologically active, there are fewer studies reported on α-diaminophosphinic acids/alkyl aryl diaminophosphinic acids contrary to widely investigated α-aminophosphinic acids. Even though derivatives of 1- aminophosphinic acids are widely investigated (Hyun-Joon and Gong-Shil, 1992; Gancarz and Wieczorek, 1978; Seyfert *et al.*, 1971; Worms and Schmidt-Dunker, 1976), the chemistry of α-aminophosphinic derivatives was reported in relatively fewer articles.

Scope of the study includes a pre-biological study for assessing antioxidant, antimicrobial and cytotoxic activities of newly synthesized α-aminophosphinic acid derivatives (table 1, fig. 1, Sarac *et al.,* 2016) and an updated study for synthesizing and developing new drug models in organic chemistry field developed on previous studies. Today the synthesis of polyfunctional compounds containing broader antimicrobial, antitumor and biologic activities is constituting an important field in organic

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syntheses. We believe that these synthesized substances will provide opportunity for new research subjects in different disciplines such as medicine, pharmacy, pharmacology, toxicology and so on and also may lead to new fields of study. Nevertheless, great majority of medical and pharmacological studies are on cancer research which is quite trending and unfortunately yet to be successful. Even though there is intensive research commencing, radical treatments have yet to be achieved for many diseases including various cancers (Bulbul, 2011). Studies for discovery and development of newer and effective compounds to effectively treat various diseases are carried on. We believe that synthesized bis-amino phosphinic acid derivatives in our study would be important for drug developments in further studies. Furthermore, introduction of this subject to the literature is imperative even though it is at the basic research level.

MATERIAL AND METHODS

Materials

Studied compounds were synthesized in the department of organic chemistry of Faculty of Science in Fırat University in Elazıg, TR. Purities were checked by IR, 1H-NMR and NMR spectra and elemental analyses. General structure of bis (α-amino alkyl) phosphinic acids derivatives studied and particular substituents were presented in previous article (Sarac *et al*., 2016). Cell culture experiments were approved by the Non-Invasive Clinical Research Ethics Committee of Cumhuriyet University in Sivas, TR (Approval Issue: 2015.01/10).

Cell proliferation assay

Human breast adenocarcinoma cell (MCF-7) and human umbilical vein endothelial cell (HUVECs) lines were provided from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS) and trypsin–EDTA were all supplied from Gibco (Invitrogen). L-glutamine, penicillin, streptomycin solutions were provided from Sigma–Aldrich. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium-5-carboxanilide) cell proliferation kit was purchased from Biotium. All other reagents were of ultrapure grade. Synthesized novel derivative compounds (4a-h) from bisphosphonic acids were prepared using different dilutions (10^{-3}-10^{-9} M) in ethanol solution containing NaOH (0.1M). Cytotoxic activities of synthesized compounds were measured by XTT cell proliferation kit (Biotium) on MCF-7 and HUVEC lines. Cells were cultured in a medium containing 10^{-3}M to 10^{-9}M concentrations of novel compounds. After 24 and 48 hours of incubation, cultured cells were washed with sterile PBS. XTT reagent incubated along cells for 4 hours and color change was measured in microplate reader at 450-500 nm, then the cell viability was defined (Gursoy and Cevik, 2014).

Antimicrobial activity

Microbial strains: Antimicrobial and antifungal activities of synthesized compounds (4a-h) were challenged to two Gram-positive and three Gram-negative bacteria and to a fungus using disk diffusion method. Challenge microorganisms were *Staphylococcus aureus* ATCC-29213, *Enterococcus faecalis* ATCC-29212, *Escherichia coli* ATCC-25922, *Pseudomonas aeruginosa* ATCC-27853, *Klebsiella pneunomoniae* ATCC-700603 and *Candida albicans* ATCC-10231. Bacterial strains were cultured overnight at 37°C in Mueller Hinton Agar (MHA-Oxoid-CM 337). Mold was cultured overnight at 30°C in Sabouraud Dextrose Agar (Oxoid-CM41). All experiments were carried out twice and the diameters of zones of bacterial growth inhibition were measured.

Disk diffusion assay

Agar disc diffusion method was utilized to determine antimicrobial activities of synthesized compounds (4a-h) (NCCLS, National Committee for Clinical Laboratory Standards, 1999). Each chemical dissolved in 1ml (1000 µg/ml) ethanol containing NaOH (0.1M) and 30µg was applied to sterile filter paper discs (6mm). Suspension of a challenge microorganism (0.1mL from stock having 10^8cells per mL) was spread on solid media plates. Filter paper discs were impregnated with 30µL of the chemical and placed on inoculated plates. These plates kept at 4°C for 2 h and were incubated at 37°C for 24h for bacteria and at 30°C for 48 h for mold. Gentamycin and nystatin were used as positive controls. All tests were carried out thrice (Atas *et al*., 2011).

Determination of antioxidant activities in MCF-7 cell cultures

Scope of our study was aimed to reveal changes in the antioxidant load of cancerous cells from applying derivatives of bis(α-amino)phosphinic acid at different concentrations in range of 1µM and 100µM on MCF-7 cell line using certain biochemical parameters and various analysis criteria, and to determine the most effective test compound concentration. Effects of synthesized bis(α-amino alkyl)phosphinic derivatives on levels of Malondialdehyde (MDA) which is the final product of lipid peroxidation in MCF-7 cancerous cells and on thiol levels, aryl-esterase (ARE) levels and paraoxanase enzyme (PON-1) levels were analyzed. Rel Assay brand commercial kits (Turkey) were used to assess antioxidant effects of synthesized chemicals on MCF-7 cell lines. Following the application of compounds on MCF-7 cultures at different concentrations in range of 1µM - 100 µM, phosphate buffer (pH=7.4; 50mM) at the rate of 1/9 (v/v) was added to cell samples first and homogenization was performed in a refrigerated environment to perform biochemical analyzes. Homogenates were centrifuged at 3000 rpm for 15 minutes after homogenization. Supernatants obtained from centrifugation were used for biochemical analyses. Method proposed by Dastan et al. (2014) in their previous articles was used for the analyzes

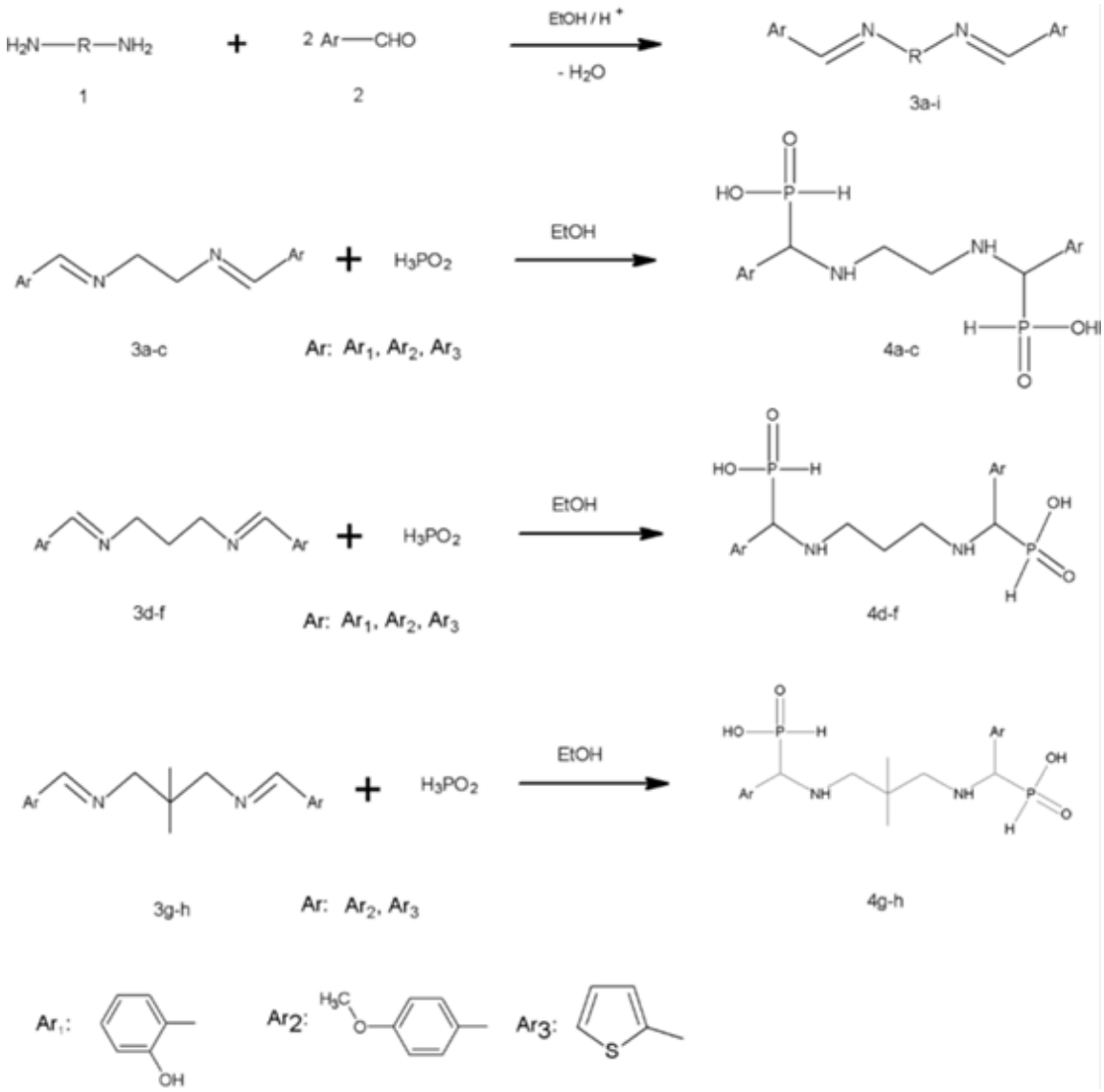


Fig. 1: Diagram showing the synthesis of bis (α-amino) phosphinic acid derivatives (4a-h) used in the study (Sarac *et al.,* 2016).

of MDA levels, Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Oxidative Stress Index (OSI) and Total Thiol Levels on MCF-7 cultures treated with derivatives of bis(α-amino) phosphinic acid. Amount of aryl-esterase enzyme in MCF-7 cultures was determined using "Rel Assay Aryl Esterase Kit" (Turkey) according to instructions from the producer company. Amount of paraoxanase enzyme in MCF-7 cultures was determined using "Rel Assay Paraoxanase Kit" (Turkey) according to instructions from the producer company.

STATISTICAL ANALYSIS

SPSS 22.0 (IBM Corporation, Armonk, New York, United States) and PAST3 (Hammer et al. 2001. Paleontological statistics) software were used for statistical analysis. Lilliefors corrected the Kolmogorov-Smirnov test and the Shapiro-Wilk test was used to test suitability of mono-variable data for normal distribution, and the Mardia (Dornik and Hansen omnibus) test was used to test suitability for multivariable normal distribution with coefficients of variation, and the Levene's test was used for variance homogeneity. The Independent-Samples T-test was used together with Bootstrap results to compare two independent groups. One-Way ANOVA (Robust Test: Brown-Forsythe) test was used together with Bootstrap results to compare more than two groups. LSD Dunnett and Games-Howell tests were used for post hoc analyses. Quantitative data were expressed as average ± standard deviation values in tables while categorical data were expressed in n (number) and percentages (%). The data were examined at the confidence level of 95% and the p-value was accepted significant when below 0.05.

Table 1: General properties of bis(α-amino alkyl) phosphinic acid derivatives (4a-h) used in the study.

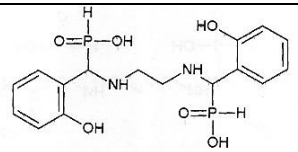
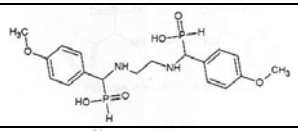
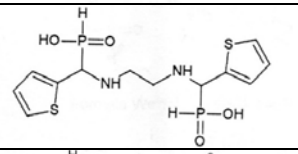
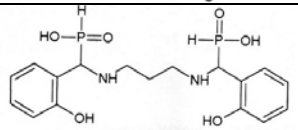
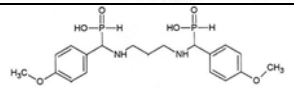
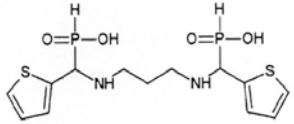
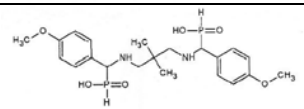
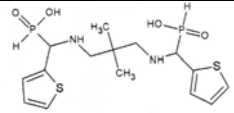
Compound code	Compound structure	Compound formula	Compound name	Mp (^{O}C)	Yield (%)	Molecular Weight (g/mol)
4a		$C_{16}H_{22}N_2O_6P_2$	*Ethane-1,2-diylbis{imino[(2-hydroxyphenyl)methanediyl]})bis phosphinic acid*	250-252 °C	60	400.303204
4b		$C_{18}H_{26}N_2O_6P_2$	*Ethane-1,2-diylbis{imino[(4-methoxyphenyl)methanediyl]})bis phosphinic acid*	200-203 °C	55	428.356364
4c		$C_{12}H_{18}N_2O_4P_2S_2$	*Ethane-1,2-diylbis[imino(thiophen-2-ylmethanediyl)]}bis phosphinic acid*	220-222 °C	43	380.359844
4d		$C_{17}H_{24}N_2O_6P_2$	*Propane-1,3-diylbis{imino[(2-hydroxyphenyl)methanediyl]})bis phosphinic acid*	217-219 °C	47	414.329784
4e		$C_{19}H_{28}N_2O_6P_2$	*Propane-1,3-diylbis{imino[(4-methoxyphenyl)methanediyl]})bis phosphinic acid*	256-258°C	45	442.382944
4f		$C_{13}H_{20}N_2O_4P_2S_2$	*Propane-1,3-diylbis[imino (thiophen-2-ylmethanediyl)]}bis phosphinic acid*	243-245°C	45	394.386424
4g		$C_{21}H_{32}N_2O_6P_2$	*2,2-dimethylpropane-1,3-diylbis[imino(4-methoxyphenyl) methanediyl)]bis phosphinic acid*	244-246°C	55	470.436104
4h		$C_{15}H_{24}N_2O_4P_2S_2$	*2,2-dimethylpropane-1,3-diyl)bis[imino(thiophen-2-ylmethanediyl)]}bis phosphinic acid*	217-219°C	49	422.439584

Table 2: Statistical comparisons of viability values obtained from application of test compounds for 24 hours on MCF-7 cultures.

Dose	MCF7 - 24 hour							
	4c	4e	4f	4g	4h	4d	4a	4b
0=I	100,0±12,3	100,0±12,3	100,0±12,3	100,0±12,3	100,0±12,3	100,0±12,3	100,0±12,3	100,0±12,3
-9=II	78,6±5,8	74,1±1,9	76,2±0,5	139,7±0,5	123,4±0,1	178,8±0,7	178,3±6,7	137,5±4,0
-8=III	72,6±3,5	73,0±0,3	75,6±0,6	133,2±8,7	106,6±5,2	168,7±3,6	145,0±1,2	133,3±0,3
-7=IV	64,8±0,4	63,4±4,7	72,0±0,4	122,7±0,8	76,3±1,9	138,8±4,4	130,9±2,5	122,3±0,1
-6=V	63,5±3,1	63,4±1,1	58,4±3,0	119,5±5,9	64,8±1,5	130,6±14,6	127,5±4,1	118,4±0,0
-5=VI	45,2±3,3	61,2±5,5	57,7±2,6	113,7±3,3	49,6±11,8	127,7±1,2	123,3±54,3	112,4±0,1
-4=VII	32,7±11,5	50,1±21,5	56,7±1,0	104,5±0,8	47,4±0,1	115,3±2,0	117,2±0,2	98,1±1,5
-3=VIII	17,9±11,9	31,1±0,3	33,5±0,1	95,7±0,3	38,2±3,0	65,7±0,4	80,1±0,6	90,1±0,2
P value	<0,001	0,001	0,001	<0,001	<0,001	<0,001	0,038	0,001
I-VIII	0,005	0,010	<0,001	<0,001	0,010	0,068	0,254	0,737
I-VII	<0,001	0,091	<0,001	<0,001	0,021	0,425	0,343	1,000
I-VI	<0,001	0,033	<0,001	<0,001	0,012	0,117	0,977	0,579
I-V	<0,001	0,057	<0,001	<0,001	0,062	0,164	0,108	0,300
I-IV	0,060	0,043	<0,001	<0,001	0,170	0,037	0,085	0,198
I-III	0,097	0,126	0,671	0,002	0,955	0,006	0,032	0,074
I-II	0,806	0,136	1,000	0,489	0,179	0,006	0,002	0,043

Table 3: Statistical comparisons of viability values obtained from application of test compounds for 24 hours on HUVEC cultures.

Dose	Huvec - 24 hour							
	4c	4e	4f	4g	4h	4d	4a	4b
0=I	100,0±4,1	100,0±4,1	100,0±4,1	100,0±4,1	100,0±4,1	100,0±4,1	100,0±4,1	100,0±4,1
-9=II	78,7±13,1	63,0±3,8	84,2±1,0	64,9±4,2	61,2±0,4	120,8±2,6	97,9±12,5	101,4±7,0
-8=III	75,7±1,7	67,5±2,4	79,2±3,1	68,2±1,3	57,1±0,5	106,1±1,6	84,6±3,6	93,2±3,8
-7=IV	69,7±3,1	67,5±7,0	78,3±0,1	70,4±4,8	55,4±9,7	99,2±3,4	78,0±0,3	88,9±2,2
-6=V	67,0±0,6	68,7±8,2	76,9±0,7	78,7±7,2	50,7±8,1	92,5±2,6	71,9±0,6	76,5±7,8
-5=VI	65,1±0,9	69,1±6,6	70,1±1,6	80,1±0,3	46,2±2,4	90,0±1,6	66,7±2,2	74,7±5,7
-4=VII	57,1±1,6	73,1±1,7	67,2±2,0	82,6±1,9	45,0±2,3	72,8±5,0	59,6±0,2	66,3±2,8
-3=VIII	55,2±1,3	73,3±2,5	64,0±2,5	84,6±2,2	31,2±3,8	64,4±1,7	46,0±1,5	64,3±4,3
P value	0,002	<0,001	<0,001	<0,001	<0,001	<0,001	<0,001	<0,001
I-VIII	0,001	0,001	<0,001	<0,001	<0,001	<0,001	<0,001	<0,001
I-VII	<0,001	0,002	0,001	<0,001	<0,001	<0,001	0,002	<0,001
I-VI	0,002	0,005	0,002	<0,001	<0,001	<0,001	0,001	<0,001
I-V	0,003	0,014	0,008	<0,001	0,002	<0,001	0,005	<0,001
I-IV	<0,001	0,006	0,011	<0,001	0,008	<0,001	0,011	0,005
I-III	0,003	<0,001	0,003	0,240	0,001	<0,001	0,015	0,071
I-II	0,246	<0,001	0,024	0,741	0,002	<0,001	1,000	0,693

OneWay ANOVA (Brown-Forsythe) - (Method:Bootsrap) Post Hoc Test: Dunnett - Games Howell All the data were shown as Mean ± standart deviation.

Table 4: MDA levels from MCF-7 lines that are treated with phosphinic acid compounds at different concentrations

Phosphinic acid compounds	MDA Value (nmol/gP)			
	Dose Groups			
	Mean ± S.E. (1µM)	Mean ± S.E. (10µM)	Mean ± S.E. (50µM)	Control
4a	3,632±0,142	3,542±0,222	3,634±0,033	3,173±0,163
4b	3,032±0,112	3,242±0,192	3,334±0,093	3,173±0,163
4c*	3,140±0,074[a]*	2,867±0,076[b]*	2,893±0,136[b]*	3,173±0,163[a]
4d	3,332±0,122	3,242±0,202	3,334±0,013	3,173±0,163
4e	3,307±0,119	3,290±0,143	3,573±0,195	3,173±0,163
4f	3,497±0,080	3,411±0,221	3,336±0,095	3,173±0,163
4g**	3,136±0,070[a]	3,738±0,127[b]	3,790±0,123[b]	3,173±0,163[a]
4h	3,340±0,174	3,167±0,076	3,193±0,036	3,173±0,163

Numbers shown with different letters in each row are significantly diverse ($P < 0.05$). S.E. means Standart Error.
A decrease was observed in the level of MDA in the groups shown with * when compared to the control group.
An increase was observed in the level of MDA in groups shown with ** when compared to the control group.

Table 5: TAS values from MCF-7 lines that are treated with phosphinic acid compounds at different concentrations.

TAS Value (mmol/L)	Dose Groups			
	Mean ± S.E. (1µM)	Mean ± S.E. (10µM)	Mean ± S.E. (50µM)	Control
4a	0,554±0,003	0,596±0,002	0,509±0,006	0,402±0,006
4b	0,454±0,013	0,496±0,012	0,409±0,016	0,402±0,006
4c	0,374±0,011[c]	0,512±0,012[b]	0,550±0,011[b]	0,402±0,006[a]
4d	0,354±0,001	0,396±0,005	0,309±0,008	0,402±0,006
4e	0,531±0,007[b]	0,501±0,005[b]	0,577±0,006[b]	0,402±0,006[a]
4f	0,512±0,001[b]	0,465±0,004[b]	0,568±0,004[b]	0,402±0,006[a]
4g	0,668±0,001[c]	0,583±0,002[b]	0,515±0,033[b]	0,402±0,006[a]
4h	0,474±0,001[b]	0,612±0,002[c]	0,650±0,001[c]	0,402±0,006[a]

Table 6: TOS levels from MCF-7 lines that are treated with phosphinic acid compounds at different concentrations.

TOS Value (µmol/L)	Dose Groups			
	Mean ± S.E. (1µM)	Mean ± S.E. (10µM)	Mean ± S.E. (50µM)	Control
4a	0,731±0,002[b]	0,888±0,001[a]	0,913±0,003[a]	0,838±0,005[a]
4b	0,631±0,012[b]	0,788±0,011[a]	0,813±0,013[a]	0,838±0,005[a]
4c	0,354±0,012[c]	0,611±0,014[b]	0,856±0,011[a]	0,838±0,005[a]
4d	0,531±0,007[c]	0,688±0,006[b]	0,713±0,008[b]	0,838±0,005[a]
4e	0,493±0,002[c]	0,655±0,003[b]	0,477±0,004[c]	0,838±0,005[a]
4f	0,720±0,003[c]	0,565±0,003[b]	0,559±0,005[b]	0,838±0,005[a]
4g	0,783±0,002[a]	0,455±0,003[c]	0,504±0,003[b]	0,838±0,005[a]
4h	0,454±0,002[d]	0,711±0,004[c]	0,956±0,001[b]	0,838±0,005[a]

Numbers shown with different letters in each row are significantly diverse (P<0.05). S.E. means Standart Error.

Table 7: OSI levels from MCF-7 lines that are treated with phosphinic acid compounds at different concentrations.

OSI Value	Dose Groups			
	Median (Max.-Min.) 1 µM	Median (Max.-Min.) 10 µM	Median (Max.-Min.) 50 µM	Control
4a	0,0013 (0,0013-0,0013)[b]	0,0015 (0,0015-0,0015)[a]	0,0018 (0,0018-0,0018)[a]	0,0022 (0,0023-0,0021)[a]
4b	0,0013 (0,0013-0,0013)[b]	0,0015 (0,0015-0,0015)[a]	0,0018 (0,0018-0,0018)[a]	0,0022 (0,0023-0,0021)[a]
4c	0,0010 (0,0010-0,0009)[b]	0,0012 (0,0012-0,0012)[a]	0,0015 (0,0015-0,0015)[a]	0,0022 (0,0023-0,0021)[a]
4d	0,0013 (0,0013-0,0013)[b]	0,0015 (0,0015-0,0015)[a]	0,0018 (0,0018-0,0018)[a]	0,0022 (0,0023-0,0021)[a]
4e	0,0009 (0,0009-0,0009)[a]	0,0013 (0,0013-0,0013)[a]	0,0008 (0,0009-0,0008)[b]	0,0022 (0,0023-0,0021)[a]
4f	0,0014 (0,0014-0,0014)[a]	0,0012 (0,0012-0,0012)[a]	0,0010 (0,0010-0,0010)[b]	0,0022 (0,0023-0,0021)[a]
4g	0,0011 (0,0011-0,0011)	0,0008 (0,0008-0,0008)	0,0009 (0,0011-0,0009)	0,0022 (0,0023-0,0021)
4h	0,0010 (0,0010-0,0009)[b]	0,0012 (0,0012-0,0012)[a]	0,0015 (0,0015-0,0015)[a]	0,0022 (0,0023-0,0021)[a]

Numbers shown with different letters in each row are significantly diverse (P<0.05).

Table 8: Total Thiol levels from MCF-7 lines that are treated with phosphinic acid compounds at different concentrations.

Total thiol value (µmol/L)	Dose Groups			
	Mean ± S.E. (1µM)	Mean ± S.E. (10µM)	Mean ± S.E. (50µM)	Control
4a	315,968±0,508[a]	376,987±0,075[a]	428,402±1,183[b]	336,260±10,838[a]
4b	305,968±0,518[a]	356,987±0,085[a]	408,402±1,193[b]	336,260±10,838[a]
4c	349,479±0,317	210,569±0,489	354,559±0,723	336,260±10,838
4d	315,968±0,508[a]	376,987±0,075[a]	428,402±1,183[b]	336,260±10,838[a]
4e	132,423±0,723[b]	341,711±0,891[a]	328,607±0,435[a]	336,260±10,838[a]
4f	354,706±0,425	358,485±0,188	350,405±0,307	336,260±10,838
4g	156,130±0,115[b]	380,902±0,419[a]	344,840±0,543[a]	336,260±10,838[a]
4h	379,479±0,317	210,569±0,289	394,559±0,223	336,260±10,838

Numbers shown with different letters in each row are significantly diverse (P < 0.05). S.E. means Standart Error.

Table 9: ARE enzyme levels from MCF-7 lines that are treated with phosphinic acid compounds at different concentrations.

ARE Value (ku/L)	Dose Groups			
	Mean ± S.E. (1µM)	Mean ± S.E. (10µM)	Mean ± S.E. (50µM)	Control
4a	16.331,333±2,028[a]	17.994,333±1,202[b]	18.258,000±2,517[b]	15.489,333±256,048[a]
4b	15.331,333±2,228[a]	16.994,333±1,402[b]	17.258,000±2,717[b]	15.489,333±256,048[a]
4c	15.553,667±2,733	15.821,333±2,528	15.393,667±1,964	15.489,333±256,048
4d	17.331,333±2,048[c]	18.994,333±1,252[b]	18.258,000±2,547[b]	15.489,333±256,048[a]
4e	16.985,667±0,882[b]	16.826,333±1,856[b]	16.275,667±0,882[b]	15.489,333±256,048[a]
4f	16.435,667±0,882[b]	17.094,000±1,528[b]	16.786,667±1,764[b]	15.489,333±256,048[a]
4g	15.934,333±1,202[a]	19.862,333±1,764[b]	20.285,000±2,309[b]	15.489,333±256,048[a]
4h	16.553,667±2,333[b]	16.821,333±2,028[b]	16.393,667±1,764[b]	15.489,333±256,048[a]

Numbers shown with different letters in each row are significantly diverse ($P < 0.05$). S.E. means Standart Error.

Table 10: PON-1 enzyme levels from MCF-7 lines that are treated with phosphinic acid compounds at different concentrations.

PON-1 Value (U/L)	Dose Groups			
	Mean ± S.E. (1µM)	Mean ± S.E. (10µM)	Mean ± S.E. (50µM)	Control
4a	3.024,000±2,309	3.232,667±1,453	2.884,000±2,309	2.450,667±79,963
4b	3.024,000±2,329	3.032,667±1,473	2.684,000±2,329	2.450,667±79,963
4c	8.140,333±2,804[c]	5.249,667±1,964[b]	2.088,333±0,882[a]	2.450,667±79,963[a]
4d	3.224,000±2,312	2.432,667±1,445	2.794,000±2,325	2.450,667±79,963
4e	1.848,000±1,155[a]	2.774,333±2,028[a]	6.879,333±5,207[b]	2.450,667±79,963[a]
4f	5.008,333±0,882[c]	5.844,667±1,764[b]	5.874,000±2,082[b]	2.450,667±79,963[a]
4g	4.949,333±2,603[c]	8.872,667±1,764[b]	2.690,667±2,728[a]	2.450,667±79,963[a]
4h	9.140,333±2,404[c]	6.249,667±1,764[b]	2.088,333±0,882[a]	2.450,667±79,963[a]

Numbers shown with different letters in each row are significantly diverse ($P < 0.05$). S.E. means Standart Error.

RESULTS

Cytotoxicity effects of phosphinic acids in MCF-7 cells

Cytotoxicity of compounds 4a–4h was evaluated on MCF-7 and HUVECs by XTT assay. Compounds of 4c, 4e, and 4f were decreased MCF-7 cell proliferation almost at all concentrations in 24 and 48 h. These chemicals inhibited MCF-7 cell proliferation in a dose- and time-dependent manner ($p<0.05$; table 2). Other compounds were presented lower activity against this cell line. Furthermore, we obtained effects of compounds on proliferation of normal HUVECs generally in high concentrations. Results are summarized in table 3.

Since cells interact with effective substance longer as incubation duration increases, it was expected that cytotoxicity of components also increases. Indeed, cytotoxicity was found increased on samples incubated for 48 h compared to samples incubated for 24 h. Results from viability rates of MCF-7 and HUVEC cell cultures treated with test substances are shown in comparison.

Antimicrobial activity

Eight synthesized compounds were tested for antibacterial and antifungal activities against six strains. Antimicrobial activity could not be detected from chemicals.

Evaluation of antioxidant activities in MCF-7 cell cultures

Evaluation of Malondialdehyde (MDA) Levels

Differences between effects of 8 different newly synthesized phosphinic acid compounds (4a-h) on MCF-7 cancerous cell lines at different concentrations were statistically insignificant compared to control. Only a slight increase was observed from 4g compound in 10µM and 50 µM concentrations compared to control group ($P<0.05$). MDA values from all doses from 4c compound were found lower compared to control group. Similar values were obtained in almost all dose groups from all other compounds compared to control (table 4; fig. 2). MDA formed from lipid peroxidation causes physiologic and metabolic disorders in the organism. Hence, it can be said that 4a and 4g compounds have pro-oxidant effects due to increasing MDA levels compared to other compounds.

Evaluation of total antioxidant status (TAS)

It was observed that there is no statistically significant difference ($P>0.05$) between TAS values in all applied concentrations of 4a, 4b, 4d compounds. TAS values in all applied concentrations of 4c, 4e, 4f and 4h compounds are found higher compared to control group, increase was according to applied doses of compounds. As for 4a and

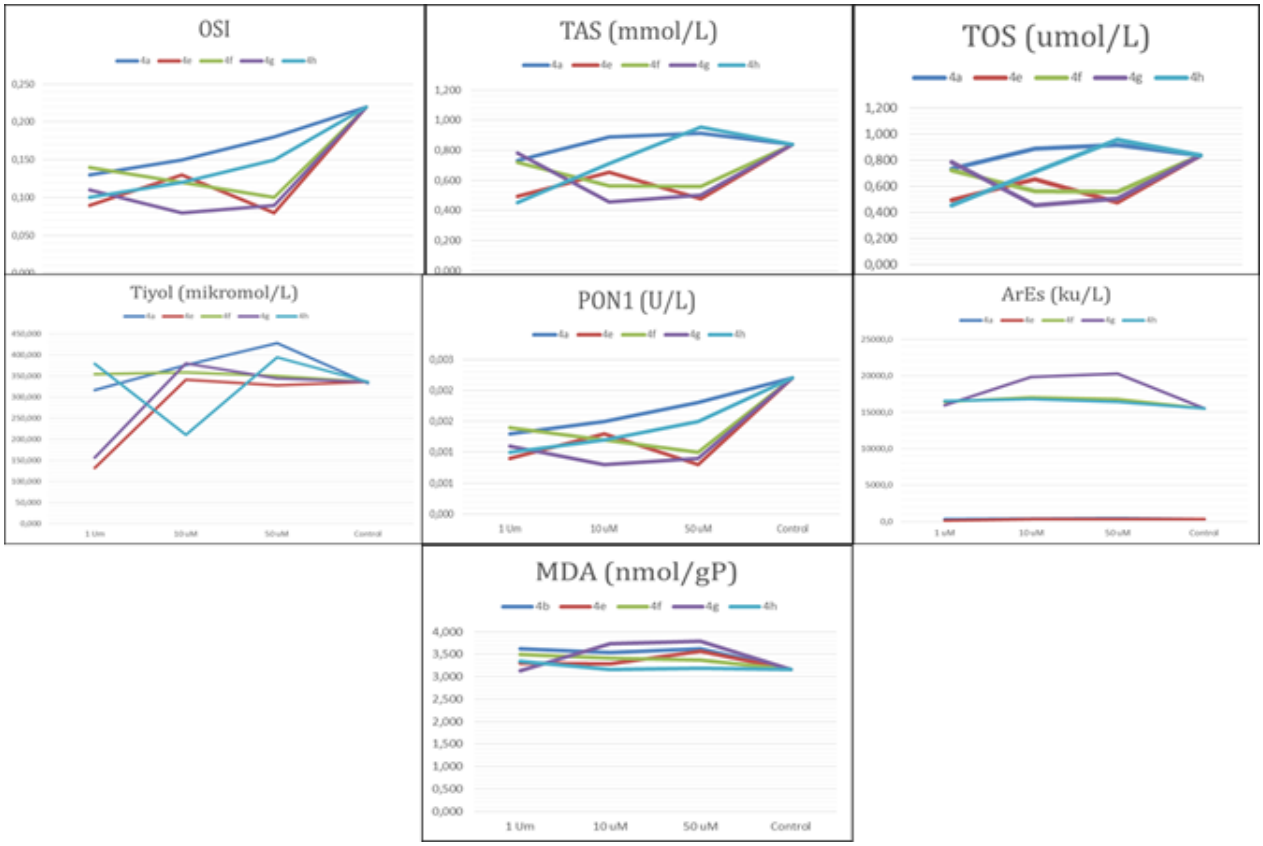


Fig. 2: Graphical representations showing the control group and various antioxidant parameters in MCF-7 cell cultures that were treated with phosphinic acid compounds at different concentrations for 24 hours.

4g compounds, it was observed that TAS values decrease as the applied dosage increases (table 5; fig. 2).

Evaluation of total oxidant status (TOS)

It was detected that there are statistically significant decreases (P<0.05) in all experimental groups (1-50 µM). Lowest statistical TOS values were obtained from groups in which chemicals applied at 1µM concentration compared to control group (P<0.05). In general, it was observed that the highest decrease in TOS levels occurred in groups in which 4e, 4f, 4g and 4h compounds applied (table 6; fig. 2).

Evaluation of the oxidative stress index (OSI)

Statistically significant (P<0.05) decrease was determined in OSI values from all experiment groups compared to control group. Statistically insignificant difference was found only between control group and dosage groups of 4g compound. In general, the lowest OSI values were obtained at concentration of 1µM from all compounds (table 7; fig. 2). It was determined that 4a, 4b, 4c, 4d and 4h compounds significantly (P<0.05) reduce OSI values in cells compared to control group. 1µM concentration groups of 4e and 4f compounds were significantly increased values compared to data from control group (P <0.05). Oxidative stress index is expressed as the ratio between total oxidant levels and total antioxidant levels, lower OSI values compared to control group would be resulted from effective antioxidant capacities of phosphinic acid compounds. Phosphinic acid compounds that lead to decreases in OSI values were further reduced damaging oxidative effects of compounds in unstable forms. As for groups which increased OSI values significantly compared to values from control group, this can be explained as cellular damage is occurring in the organism due to increased formation and amounts of lipid peroxidation and reactive oxygen species.

Evaluation of total thiol levels

It was observed that there were statistically significant changes (P<0.05) in all applied concentration groups (1 µM –50µM) of 4a, 4b, 4d, 4e, 4g compounds. As for 4c, 4f, 4h compounds, no difference was found compared to data of control group. The highest increase was observed in 50µM concentrations of 4b and 4d compounds. The lowest total thiol values were obtained from 1 µM concentrations of 4e and 4g compounds. (table 8; fig. 2). Thiols in biological systems have countless functions and primarily play an essential part in the coordination of antioxidant defense mechanisms. Hence, it is expected that the antioxidant levels increase with the increase in thiol levels in organisms.

Evaluation of aryl esterase (ARE) and paraoxanase (PON-1) enzyme levels

When control group was compared to ARE levels from breast cancer cell lines that were treated with phosphinic acid compounds at different concentrations (1μM-50μM), it was observed that there were statistically significant changes caused by all phosphinic acid derivatives excluding 4c compound (P<0.05). The highest ARE value was obtained from 4g compound while the lowest ARE value was obtained from 1 μM concentrations of 4b and 4c compounds (table 9; fig. 2). According to PON-1 levels in breast cancer cell lines, it was observed that there were statistically significant changes (P<0.05) from 4c, 4f, 4e, 4g compounds. As for 4a, 4b, 4d, 4h compounds, no statistically significant difference was found when compared to data of control group. The highest PON-1 value was obtained from 4c and 4g compounds while the lowest PON-1 level was obtained from 1μM concentration of 4e compound (table 10; fig. 2).

DISCUSSION

It is reported that Aryl Esterase (ARE) activity is an indicator of the actual protein concentration independent of changes in PON1 activity (Mackness *et al.,* 1991). While methods used in measurements of paraoxanase and aryl-esterase activity are basically the same, solutions at different pH values are being used and also reactions are forming at different temperatures (Hernandez *et al*., 1993; Kelso *et al.,* 1994; Mackness *et al.,* 1993). Spectrophotometric measurements of 4-nitrophenol as substrate which is a result of enzymatic hydrolysis of organophosphates such as paraoxane is taken as a basis in measurements of paraoxanase activity. Phenyl acetate is used for measurements of aryl-esterase as substrate in lieu of paraoxane and resulting phenol is measured. Paraoxanase (PON-1) enzyme has glycoprotein structure and it is an esterase that hydrolyzes aromatic carboxylic acid esters and tightly bound to HDL (Hernandez *et al*., 1993; Kelso *et al.,* 1994; Mackness *et al.,* 1991; 1993). It is known that HDL reduces lipid peroxides from oxidation of LDL and thus suspends the accumulation of lipid peroxides with mentioned enzymatic mechanisms (Eckerson *et al.,* 1983; Mackness *et al*., 1991; 1993; Navab *et al.,* 1997; Odowara *et al*., 1997; Heinecke and Lusis, 1998).

Results are proposing that some of the synthesized bis (α-amino) phosphinic acid compounds have weak antitumor effects by inhibiting cell proliferation, inducing apoptosis and inhibiting cell migration. Besides, synthesized compounds have effects on normal HUVECs which is suggesting the cytotoxicity when used as anticancer agents. Endothelial cells are the primary target for many chemical agents. Many chemical anticancer agents cannot be used in clinic for their cytotoxicity on endothelial cells. Nevertheless, design of novel compounds are crucial for developing anticancer therapeutics. Therefore, presented novel bis(α-amino)phosphinic acids derivatives may enable drug development for pharmaceutical applications on human breast cancer in future by providing a starting template for the synthesis of effective compounds.

REFERENCES

Atas AD, Goze I, Alim A, Akkus Cetinus S, Vural N, Goze HM and Korkoca H (2011). Chemical composition, antioxidant, antimicrobial activities of the essential oil of *salvia hypargeia* fisch. & mey. *J. Essent. Oil Bear.Plants*, **14**(3): 289-296.

Bulbul D and Ark M (2011). Investigation of possible anticancer activity of pyrazole derived compounds. *Gazi University Institute of Health Sciences Department of Pharmacology, Ankara, Turkey*. Pp. 1-113.

Cates LA and Li VS (1985). Phosphinic Acid analogues of methylaspartic and methylglutamic acids as antibacterials. *Pharm. Res.,* **2**: 135-136.

Collinsova M and Jiracek J (2000). Phosphinic acid compounds in biochemistry, biology and medicine. *Curr. Med. Chem.,* **7**: 629-647.

Dastan SD, Dastan T, Gulhan MF, Kirkbes A and Talas ZS (2014). Biochemical changes in muscle and gill tissues of rainbow trout treated with various concentrations of pollen extract. *ROAVS,* **4**(10): 540-544.

Eckerson HW, Wyte CM and LaDu BN (1983). The human serum paraoxonase/arylesterase polymorphism. *AJHG,* **35**: 1126-1138.

Gancarz R and Wieczorek JS (1978). A Useful Method for the Preparation of 1-Aminoalkane phosphonic acids. *Synthesis,* **625**.

Gittens SA, Bansal G, Zernicke RF and Uludag H (2005). Designing proteins for bone targeting. *Adv. Drug Dlv. Rev.,* **57**: 1011-1036.

Gursoy N and Cevik O (2014). Design, characterization and *in vitro* evaluation of SMEDDS containing an anticancer peptide, linear LyP-1. *Pharm. Develop. Tech.,* **19**(4): 486-490.

Hammer Q, Harper DAT and Ryan PD (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia. Electronica.,* **4**(1): 4-9.

Heinecke IW and Lusis AJ (1998). Paraoxonase-gene polymorphisms associated with coronary heart disease: Support for the oxidative damage hypothesis? *AJHG,* **62**: 20-24.

Hernandez AF, Pla A, Valenzuela A, Gil F, Hougen HP and Villanueva E (1993). Paraoxonase activity in human pericardial fluid and its relationship to coronary artery disease. *Int. J. Legal Med.,* **105**: 321-324.

Hyun-Joon H and Gong-Shil N (1992). An efficient synthesis of anilinobenzylphosphonates. *Synth. Commun.,* **22**: 1143.

Kaboudin B and As-Habei N (2003) Microwave-assisted synthesis of α-aminophosphinic acids from hypophosphorus acid salts under solvent free conditions. *Tetrahedron Lett.,* **44**: 4243-4245.

Kaboudin B, Haruki T, Yamaghishi T and Yokomatsu T (2007). Diastereoselective Addition α -Substituted α-Amino-H-Phosphinates to Imines Using Yb(OTf)3 as an Efficient Lewis Acid Catalyst. *Tetrahedron,* **63**: 8199-8205.

Kaboudin B and Jafari J (2008). One-Pot Synthesis of 1-Aminophosphinic Acids Using 50% Hypophosphorus Acid under Microwave Irradiation. *J. Iranian Chem. Soc.,* **5**: 97-102.

Kafarski P, Lejczak B, Tyka R, Koba L, Pliszczak E and Wieczorek P (1995). Herbicidal activity of phosphonic, phosphinic, and phosphonous acid analogues of phenylglycine and phenylalanine. *J. Plant Growth Reg.,* **14**: 199-203.

Katoh M, Hiratake J, Kato H and Oda J (1996). Mechanism-based inactivation of *E. coli* γ-glutamylcysteine synthetase by phosphinic acid- and sulfoximine-based transition-state analogues. *Bioorg. Med. Chem. Lett.,* **6**: 1437-1442.

Kelso GJ, Stuart WD, Richter RJ, Furlong CE, Jordan-Starck TC and Harmony JA (1994). Apolipoprotein J is associated with paraoxonase in human plasma. *Biochem.,* **33**: 832-839.

Kukhar VP and Hudson HR (2000). Aminophosphonic and Aminophosphinic Acids: Chemistry and Biological Activity. Wiley Publishing, Chichester, UK. Pp. 634.

Latajka R, Krezel A, Mucha A, Jewginski M and Kafarski P (2008). Conformational investigations of bis (a-aminoalkyl) phosphinic acids and studies of the stability of their complexes with Cu(II). *J. Mol. Struc.,* **877**: 64-71.

Luckman SP, Coxon FP, Ebetino FH, Russell RG and Rogers MJ (1998). Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: Evidence from structure-activity relationships in J774 macrophages. *J. Bone Miner. Res.,* **13**: 1668-1678.

Mackness MI, Arrol S and Durrington PN (1991). Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett.,* **286**: 152-154.

Mackness MI, Arrol S, Abbott C and Durrington PN (1993). Protection of low density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxanase. *Atherosclerosis,* **104**: 129-135.

Navab M, Hama-Levy S, Van Lenten RI, Fonarow GC, Cardinez CJ, Castellani LW, Brennan ML, Lusis AI, Fogelman AM and LaDu BN (1997). Mildly oxidized LDL induces an increased apolipoprotein J/paraoxonase ratio. *J. Clin. Invest.,* **99**: 2005-2019.

NCCLS (National Committee for Clinical Laboratory Standards) (1999). Performance standards for antimicrobial susceptibility testing. *9th International Supplement* Wayne Pa. M100-S9.

Odowara M, Tachi Y and Yamashita K (1997). PON1 polymorphism (Gln 192-Arg) is associated with coronary heart disease in Japanese non-insulin-dependent diabetes mellitus. *JCEM.,* **82**: 2257-2260.

Sanders JM, Gomez AO, Mao J, Meints GA, Van Brussel EM, Burzynska A, Kafarski P, Gonzales-Pacanowska D and Oldfield E (2003). 3-D QSAR investigations of the inhibition of Leishmania major farnesyl pyrophosphate synthase by bisphosphonates. *J. Med. Chem.,* **46**: 5171-5183.

Sarac K, Orek C, Cetin A, Dastan T, Koparir P, Dastan SD and Koparir M (2016). Synthesis and In Vitro Antioxidant Evaluation of New bis α-Aminoalkylphosphinic Acids Derivatives. *Phosphorus Sulfur Silicon and Relat. Elem.,* DOI: 10.1080/10426507.2016.1192620.

Seyferth D, Marmor RS and Hilbert P (1971). Reactions of dimethylphosphono-substituted diazoalkanes. (MeO)2P(O)CR transfer to olefins and 1,3-dipolar additions of (MeO)2P(O)C(N2)R. *J. Org. Chem.,* **36**: 1379-1386.

Worms KH and Schmidt-Dunker M (1971). Phosphonic acids and derivatives. *In Organic Phosphorus Compounds, Vol. 7*; Kosolapoff GM, Maier L, Editors. Wiley, New York, USA. pp. 876.

Ye Y, Liu M, Kao JLF and Marshall GR (2008). Design, synthesis, and metal binding of novel *Pseudo*-oligopeptides containing two phosphinic acid groups. *Biopolymer,* **89**: 72-85.