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Synthesis, cytotoxicity, and promising anticancer potential of novel β-amino- and β-iminophosphonates

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ABSTRACT

New series of β-amino- and β-iminophosphonates were produced via a nucleophilic addition reaction of benzyl amine and 2-aminopirydine to 1,2-alkadienephosphonates. Cytotoxicity and anticancer potential of newly synthetisized compounds were investigated. *Triticum aestivum* L. root growth inhibition test and *Allium cepa* L. assay were used to assess the cytotoxic potential of phosphonates. Anticancer activity on human fibrosarcoma cell line HT 1080 was assessed. The antiproliferative effect of compounds was determined by WST-1 test, and nuclear morphology changes in cancer cells stained with Giemsa were evaluated. After treatment with β-iminophosphonate (E,Z)(3-Methyl-2-(pyridin-2-ylimino)butyl)diphenylphosphine oxide (4e) proliferation/viability of cancer cells was strongly inhibited and in 65.61% of the cells signs of early apoptosis were observed. The results reported here show once again phosphonates as versatile substrates in synthetic organic chemistry, especially as fruitful starting compounds for the achievement of molecular diversity. Among tested compounds β-iminophosphonate '(E,Z)(3-Methyl-2-(pyridin-2-ylimino)butyl) diphenylphosphine oxide (4e) showed anticancer activity against human fibroblastoma cell line HT 1080.

INTRODUCTION

Cancer death rate has continued to decline over the last years due to advances in chemotherapy (DeVita and Chu, 2008). At the same time, the increasing cancer rates and specific features of different types of cancer create a need for the search of new effective chemical compounds (Skoupilova *et al*., 2019). Discovery of new drugs requires evaluation of their influence on different cancer hallmarks (Ediriweera *et al*., 2019). Uncontrolled proliferation, an important part of cancer development, is a target of cancer treatment (Feitelson *et al*., 2015; Hanahan and Weinberg, 2011; Stewart *et al*., 2003). The major concern of chemotherapy is the side effects of existing drugs (Baldo and Pham, 2013). Screening of synthetic compounds inspired by natural products represents a promising approach in cancer therapy (Kandekar *et al*., 2013).

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Organophosphorus compounds represent significant importance as biologically active agents. α-aminophosphonates are among the most common organophosphorus compounds (Abdel-Megeed *et al*., 2012). They are analogs of natural amino acids (Tajti and Keglevich, 2018). Diverse biological activities of new α-aminophosphonates are reported including their great potential as antitumor agents (Azaam *et al*., 2018; Azzam *et al*., 2020; Chinthaparthi *et al*., 2013; Chukka *et al*., 2018; Guo *et al*., 2015; Kandekar *et al*., 2013; Kraicheva *et al*., 2018; Liu *et al*., 2017; Naydenova *et al*., 2010). Data on synthesis and bioactivity of β-aminophosphonates (Rapp *et al*., 2014) and β-iminophosphonates are extremely scarce. It is well known that biological activity depends on the chemical structure (Lichota and Gwozdzinski, 2018). Iminophosphonates are also object of interest being common precursors of aminophosphonates (Motevalli *et al*., 2015).

Alkadienephosphonates are famous building blocks in organic synthesis (Enchev, 2010; Enchev, 2012). An approach for the synthesis of bioactive substances is the amine nucleophilic addition to different alkadienephosphonates (Altenbach and Korff, 1981; Brel, 2007; Brel, 2009; Enchev, 2005; Enchev, 2017; Khusainova *et al*., 2005, 2007; Mazzuca *et al*., 2015; Pavan and Swamy, 2011; Pudovik and Khusainova, 1966). The first example

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of the reaction of amines with substituted allenephosphonic acid derivatives was published in 1966 (Pudovik and Khusainova, 1966) when it was shown that diethylamine and piperidine add to dialkyl 3-methyl-1,2-butadienephosphonates on the 1,2-double bond to afford 1,2-aducts. More recently, nucleophilic additions to allene phosphonium salts, phosphine oxides, and diethyl allene phosphonate have been reported to yield a wide variety of primary amines. For example, the addition of aminoethanol occurs at the 2,3-double bond, with isolation of the 2,3-adducts in all cases (Palacios *et al*., 1996). Having in mind all these facts and the procedures described previously, we have investigated the same reaction applying an atom economy, environment-friendly, and easy for industrial application synthetic protocol, as well as different starting 1,2-alkadienephosphonates. All the starting compounds have never been studied in the discussed reaction. The chosen synthetic protocol has involved room temperature, inner atmosphere, and no solvents.

The present work focused on (1) synthesis of β-amino- and β-iminophosphonates via a nucleophilic addition reaction of benzylamine and 2-aminopirydine to 1,2-alkadienephosphonates, i.e., dialkyl esters and amidoesters, as well as dimethyl- and diphenylphosphine oxides, and (2) investigation of cytotoxicity and anticancer potential of synthesized compounds.

MATERIALS AND METHODS

Synthesis

NMR spectra were recorded on Brucker Avance II+600 spectrometer in CDCl3 solution. The IR (Infrared spectroscopy) spectra were run on FT-IRAffinity-1 Shimadzu (Shimadzu Corp., Japan) spectrophotometer. Elemental analyses were carried out by the Microanalytical Service Laboratory of Faculty of Chemistry and Pharmacy, University of Sofia, using Vario EL3 CHNS (O) (Elementar Analysensysteme GmbH, Hanau, Germany). Column chromatography was carried out on Kieselgel F_{254}60 (70–230 mesh ASTM (American Standard Test Sieve Series), 0.063-0.200 nm, Merck).

General procedure for preparation of 1a–e

Compounds **1a-e** were synthesized according to the procedures described earlier (Angelov and Enchev, 1987; Angelov *et al*., 1979, Grissom and Huang, 1995; Ignatiev *et al*., 1966).

General procedure for preparation of 2-9

To 5 mmol of the corresponding 1,2-alkadienephosphinate **1a-e**, 8 mmol of benzylamine or 2-aminopyridine was added under argon and stirred for 12–24 hours at room temperature*. The reaction was monitored by IR and TLC (Thin Layer Chromatography). After complete disappearing of the characteristic band for the allene system in the IR spectra of the reaction mixtures, the latest was purified by column chromatography (hexane: EA = 4:1), followed by recrystallization (hexane/CH_2Cl_2)

*Compounds **2a-5a** were prepared at the same protocol, but in boiled acetonitrile (Mazzuca *et al*., 2015).

2a, (*E,Z*)Dimethyl-(2-(benzylamino)-3-methylpent-1-en-1-yl)phosphonate, 65%, white solid, IR spectrum, *v* cm^{-1}: 1,215 P=O, 1,053 P-O-C, 1,643 C=C, 1,495 Ph, 1,589 N-H, 3,292 =C-H; ^{1}H NMR ($CDCl_3$) δ/ppm: 1.68[q, $^3J_{HH}$ 7.0Hz, $^3J_{HH}$ 8.0Hz, 2H(CH_2-CH_3)]; 1.40[d, $^3J_{HH}$ 6.8Hz, 3H, $CHCH_3$)]; 0.89 [t,$^3J_{HH}$ 8.0Hz, 3H, (CH_2-CH_3)]; 3.09 [t, $^2J_{HH}$ 5.8Hz, 1H(CH)]; 3.70 (d, $^3J_{HP}$ 12.2Hz, 6H(CH_3O); 3.76 (s, 1H, NH), 7.49–7.25(m, 5H, arom.); ^{13}C{H} NMR ($CDCl_3$) δ/ppm:19.85 ,18.93, 11.88, 11.75, 29.25, 28.21, 46.08, 47.5, 73.12, 71.24, 126.93–130.9 (C-arom); ^{31}P{H} NMR δ/ppm: 25.44., Found, %: N 4.65; P 10.34. $C_{15}H_{24}O_3$NP. Calculated, %: N 4.70; P 10.42. *M* 297.312.

2b, (*E,Z*)2-(Benzylamino)-3-methylpent-1-en-1-yl) dimethylphosphine oxide, 75%, white solid, IR spectrum, *v,* cm^{-1}: 1,217 P=O, 1,582 N-H, 1,602 C=C, 1,490 Ph, 3,292 =C-H; ^{1}HNMR ($CDCl_3$) δ/ppm: 0.93(m, 6H, 2XCH_3-P); 1.68 (q, $^3J_{HH}$ 7.0Hz, $^3J_{HH}$ 8.0Hz, 2H[CH_2-CH_3)]; 1.40 [d, $^3J_{HH}$ 6.8Hz, 3H($CHCH_3$)]; 0.89 [t,$^3J_{HH}$ 8.0Hz, 3H(CH_2-CH_3)]; 3.09 [t, $^2J_{HH}$ 5.8Hz, 1H(CH)]; 3.76 (s, 1H, NH), 7.49–7.25 (m, 5H, arom.); ^{13}C{H} NMR ($CDCl_3$) δ/ppm: 18.00, 17.91, 14.20, 14.22, 22.00, 21.07, 38.60, 39.5, 60.41, 67.24, 126.93–130.9(C-arom); ^{31}P{H} NMR δ/ppm: 26.48. Found, %: N 5.19; P 11.53. $C_{15}H_{24}$ONP. Calculated, %: N 5.28; P 11.67. *M* 265.312.

2c, (*E,Z*)Methyl-P-(2-(benzylamino)-3-methylpent-1-en-1-yl)-N,N-diethylphospho-namidate, 70%, white solid, IR spectrum, *v*, cm^{-1}: 1,209 P=O, 1,045 P-O-C, 1,589 N-H, 1,475 Ph, 1,690 C=C, 3,100 =C-H; ^{1}HNMR ($CDCl_3$) δ/ppm: 2.59 (m, 4H,$^3J_{HP}$13.6Hz, $^3J_{HH}$ 7.2Hz, CH_3CH_2N); 1.02 (tr, 6H, $^3J_{HH}$ 7.2Hz, CH_3CH_2N); 1.68 [q, $^3J_{HH}$ 7.0Hz, $^3J_{HH}$ 8.0Hz, 2H(CH_2-CH_3)]; 1.40 [d, $^3J_{HH}$ 6.8Hz, 3H, $CHCH_3$)]; 0.89 [t,$^3J_{HH}$ 8.0Hz, 3H, (CH_2-CH_3)]; 3.09 [t, $^2J_{HH}$ 5.8Hz, 1H(CH)]; 3.76 (s, 1H, NH); 3.40[d, $^3J_{HP}$ 10.25Hz, 3H(CH_3O)]; 7.49–7.25 (m, 5H, arom.); ^{13}C{H} NMR ($CDCl_3$) δ/ppm: 18.19, 17.96, 14.21, 14.25, 22.00, 21.25, 38.78, 38.74, 50.18, 50.24, 126.93–130.9(C-arom); ^{31}P{H} NMR δ/ppm: 28.33. Found, %: N 8.18; P 8.96. $C_{18}H_{31}N_2$ O_2P. Calculated, %: N 8.27; P 9.15. M 338.43.

2d, (*E,Z*)2-(Benzylamino)-3-methylpent-1-en-1-yl) diphenylphosphine oxide, 69%, white solid, IR spectrum, *v*, cm^{-1}: 1,155 P=O, 1,643 C=C, 3,223 =C-H, 1,556 N-H, 1,436-1,452 3Ph; ^{1}HNMR ($CDCl_3$) δ/ppm: 1.68[q, $^3J_{HH}$ 7.0Hz, $^3J_{HH}$ 8.0Hz, 2H(CH_2-CH_3)]; 1.40 [d, $^3J_{HH}$ 6.8Hz, 3H, $CHCH_3$)]; 0.89 [t,$^3J_{HH}$ 8.0Hz, 3H, (CH_2-CH_3)]; 3.09 [t, $^2J_{HH}$ 5.8Hz, 1H(CH)]; 3.76(s, 1H, NH); 7.19–7.80 (m, 15H, arom.); ^{13}C{H} NMR ($CDCl_3$) δ/ppm: 21.85, 24.40, 14.88, 14.75, 27.25, 22.21, 34.25, 32.21, 60.02, 59.24, 126.93–130.9(C-arom); ^{31}P{H} NMR δ/ppm: 28.98. Found, %: N 3.47; P 7.76. $C_{25}H_{28}$ONP. Calculated, %: N 3.59; P 7.95. *M* 389.444.

4a, (*E,Z*)Dimethyl-(3-methyl-2-(pyridin-2-ylimino) pentyl)phosphonate, 71%, white solid, IR spectrum, *v,* cm^{-1}: 1,155 P=O, 1,033 P-O-C, 1,693 C=N, ^{1}HNMR ($CDCl_3$) δ/ppm: 1.68[q, $^3J_{HH}$ 7.0Hz, $^3J_{HH}$ 8.0Hz, 2H(CH_2-CH_3)]; 1.40 [d, $^3J_{HH}$ 6.8Hz, 3H, $CHCH_3$)]; 0.89[t,$^3J_{HH}$ 8.0Hz, 3H, (CH_2-CH_3)]; 2.25 (d, J_{HP} 12.25, 2H, CH_2-P); 3.09 [t, $^2J_{HH}$ 5.8Hz, 1H(CH)]; 3.70 (d, $^3J_{HP}$ 12.2Hz, 6H(CH_3O); 7.49–7.25 (m, 4H, arom.); ^{13}C{H} NMR ($CDCl_3$) δ/ppm: 9.05, 18.8, 29.8, 42.21[d, $^1J_{PC}$ 64.4Hz(CH_2-P)], 52.84, 109.35, 113.28, 138.12, 146.52, 155.0, 158.39; ^{31}P{H} NMR δ/ppm: 27.35. Found, %: N 9.65; P 10.34. $C_{13}H_{21}N_2O_3$P. Calculated, %: N 9.85; P 10.89. *M* 284.268.

4b, (*E,Z*)Dimethyl(3-methyl-2-(pyridin-2-ylimino) pentyl)phosphine oxide, 75%, white solid, IR spectrum, *v,* cm^{-1}:1,155 P=O, 1,693 C=N, ^{1}HNMR ($CDCl_3$) δ/ppm: 1.06(d, J_{HH} 6.87Hz, 3H, CH_2CH_3); 1.32(t, J_{HH} 7.43Hz, 3H, CH-CH_3); 2.03 (d, J_{HP}26.16Hz, 6H, 2CH_3); 2.93(m, 1H, CH); 3.03 (q, J_{HH} 27.42Hz,

2H, CH_2CH_3); 3.6 (d, J_{HP} 12.37Hz, 2H P-CH_2); 6.52–7.86 (m, 4H arom.); $^{13}C\{H\}$ NMR ($CDCl_3$) δ/ppm: 9.21, 16.38, 19.6, 38.4[d, $^1J_{PC}$ 58.8Hz(CH_2-P)], 39.98, 50.4, 102.92, 114.68, 137.38, 149.0, 153.48, 155.0; $^{31}P\{H\}$ NMR δ/ppm: 16.31. Found, %: N 10.96; P 12.22; $C_{13}H_{21}N_2OP$; Calculated, %: N 11.1; P 12.28. *M* 252.268.

4c, (*E,Z*)Methyl-N,N-diethyl-P-(3-methyl-2-(pyridin-2-ylimino)pentyl)phosphonamidate, 70%, white solid, IR spectrum, *v,* cm^{-1}: 1,209 P=O, 1,045 P-O-C, 1,589 N-H, 1,475 Ph, 1,690 C=C, 3,100 =C-H; ^{1}HNMR ($CDCl_3$) δ/ppm: 2.59 (m, 4H,$^3J_{HP}$13.6Hz, $^3J_{HH}$ 7.2Hz, CH_3CH_2N); 1.02 (tr, 6H, $^3J_{HH}$ 7.2Hz, CH_3CH_2N); 1.68 [q, $^3J_{HH}$ 7.0Hz, $^3J_{HH}$ 8.0Hz, 2H(CH_2-CH_3)]; 1.40 [d, $^3J_{HH}$ 6.8Hz, 3H, $CHCH_3$)]; 0.89 [t,$^3J_{HH}$ 8.0Hz, 3H, (CH_2-CH_3)]; 3.09 [t, $^2J_{HH}$ 5.8Hz, 1H(CH)]; 3.40 [d, $^3J_{HP}$ 10.25Hz, 3H(CH_3O)]; 6.52–7.86 (m, 4H arom.); $^{13}C\{H\}$ NMR ($CDCl_3$) δ/ppm: 18.19; 17.96; 14.21; 22.00; 21.25; 50.18; 50.24, 126.93–130.9; $^{31}P\{H\}$ NMR δ/ppm: 16.38. Found, %: N 12.68; P 9.26. $C_{16}H_{28}N_3O_2P$. Calculated, %: N 12.9; P 9.51; M 325.354.

4e, (*E,Z*)(3-Methyl-2-(pyridin-2-ylimino)butyl) diphenylphosphine oxide, 75%, white solid, IR spectrum, *v,* cm^{-1}:1,155 P=O, 1,693 C=N, ^{1}HNMR ($CDCl_3$) δ/ppm: 2.03 (d, J_{HP} 26.16Hz, 6H, $2CH_3$); 2.93 (m, 1H, CH); 3.6(d, J_{HP} 12.37Hz, 2H P-CH_2); 6.52–7.86 (m, 4H arom.); $^{13}C\{H\}$ NMR ($CDCl_3$) δ/ppm: 9.21, 16.38, 19.6, 38.4[d, $^1J_{PC}$ 58.8Hz(CH_2-P)], 73.12; 71.24, 126.93–130.9; $^{31}P\{H\}$ NMR δ/ppm: 16.44. Found, %: N 7.66; P 8.55; $C_{22}H_{23}N_2OP$; Calculated, %: N 7.89; P 8.73. M 354.588.

Biology

Compounds tested

The solubility of newly synthesized compounds was evaluated. Water solutions of β-iminophosphonates **4a**, **4c**, and **4e** were prepared just before each treatment. β-aminophosphonates **2a** and **2c** were solubilized in methanol.

Root growth inhibition test

Twenty seeds of *Triticum aestivum* L. were placed on filter paper in Petri dishes (10 cm in diameter). Based on a preliminary study (data not shown), *influence on root elongation was evaluated at concentrations ranging* as follows: compound **4a** 100–1,000 µg/ml; compound **4c** 100–1,000 µg/ml; compound **4e** 200–1,000 µg/ml; compound **2a** 200–1,000 µg/ml; and compound **2c** 50–1,000 µg/ml. Distilled water and 0.40 µg/ml methanol were used as untreated controls. Five ml of each solution or of controls was applied to the seeds. The dishes were sealed and incubated at 25°C ± 1°C for 96 hours. The length of the roots of germinated seeds was measured in mm. Seeds that did not germinate were not included in the root elongation test. A growth curve was drawn based on the obtained values plotted as growth in the percentage of control (ordinate) against test concentrations (abscissa). From the growth curve, the effective concentration that decreased root growth about 50% (EC50) when compared to the negative control group (distilled water, 100%) was obtained. Three replications of each treatment were carried out.

Allium cepa test

A commercial variety of common onion (*Allium cepa*) was used. Equal-sized healthy onion bulbs were chosen. The outer scales of the bulbs and the old dry roots were carefully removed without destroying the root primordia. The bulbs were kept for root germination in distilled water for 48 hours. Bulbs with new roots and length of about 1 cm were placed in solvents tested at concentrations lower than EC50 values. The treatment duration was 48 hours at 25°C ± 1°C. Distilled water and 0.40 µg/ml methanol were used as controls. After treatment, the roots were fixed in Clarke's fixative (95% ethanol: acetic acid glacial, 3:1) for 90 minutes, hydrolyzed in 3N HCl for 8 minutes, and in 45% acetic acid (CH_3COOH) for 30 minutes at room temperature, and stained for 40 minutes in 2% acetoorseine. After staining, the terminal root tips (1–2 mm) were cut off and squashed in 45% CH_3COOH. At least 1,000 cells of each root meristem were analyzed. The microscopic analysis included the estimation of the mitotic and phase indices. The mitotic index was determined as a ratio between the number of cells in mitosis and the total number of analyzed cells. The index of each phase of the mitotic division was calculated as a ratio between the cell number in the respective period and the number of dividing cells. The analysis was carried out using an optical microscope (BOECO, 99 Germany).

In vitro cytotoxicity assay

Cell lines and culture conditions

The human fibrosarcoma cell line HT 1080 cells were maintained as adherent in controlled environment: DMEM (Dulbecco's Modified Eagle Medium) medium, supplemented by 10% heat-inactivated fetal calf serum, and 100 U/ml penicillin/streptomycin mixture, in an incubator at 37°C, 5% CO_2, and humidified atmosphere. In order to keep cells in log phase, the cultures were subcultured in a fresh medium 2 or 3 times/week. The treated cells were at a density of 1×10^5 cells per ml. The complete growth medium and 0.26 µg/ml methanol were used as untreated controls. The cells were treated with compounds for 48 hours at concentrations lower than EC50 values.

WST-1 cell proliferation/viability assay

Cell viability was assessed using water-soluble tetrazolium salt 1 (WST-1). The assay principle is based upon the reduction of WST-1 to formazan in the mitochondria of living cells. Exponentially growing cells were seeded in 96-well flat-bottomed microplates (200 µl/well). Four hours before the end of incubation time, cell proliferating reagent WST-1 (20 µl/well) was added to the culture media. Microplates were further incubated for 4 hours at 37°C. The absorbance of the formazan product was quantitated at 450 nm using an ELISA reader. The cell survival fractions were calculated as a percentage of the control (control = 100%). All the treatments were carried out in triplicate.

Evaluation of apoptosis by nuclear morphology

The cells at a density of 0.5×10^5 cells per ml were placed at 25 cm^2 matrix. After 24 hours, growth media were replaced with media containing tested compounds. 5-Fluorouracil was used as a positive control. After treatment for 48 hours, the cells were harvested and fixed in 3 ml methanol:acetic acid glacial (3:1) for 30 minutes at 4°C–8°C. Then, the cells were centrifuged, resuspended in 0.2 ml fixative, placed on glass slides, and stained with Giemsa. The slides were examined in order to establish nuclear morphological features of apoptosis, pyknosis (irreversible condensation of the chromatin causing nuclei to shrink in size), karyorrhexis (destructive fragmentation

of a pyknotic nucleus), and karyolysis (nuclear fading caused by dissolution of the chromatin) (Naipal *et al*., 2016). The analysis was carried out using optical microscope (BOECO, 99 Germany).

Statistical analysis

In the root inhibition test, an experimental unit was the root. The calculations were carried out on the assumption that roots used in each treatment made one sample. In *Allium cepa* test, an experimental unit was the cell, instead of the root. The calculations were carried out on the assumption that all the cells of the nine root meristems of three bulbs made one sample. In the cell proliferation/viability test, an experimental unit was a microplate well. The calculations were carried out on the assumption that wells used in each treatment made one sample. The viabile cells and apoptosis induction evaluation were conducted in two replicate flasks and each flask is one sample. The samples were tested against the control sample. The values for each concentration tested represent the average (mean ± SD). Student's *t*-test was carried out with $p \leq 0.05$ taken as athe significance level.

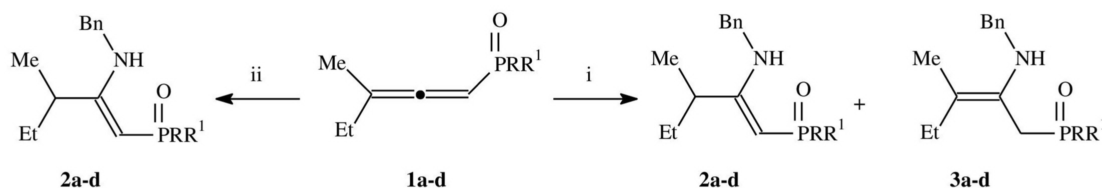
RESULTS AND DISCUSSION

Chemistry

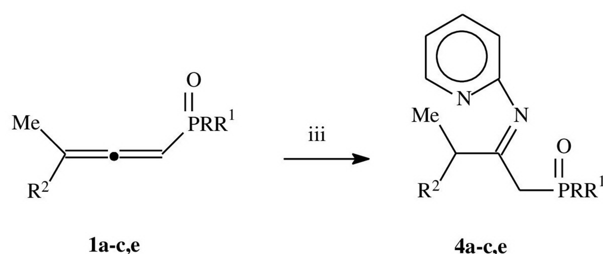
1,2-Alkadienephosphonates **1a-e** (Table 1) react with benzylamine and 2-aminopyridine at room temperature, neat and with stirring under argon for 24 hours (Scheme 1). The discussed reaction followed the well-documented mechanism that involved initial attack of the nucleophile onto central carbon of the allenephosphonate system of double bonds and subsequent protonization of the resulting carbanion (Pudovik and Khusainova, 1966). The reaction progress was monitored by IR and TLC. The end of the reaction was indicated by the complete disappearance of the characteristic band for allene chromophore in the IR spectra of the reaction mixtures, as well as by the dramatic change in the aggregate state of them. In all cases, crystalline products were obtained. In all cases, reaction with benzylamine produced both 1,2- and 2,3-adducts, both in their *E* and *Z* isomers. When the reaction was carried out in boiling acetonitrile, only 2,3-adduct was obtained as a mixture of *E* and *Z* isomers, in an approximate

Table 1. Structural elements of synthesized β-amino- and β-iminophosphonates **2a-5a**, **6-9**.

N	Compound	Precursor amine	Precursor phosphonate
2a	Dimethyl-(2-(benzylamino)-3-methylpent-1-en-1-yl)phosphonate	Benzylamine	Dimethyl (3-methylpenta-1,2-dien-1-yl)phosphonate
b	2-(Benzylamino)-3-methylpent-1-en-1-yl)dimethylphosphine oxide	Benzylamine	Dimethyl(3-methylpenta-1,2-dien-1-yl)phosphine oxide
c	Methyl-P-(2-(benzylamino)-3-methylpent-1-en-1-yl)-N,N-diethylphospho-namidate,	Benzylamine	Methyl-N,N-diethyl-P-(3-methylpenta-1,2-dien-1-yl)phosphonamidate
d	2-(Benzylamino)-3-methylpent-1-en-1-yl)diphenylphosphine oxide	Benzylamine	(3-Methylpenta-1,2-dien-1-yl)diphenylphosphine oxide
4a	Dimethyl-(3-methyl-2-(pyridin-2-ylimino)pentyl)phosphonate	2-Aminopiridyne	Dimethyl (3-methylpenta-1,2-dien-1-yl)phosphonate
b	Dimethyl(3-methyl-2-(pyridin-2-ylimino)pentyl)phosphine oxide	2-Aminopiridyne	Dimethyl(3-methylpenta-1,2-dien-1-yl)phosphine oxide
c	Methyl -N,N-diethyl-P-(3-methyl-2-(pyridin-2-ylimino)pentyl)phosphonamidate	2-Aminopiridyne	Methyl-N,N-diethyl-P-(3-methylpenta-1,2-dien-1-yl)phosphonamidate
e	3-Methyl-2- (pyridin-2-ylimino)butyl)diphenylphosphine oxide	2-Aminopiridyne	(3-Methylbuta-1,2-dien-1-yl)diphenylphosphine oxide



(a)R=R^1=OMe; (b)R=R^1=Me; (c)R=OMe, R^1=NEt$_2$; (d)R=R^1=Ph



(a)R=R^1=OMe, R^2=Et; (b)R=R^1=Me, R^2=Et; (c) R= OMe, R^1=NEt$_2$, R^2=Et; (e) R=R^1=Ph, R^2=Me

Scheme 1. Interaction of alkadienephosphonates **1a-e** with benzylamine and 2-aminopyridine.

2:1 isomeric ratio (Palacios *et al*., 1996), but it was not possible to separate either isomer in pure form by recrystallization (Mazzuca *et al*., 2015).

In the case of 2-aminopiridyne as a reactant, the reaction was carried out at room temperature and neat. Here, the intermediate enamines isomerized to β-iminoderivatives.

Compounds **2a-d** and **4a–c,e** were purified by column chromatography and recrystallization. The spectral investigations of the reaction mixtures show no signals for allenephosphonate fragments; that is, in the chosen reaction conditions, all the starting alkadienephosphonates have been completely consumed.

Biological activity

Drug discovery includes biological testing of numerous chemical compounds. At the same time, ethical issues encouraged researchers to reduce the number of animals used in experiments (Ferdowsian and Beck, 2011). Nowadays, alternative tools for the evaluation of bioactivity of different compounds are widely used. In the present study, *Triticum aestivum* L. root growth inhibition test, *Allium cepa* L. assay, and *in vitro* assays using human fibrosarcoma cell line HT 1080 were used in order to assess the cytotoxic potential of newly synthesized phosphonates. It is known that even bioactive compounds with proven health benefits failed to be used commercially because of low solubility (Recharla *et al*., 2017). As the first step of this study, an analysis of the solubility of phosphonates was carried out. The results showed that the cytotoxic activity of five compounds could be studied. Water solutions of β-iminophosphonates **4a**, **4c**, and **4e** and methanol solutions of β-aminophosphonates **2a** and **2c** were used in biological tests.

Root growth inhibition test

In the present study, root growth inhibition of *T. aestivum* was used for the evaluation of the general toxicity of phosphonates tested (Jităreanu *et al*., 2019). All compounds inhibited root elongation in comparison with the control (Table 2). The growth inhibitory effect increased with concentrations. The effective concentrations that cause 50% of root length as compared to control (EC50) were determined.

We observed differences in EC50 values of compounds. EC50 values of β-iminophosphonates and β-aminophosphonate **2a** were in the range of 340–397 µg/ml. EC50 value of β-aminophosphonate **2c** was much lower, (140 µg/ml). Root elongation is a macroscopic effect which reflects different mechanisms of cytotoxic influence (Olaru *et al*., 2019). This finding could explain the differences between the degrees of the negative influence of phosphonates tested.

Allium cepa test

Table 3 presents the effect of phosphonates tested on the rate of cell division in root meristematic cells of *A. cepa*. We chose to test the effect of compounds at concentrations lower than estimated EC50 values. The results revealed the inhibition of cell proliferation upon treatment with two compounds, **4c** and **4e**. Upon treatment with compound **4c**, the mitotic index was decreased by about twofold ($p \leq 0.05$) in comparison with the negative control. The cytostatic effect of compound **4e** was much stronger; cell division was completely inhibited. Çelik and Aslantürk (2010) summarized data about reduction of mitotic index, lethal effects if the decrease in the mitotic index is below 22% of the control and sublethal effects in case of a decrease below 50%. The cytostatic effect can be due to numerous molecular events, including a blockage of specific cell cycle proteins, inhibition of DNA synthesis, or cell metabolism alterations (El-Ghamery *et al*., 2000; Karaismailoglu, 2017; Kumari *et al*., 2011; Liman *et al*., 2019; Recep, 2020).

Disturbance of cell cycle kinetics also serves as an indicator for cytotoxicity (Amin, 2002; Liman *et al*., 2012). We observed a notable change of phase indices value after treatment with compounds **2a** and **2c**. Treatment with compound **2a** significantly decreased the percentage of metaphase and increased to some extent the percentage of the prophase. This

Table 2. Growth inhibition of *Triticum aestivum* roots exposed to different concentrations of compounds for 96 hours.

Sample	Dose (µg/ml)	mean	±SD	%	EC50(µg/ml)
Control-1	0	75.29	12.40	100.00	
4a	100	57.62	12.28	76.53*	349
	200	45.79	10.56	60.82*	
	400	34.56	9.16	45.90*	
	600	26.72	5.30	35.49*	
	800	22.57	4.16	29.98*	
	1,000	20.75	4.45	27.57*	
4c	100	54.69	11.11	72.63*	327
	200	39.40	9.07	52.32*	
	400	29.89	5.89	39.70*	
	600	24.61	4.84	32.69*	
	800	21.16	4.21	28.11*	
	1,000	15.44	3.18	20.51*	
4e	200	57.81	12.24	76.79*	397
	400	33.33	6.11	44.27*	
	600	26.67	5.97	35.43*	
	800	21.05	4.68	27.96*	
	1,000	16.99	4.88	22.57*	
Control-2	0.40	67.00	14.50	100.00	
2a	200	45.59	10.92	68.04*	340
	400	28.02	5.07	41.82*	
	600	23.63	3.93	35.27*	
	800	19.27	4.70	28.76*	
	1,000	17.14	4.25	25.59*	
2c	50	58.72	11.87	87.64	140
	100	40.42	7.98	60.33*	
	200	16.57	2.85	24.73*	
	400	13.62	2.68	20.33*	
	500	11.57	2.72	17.27*	
	600	10.26	1.98	15.31*	
	800	8.93	1.79	13.33*	
	1,000	7.32	1.56	10.92*	

Control 1: distilled water.
Control 2: distilled water with methanol.
EC50 =: half maximal effective concentration; data are expressed as means ± standard deviation (mean ± SD).
*$p \leq 0.05$.

probably is due to a blockage of cell division at the end of the prophase (El-Ghamery *et al*., 2000). The treatment with compound **2c** caused a significant decrease in the percentage of telophase.

Reduction in the mitotic index and the changes of the mitotic phase frequencies are used as signs for negative action on cell division. *Allium cepa* is considered one of the most sensitive plant systems to determine the cytotoxic effects of a large number of compounds (Casillas-Figueroa *et al*., 2020).

In vitro cytotoxicity assay

In the present study, the influence of newly synthesized phosphonates on human fibrosarcoma cell line HT 1080 proliferation/viability was evaluated. Colorimetric assay based on the reduction of a tetrazolium salt to formazan by mitochondrial enzyme was used. The results are summarized in Table 4. Cell viability after compounds treatment are similar to viability of untreated control. Only β-iminophosphonate **4e** exerted a significant negative impact on HT 1080 cells.

Effects on nuclear morphology

Chemotherapeutic agents are used as tools that promote the death of cancer cells. The number of cancer cells can be reduced by direct toxicity or by induction of apoptosis (Gerl and Vaux, 2005). Cell-based drug screens based on viability assays, such as WST-1, evaluate growth inhibition. It is known that tetrazolium dye reduction assays measure mitochondrial dehydrogenase activity. But, as pointed out by Eastman (2017), rapid change of enzyme activity could not correlate with the cell number. Morphological alterations of cell nucleus are microscopic changes used as marker signs of apoptosis (da Mota *et al*., 2012; Mahdi *et al*., 2015).

The ability of studied phosphonates to induce apoptosis was assessed by evaluation of nuclear morphology changes in cancer cells stained with Giemsa (Table 5). Three stages of the nucleus at the early stage of apoptosis were established, karyolysis, pyknosis, and karyorrhexis. Only β-iminophosphonate **4e** exerted a significant effect; in 65.61% of fibroblastoma cells were observed signs of early apoptosis, mainly due to karyolysis.

It should be noted that pyknosis was established after treatment with all phosphonates, but also in negative and positive control. Some cells revealed also a sign of karyorrhexis. Probably, alterations of a cancer cell's nucleus could be a reason for these results (Fischer, 2020).

The present study confirmed numerous data that *Triticum* and *Allium* tests represent useful tools if coupled with other cytotoxicity tests (Olaru *et al*., 2019). For example, compound **4c** exerted a negative effect on plant cell division, but *in vitro* test had no significant influence on cell proliferation/viability. Moreover, our results are in accordance with the statement of Eastman (2017) that declining cell vitality does not always lead to cell death; compound **4e** inhibited cell vitality by 97.76%, but apoptosis is initiated in only 65.61% of fibroblastoma cells.

Discovery of novel anticancer drugs depends on the synthesis and testing of new compounds with diverse chemical structure. So, anticancer properties and the structure–activity relationships should be established. For example, in a recent study on the biological activity of newly synthesized bicyclic α-iminophosphonates, none of the compounds exerted any cytotoxicity (Abás *et al*., 2020). Wermuth *et al*. (1998) define the pharmacophore as "an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response." In the present study, only β-iminophosphonate **4e** revealed significant antiproliferative and cytotoxic activity. One of the structural differences between compounds **4a**, **4c**, and **4e** is the substituents at phosphorus atom: two methoxy groups (compound **4a**), methoxy and diethylamino

Table 4. Effect of treatment with phosphonates tested (for 48 hours) on human fibrosarcoma cell line HT 1080-WST-1 cell proliferation/viability assay.

Sample	Dose (µg/ml)	Absorbtion		cell viability (%)
		mean	±SD	
Control-1		1.907	±0.259	100.00
4a	246	1.876	±0.228	98.34
4c	168	1.778	±0.170	93.22
4e	300	0.043	±0.010	2.24*
Control-2	0.26	1.903	±0.303	100.00
2a	240	1.912	±0.263	100.44
2c	92	1.852	±0.240	97.30

Control 1: complete growth medium.
Control 2: complete growth medium with methanol; date are expressed as the mean ± SD
SD – standard deviation.*$p \leq 0.05$.

Table 3. Effect of treatment with phosphonates tested (for 48 hours) on mitotic index and phase indices in root tip meristematic cells of *Allium cepa* L.

Sample	Dose µg/ml	Mitotic index (% ± SD)	Prophase index (% ± SD)	Methaphase index (% ± SD)	Anaphase index (% ± SD)	Telophase index (% ± SD)
Control-1	0	5.24 ± 0.87	46.47 ± 8.70	25.46 ± 5.84	10.89 ± 3.13	17.17 ± 5.90
4a	246	5.15 ± 0.84	50.79 ± 8.42	26.32 ± 3.64	10.48 ± 5.39	12.41 ± 4.39
4c	168	3.01 ± 0.18*	39.83 ± 6.16	26.36 ± 6.03	11.82 ± 2.58	21.99 ± 5.39
4e	300	-	-	-	-	-
Control-2	0.40	5.52 ± 1.31	45.93 ± 9.74	26.26 ± 7.26	11.90 ± 5.02	15.91 ± 4.12
2a	240	4.9 ± 1.81	55.77 ± 12.40	16.97 ± 6.53*	13.30 ± 4.74	13.96 ± 3.71
2c	92	4.48 ± 0.93	47.91 ± 6.97	28.07 ± 6.77	14.35 ± 5.82	9.68 ± 5.07*

Control 1: distilled water.
Control 2: distilled water with methanol; date are expressed as the mean ± SD.
SD = standard deviation.
*$p \leq 0.05$.

Table 5. Effect of treatment with phosphonates tested (for 48 hours) on human fibrosarcoma cell line HT 1080-microscopic marker signs of early apoptosis.

Sample	Dose (µg/ml)	Analyzedcells	Nuclear morphology changes			Total
			Pyknosis	Karyorrhexis	Karyolysis	(% ± SD)
Control-1		613	26	6	0	5.22 ± 1.03
4a	246	604	17	1	4	3.64 ± 0.85*
4c	168	611	26	1	1	4.58 ± 0.97
4e	300	602	30	4	361	65.61 ± 11.47*
Control-2	0.26	613	7	1	1	1.47 ± 0.26
2a	240	704	11	0	0	1.56 ± 0.29
2c	92	665	17	1	0	2.71 ± 0.51*
PC	50	639	21	0	3	3.76 ± 0.19

Control 1: complete growth medium; Control 2: complete growth medium with methanol; PC =: positive control (5-fluorouracil); date are expressed as the mean ± SD; SD – standard deviation.
$^{*}p \leq 0.05$.

groups (compound **4c**), and two phenyl groups (compound **4e**). Replacement of the one CH_3O group in compound **4a** with one $(C_2H_5)_2N$ group to the phosphorus atom in compound **4c** did not increase the anticancer activity of the compound. Hence, it could be speculated that the presence of two phenyl groups at phosphorus in the molecule of compound **4e** is connected with its biological activity. Among the tested compounds, only **4e** has two aromatic rings in its molecule and 2-aminopyridine moiety as well.

Particularly, aromatic rings are extensively used in drugs due to their well-known synthetic and modification paths (Aldeghi *et al*., 2014). For example, at least, one aromatic ring can be found in 99% of a database containing more than 3.500 evaluated by the medicinal chemistry department of Pfizer, AstraZeneca, and GlaxoSmithKlin (Roughley and Jordan, 2011). Still, little is known about their chemical features in biological solution, such as H-bonds availability, the lifetime of H-bonds, solvent accessibility, and conformational ensemble. In this sense, molecular dynamic simulations can provide useful information with atomistic resolution and access the aforementioned features of chemical groups in water, providing fundamental data to drive medicinal chemistry approaches. It is also well known that simple or complex structures with grafted moiety 2-aminopyridine are effective as antitumoral, anti-Alzheimer, antidiabetic, antimicrobial, antiviral, analgesic, anti-inflammatory, antiparasitic, antimalarial, antihistaminic, anticonvulsant, Renin inhibitors, n-NOS-inhibitors, CXCR1/2 inhibitors, JNK1 inhibitors, PKC inhibitors, and Syk-inhibitors and also with cardiac activity (Marinescu, 2017).

The present study contributes to limited data about the synthesis and bioactivity of β-aminophosphonates and β-iminophosphonates. Results confirmed the well-known relationship between the chemical structure and biological activity (El-Saidia *et al*., 2020; El-Sayed *et al*., 2019; Lichota and Gwozdzinski, 2018; Mohamed *et al*., 2010). Established influence on cell proliferation of some of novel β-amino- and β-iminophosphonates revealed a possibility to discover *new* anticancer drugs.

CONCLUSION

The results reported here show once again phosphonates as versatile substrates in synthetic organic chemistry, especially as fruitful starting compounds for the achievement of molecular diversity.

Among the compounds tested, β-iminophosphonate 3-Methyl-2- (pyridin-2-ylimino)butyl)diphenylphosphine oxide (4e) showed anticancer activity against human fibroblastoma cell line HT 1080.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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