Abbod *et al. Chem. Biol. Technol. Agric.* (2021) 8:48
https://doi.org/10.1186/s40538-021-00246-3

Chemical and Biological Technologies in Agriculture

RESEARCH Open Access

A study of fungicidal and anti-phenol oxidase activity of some α-amino phosphonate derivatives

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Abstract

Background: Developing new pesticides with multi-function may be a suitable strategy to save time and cost and reduce the emergence of resistant strains of pests and pathogens. The organophosphorus derivatives have not been widely used in agriculture as fungicides. In this work, a series of six α-amino phosphonate derivatives were prepared and tested for their fungicidal and anti-phenol oxidase activities.

Results: The prepared compounds revealed a promising anti-fungal activity against *Macrophomina phaseolina* and *Pythium aphanidermatum*, especially **M4**, with MIC of 62 mg/L for *M. phaseolina*. **M4** did not affect the fungus permeability rate of the cell membrane; however, it displayed a significant efficiency on mycelial soluble protein content. **M4** and **M3** with a hydroxyl group on the aniline moiety exhibited an observed anti-phenol oxidase activity. **M4** inhibited the enzyme at 1 mg/mL. The DFT theoretical study revealed a significant correlation between the substituents of aniline moiety and the bioactivity of the studied compounds. The negative charge conspicuously influenced the anti-phenol oxidase activity.

Conclusions: Our findings suggest the studied compounds as bases to design more effective α-amino phosphonate fungicides with additional anti-phenol oxidase activity.

Keywords: Organophosphorus, Phenol oxidase, DFT, *Macrophomina phaseolina*, *Pythium aphanidermatum*

Background

There is a vast amount of literature that refers to the high expense and time consumption of developing a new antifungal agent [1–3]. The primary defect in using inorganic compounds as fungicides is that they entail a slow disintegration and toxic residues [4]. Historically, most organophosphorus derivatives have been limited to use in agriculture as insecticides, and nowadays, increasing attention is paid to develop new organophosphorus derivatives as fungicides [4]. Cerezin, Kitazin, and Pyrazophos are examples of the few organophosphorus compounds which are used as fungicides [4].

Phenol oxidase (PO) interferes with several biological pathways in insects like melanization and sclerotization cascades [5–9]; also, it is considered a critical defense tool against pathogens attack [6]. Therefore, inhibition of PO may serve as a good strategy for insects control by making their immune system feeble against pathogens [10].

Several studies have been carried out on the biological activity of α-amino phosphonate derivatives [11–13]. The similarity between α-amino-phosphonic acid and its ester derivatives with natural amino acids has made it possible to use them as drugs to inhibit the activity of some enzymes [14, 15].

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The present paper aims to introduce a series of α-amino phosphonate derivatives with both antifungal and antiphenol oxidase activity. The fungicidal activity was assayed against two important plant pathogens belong to true fungi and Oomycetes (*Macrophomina phaseolina* and *Pythium aphanidermatum*). Also, the PO inhibitory activity was investigated against the *Galleria mellonella* PO enzyme. The density functional theory calculations were used to explain their bioactivities.

Materials and methods

Chemicals

All chemicals and solvents were purchased from Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany). Thiophanate-methyl 70% WP fungicide was obtained from commercial sources.

General procedures for synthesis α-amino phosphonate derivative

Kabachnike Fields reaction and Pudovik reaction are the most versatile methods for preparing α-amino phosphonate derivative; these methods are considered useful pathways for prepare the construction of P–C–N bonds [16, 17]. The first step of our procedures was refluxing equimolar amounts (10 mmol) of benzaldehyde and aniline derivatives at 70 °C for 8 h in the presence of tetrahydrofuran (THF). After the reacting mixture was cooled to room temperature, the diethyl phosphite (10 mmol) was added to the prepared imine and continue to reflux the mixture for another 18–24 h to obtain the final compound. The precipitate was filtered, and after evaporating the solvent the product was washed with water (Fig. 1). All synthesized compounds were elucidated based on the IR and NMR (^{1}H, ^{13}C, and ^{31}P) spectroscopy.

IR spectra (KBr pellets) were obtained with a Shimadzu, IR-60 model spectrometer. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker (Avance DRS) 500 MHz spectrometer and chemical shifts were determined relative to TMS and 85% H_3PO_4, respectively, as external standards. Elemental analysis was performed on a Flash EA 1112 Thermo Finnigan instrument. Melting points were determined on an Electrothermal IA 9100 digital melting point apparatus.

M1; Diethyl(2-chlorophenyl)(2-chlorophenylamino) methylphosphonate

Mp: 110–112 °C. Light yellow powder, ^{1}H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.25 (m, 6H, CH_3), 4.10 (m, 4H, CH_2), 6.96–8.44 (m, 8H, Ph), 8.23 (m, 1 H, CH-P), 10.40 (N–H). ^{13}C NMR (125.77 MHz, d-DMSO, ppm)): δ = 29.7 (s, 2 C, CH_3), 46.4 (d, 2 C, CH_2), 61.5 (s, 1C, CH-P), 113–135.1 (C_{Ph}), 152.7 ($C_{ipso\text{-}CH}$), 158.6 ($C_{ipso\text{-}NH}$), 189. (C_{Cl}). ^{31}P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.29 ppm. IR data (KBr, cm^{-1}): 3437 (υ_{N-H}); 3059 w (CH_{Ar}); 2922 s (CH_{Aliph}); 1615 s (υ_{Ar}); 1459 s (υ_{Ar}); 1266 s ($\upsilon_{P=O}$); 1043 s (υ_{P-O}). Anal. calcd. for $C_{17}H_{20}Cl_2NO_3P$: C, 52.59; H, 5.19; N, 3.61%. Found: C, 52.83; H, 5.42; N, 3.37%.

M2; Diethyl(2,4-dichlorophenyl)(2,4-dichlorophenylamino) methylphosphonate

Mp: 130–133 °C. Light yellow powder. ^{1}H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.24 (m, 6H, CH_3), 4.01 (m, 4H, CH_2), 6.59–7.78 (m, 6H, Ph), 8.13 (m, 1 H,

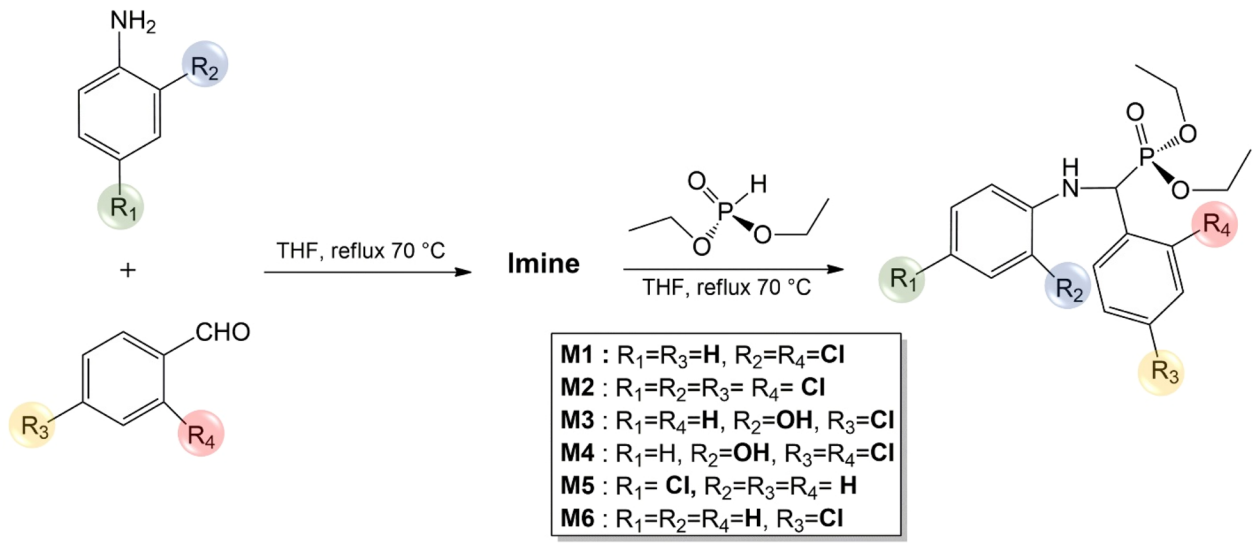

Fig. 1 The synthesis process of target compounds **M1–M6**

CH-P), 10.31 (N–H). ^{13}C NMR (125.77 MHz, d-DMSO, ppm)): δ = 29.7 (s, 2 C, CH_3), 46.4 (s, 2 C, CH_2), 61.5 (m, 1 C, CH-P), 113–135.5 (C_{ph}), 157.7 ($C_{ipso-CH}$), 158.6 ($C_{ipso-NH}$), 191.5 (C_{Cl}). ^{31}P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.34 s (d, $^2J_{P-H}$ = 11.9 Hz), ppm. IR data (KBr, cm^{-1}): 3339 (m υ_{N-H}); 2982 s (CH_{Ar}); 2922 s (CH_{Aliph}); 1617; 1583 (υ_{Ar}); 1259 s ($\upsilon_{P=O}$); 1037 s (υ_{P-O}). Anal. calcd. for $C_{17}H_{18}Cl_4NO_3P$: C, 44.67; H, 3.97; N, 3.06%. Found: C, 44.35; H, 3.84; N, 3.17%

M3; Diethyl(4-chlorophenyl)(2-hydroxyophenylamino) methylphosphonate

Mp: 102–106 °C. Brownish-yellow powder. ^{1}H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.34(m, 6H, CH_3), 4.12(m, 4H, CH_2), 6.43–7.46 (s, 1H, OH), 0.6.43–8.64 (m, 8H, ph), 8.03 (m, 1H, CH-P), 9.97 (N–H). ^{13}C NMR (125.77 MHz, d-DMSO, ppm)): δ = 39.9 (s, CH_3), 45.5 (s, CH_2), 64.9 (m, 1 C, CH-P), 115.6–138 (C_{Ph}), 158.6 ($C_{ipso-CH}$), 156.1($C_{ipso-NH}$), 173.3 (C_{OH}), 190.9 (C_{Cl}). ^{31}P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.136 s (d, $^2J_{P-H}$ = 11.8 Hz), ppm. IR data (KBr, cm^{-1}): 3379b (υ_{O-H}); 3304 m (υ_{N-H}); 3042 w (CH_{Ar}); 2925 w (CH_{Aliph}); 1623 s;1582 s (υ_{Ar}); 1230 s; 1196 s ($\upsilon_{P=O}$); 1084 s (υ_{P-O}). Anal. calcd. for $C_{17}H_{21}ClNO_4P$: C, 55.22; H, 5.72; N, 3.79%. Found: C, 55.34; H, 5.80; N, 3.88%.

M4; Diethyl(2,4-dichlorophenyl)(2-hydroxyophenylamino) methylphosphonate

Mp: 115–117 °C. Yellow powder. ^{1}H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.38 (m, 6H, CH_3), 4.14(m, 4H, CH_2), 6.92 (s,1H, OH), 8.21 (1H, CH-P), 7.21–7.88 (m, 7H, ph), 9.10 (N–H), ^{13}C NMR (125.77 MHz, d-DMSO, ppm)): δ = 39.8 (s, 2 C, CH_3), 46.5 (s,2 C, CH_2), 64.5 (m, 1 C, CH-P), 115.3–138.1 (C_{ph}), 151.9 ($C_{ipso-CH}$), 155.1($C_{ipso-NH}$), 173.0 (C_{OH}), 190.9 (C_{Cl}). ^{31}P NMR (202.46 MHz, d-DMSO, ppm): δ 0.29 s (d, $^2J_{P-H}$ = 9.8 Hz), ppm. IR data (KBr, cm^{-1}): 3461 m (υ_{O-H}); 3339 m (υ_{N-H}); 2983:2920 m (CH_{Ar}); 3076 w (CH_{Aliph}); 1475 s;1616 s (υ_{Ar}); 1258 s ($\upsilon_{P=O}$); 1095 s (υ_{P-O}). 1475: 1616 s ($_{C=C}$). Anal. calcd. for $C_{17}H_{20}Cl_2NO_4P$: C, 50.51; H, 4.99; N, 3.47%. Found: C, 50.78; H, 4.85; N, 3.36%

M5; Diethyl(phenyl)(4-chlorophenylamino) methylphosphonate

Mp: 84–87 °C. Dark yellow powder. ^{1}H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.24 (m, 6H, CH_3), 4.01(m 4H, CH_2), 6.64, 7.75 (m, 9H, Ph), 8.03 (m, 1H, CH-P), 8.53 (N–H). ^{13}C NMR (125.77 MHz, d-DMSO, ppm)): δ = 22.7 (s, 2 C, CH_3), 29.72 (s,2 C, CH_2), 77.04 (m, 1 C, CH-P), 120.8–137.3 (C_{ph}), 151.68 ($C_{ipso-CH}$), 158.8 ($C_{ipso-NH}$), 191.8 (C_{Cl}). ^{31}P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.28 s (d, $^2J_{P-H}$ = 13.1 Hz), ppm. IR data (KBr, cm^{-1}): 3330b (υ_{N-H}); 3266b (CH_{Ar}); 3202 (CH_{Aliph}); 1610 s–1630 (υ_{Ar}); 1201 m ($\upsilon_{P=O}$); 1088 m (υ_{P-O}). Anal. calcd. for $C_{17}H_{21}ClNO_3P$: C, 57.71; H, 5.98; N, 3.96%. Found: C, 57.59; H, 5.85; N, 3.98%

M6; Diethyl(4-chlorophenyl)(phenylamino) methylphosphonate

Mp: 87–90 °C. Yellow powder.^{1}H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.37 (m, 6H, CH_3), 4.16(m, 4H, CH_2),7.10–7.79 (m, 9H, ph), 8.11 (1H, CH-P), 8.45 (N–H). ^{13}C NMR (125.77 MHz, d-DMSO, ppm)): δ = 16.11 (s, 2 C, CH_3), 30.3 (s 2 C, CH_2), 63.6 (m, 1 C, CH-P), 116.23–150.5 (C_{ph}), 151.9 ($C_{ipso-CH}$), 156.1($C_{ipso-NH}$), 160.72 (C_{Cl}). ^{31}P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.30 (d, $^2J_{P-H}$ = 12.8 Hz), ppm. IR data (KBr, cm^{-1}): 3376b; (υ_{N-H}); 2978 w (CH_{Ar}); 2930b (CH_{Aliph}); 1491 m—1626 m (υ_{Ar}); 1245 s ($\upsilon_{P=O}$); 1084 s (υ_{P-O}).

Fungal and oomycete strains

We used *Macrophomina phaseolina* Mph44, which was isolated and identified previously, and its high virulence was confirmed [18]. It was originally obtained from melons with charcoal rot disease in Khorasan province. *Pythium aphanidermatum* 8P isolate was used due to its high pathogenicity [19]. It was isolated from sugar beet fields of west Azarbaijan province, Iran [19].

Fungicidal activity study

Mycelial growth and microsclerotia production inhibition assays

To investigate the fungicidal activity of the synthesized compounds, increasing concentrations were tested against the fungus using the poison food technique [20]. Briefly, different concentrations of the compounds were added to Petri plates containing a Potato Dextrose Agar (PDA) medium, then a mycelial disc (6 mm diameter, 3 to 5 days old) of the fungus placed in the center of the plate, and incubated at 27 °C for 4 days. The mycelial growth inhibition was calculated using Eq. (1) [21, 22].

$$\text{Mycelial growth inhibition (MGI)\%} = \left[\frac{C - T}{C}\right] \times 100 \quad (1)$$

In which *C* is the diameter (mm) of the fungal colony in control, and *T* is the diameter (mm) of the fungal colony in the presence of the tested compound. Thiophanate-methyl 70% WP was used as a positive control.

Cell membrane relative permeability rate

The relative permeability rate of the cell membrane was evaluated using the procedures of Kobno et al. [23] with some modifications. Five mycelial discs (7 days old) of the fungus were incubated into Czapek-Dox Broth medium for 5 days. Then the harvested mycelia were washed with

double distilled water and 0.5 g of the mycelia was placed in a 50 mL tube containing 150 µg/mL of each tested compound. The conductivities were sequentially measured at 0 (J_0), 5, 10, 30, 60, 180, 360 min (J_1). After boiling which was followed by cooling, the conductivity (J_2) was measured. The permeability ($P\%$) were calculated by the formula: $P\% = [(J_1\text{-}J_0)/(J_2\text{-}J_0)] \times 100$ [23].

Preparing of the mycelium crude extract

The mycelium crude extract was prepared using the procedures of Wu et al. (2005) [24] with some modifications. Five mycelial discs (6 mm diameter, 7 days old) of the fungus were placed in an Erlenmeyer flask containing 90 mL of sterilized Czapek-Dox Broth medium and incubated in a rotary shaker (120 rpm, 27 °C for 15 days), then the tested compound was dripped into the culture medium at a concentration of 100 µg/mL. The mycelium was filtered, collected orderly at 0.5, 1, 3, 6, 12, and 24 h and washed by water. Water was soaked with a filter paper, and the dried mycelium was weighed, and preserved at − 20 °C. 0.25 g of the dry mycelium was mixed with Tris–HCl buffer (2 mL, 50 mmol/L, pH = 7) in the mortar, triturated into paste quickly, and then centrifuged at 4 °C and 15,000*g* for 30 min. The clear upper layer was preserved at − 20 °C. Every treatment had three repetitions [24].

Mycelial soluble protein content

The mycelial soluble protein content was evaluated using a modified method of Hatada et al. [25]. 150 µL of the mycelial extract was mixed with Coomassie brilliant blue G-250 solution (3 mL), after 5 min, the absorbance value of the mixture was measured at 595 nm. Tris–HCl buffer served as the control [25].

Anti-phenol oxidase activity

Phenol oxidase inhibitory activity was measured using Ullah et al. [26] method with some modifications. The Haemolymph of the fifth instar larvae of *Galleria mellonella* was collected by placing them at − 20 °C for 10 min. They were surface sterilized with 70% EtOH, cut at the abdominal prolegs, and blended into a pre-chilled sterile polypropylene tube. Then it diluted with 10 mM phosphate-buffered saline solution and kept on ice. Then it centrifuged at 4 °C and 15,000*g* for 15 min. The supernatant was collected and used as an enzyme source. Different concentrations of the tested compounds (50, 250, 500, and 1000 µg/mL dissolved in DMSO) were prepared, then phenol oxidase (PO) inhibition was determined using a L-3,4-dihydroxyphenylalanine (L-DOPA) substrate-based assay carried out in a microplate,100 µL of distilled water, 20 µL PBS buffer, 20 µL of the substrate, 20 µL of the tested compound were added to each well then 20 µL of the enzyme source was added. The absorbance was detected at 490 nm for 30 min [26]. Three replicates were used for each treatment, and Kojic acid was used as a positive control. The tested compounds (50, 250, 500, and 1000 µg/mL dissolved in DMSO) were prepared, then phenol oxidase (PO) inhibition was determined using a L-3,4-dihydroxyphenylalanine (L-DOPA) substrate-based assay carried out in a microplate,100 µL of distilled water, 20 µL PBS buffer, 20 µL of the substrate, 20 µL of the tested compound were added to each well then 20 µL of the enzyme source was added. The absorbance was detected at 490 nm for 30 min [26]. Three replicates were used for each treatment, and Kojic acid was used as a positive control.

Density functional theory (DFT) analysis

All the calculations were carried out using density functional theory (DFT) as implemented in the Gaussian 09 package [27] Geometries were optimized using the B3LYP functional with the 6-31G** basis set. The energies were reevaluated by additional single point calculations at each optimized geometry using the 6-311G** ++ basis set. [27–30]. The natural population was calculated using the NBO program (NBO Version 3.1) [31].

Statistical analysis

The experiments were carried out in a completely random design with three replications. Data were analyzed using analysis of variance (ANOVA), and mean comparison was conducted by the least significant difference (LSD) using SAS software [32] using a personal computer. The results are presented as means and their standard errors (SE).

Results and discussion

Anti-fungal activity

The synthesized compounds were evaluated for their fungicidal activity against two important plant pathogens, including *Macrophomina phaseolina* from the true fungi and *Pythium aphanidermatum* from the Oomycetes. The true fungi and Oomycetes differ from each other in several points [1]. Thus, using these pathogens in our assays may help to have a clear view of how these compounds can affect the selected pathogens.

The compounds were tested at concentrations up to 400 mg/L. **M1** and **M2** showed significant activity against both pathogens at all of the tested concentrations. **M4** and **M3** exhibited full inhibition of *M. phaseolina* at concentrations from 400 to 100 mg/L. Moreover, **M4** inhibited the growth of *P. aphanidermatum* at any of the tested concentrations; thus, we have tested these compounds at lower doses (Table 1).

Table 1 Growth inhibition values of the tested compounds

Plant pathogens	Concentration (mg/L)	M1	M2	M3	M4	M5	M6
M. phaseolina	400	100 ± 0*	100 ± 0	100 ± 0	100 ± 0	42 ± 2.36	34 ± 2.08
	300	94 ± 1.52	96 ± 1	100 ± 0	100 ± 0	0	0
	200	90 ± 1	95 ± 1.5	100 ± 0	100 ± 0	0	–
	150	85 ± 3.06	91 ± 1.32	96 ± 2.64	100 ± 0	–	–
	100	62.5 ± 2.3	82.5 ± 1.44	90 ± 1.3	100 ± 0	–	–
	50	–	–	55 ± 3.05	90 ± 2	–	–
	25	–	–	–	63 ± 1.15	–	–
P. aphanidermatum	400	100 ± 0	100 ± 0	100 ± 0	100 ± 0	90 ± 2.51	0
	300	90 ± 1.73	100 ± 0	100 ± 0	100 ± 0	0	0
	200	67.5 ± 1.32	95 ± 1	100 ± 0	100 ± 0	0	–
	100	23 ± 1.52	93 ± 0.76	35 ± 4.04	100 ± 0	–	–
	50	–	–	0	25 ± 1.81	–	–
	25	–	–	–	20 ± 1.52	–	–

*Inhibition was measured experimentally (mean ± SE), replicate number $n = 3$

–: The compound was tested at this concentration

The MIC of **M4** was about 62 mg/L for *M. phaseolina* and less than 100 mg/L for *P. aphanidermatum*, while **M3** had MICs about 175 and 200 mg/L. **M5** displayed 90% ± 2.51 inhibition of *P. aphanidermatum* at 400 mg/L, while **M6** showed weaker activity against the pathogens compared to other compounds (Table 1). As can be noted from Table 1, there are no significant differences in the fungicidal activity on both *M. phaseolina* and *P. aphanidermatum*. These findings suggest that the tested compounds may interfere with the same targets within the pathogens or might have multiple modes of action. Consequently, future studies are required to understand their mode of action on both True fungi and Oomycetes. All tested compounds showed a level of fungicidal activity against *M. phaseolina*; thus, we studied the impact of different R-substituents on their activity. Figure 1 and Table 1 indicate that all compounds having substituents on both rings (i.e., **M1**, **M2**, **M3**, and **M4**) showed higher activity. In contrast, the compounds contain only one substituent displayed a weak comparative activity, which implies that both rings and their substituents are essential to improve their fungicidal activity. **M5** with a Cl-substituent on the aniline moiety showed higher activity than **M6** with Cl-substituent on the phenyl moiety. Additionally, when OH-substituent replaces the Cl-substituent on the aniline moiety as in **M3** and **M4**, we observed a significant increase in the fungicidal activity. Furthermore, having a hydroxyl group on the aniline increased the fungicidal activity more than chlorines. It may suggest that the aniline ring and its substituents have a higher impact on the fungicidal activity than phenyl ring substituents.

The effect on the relative permeability rate of the cell membrane

The relative permeability rate of the cell membrane of *M. phaseolina* was detected in the presence and absence of **M2** and **M4** to evaluate the cytotoxic impact of synthesized derivatives at 150 µg/mL. In the case of **M2**, the relative permeability rate of the cell membrane was higher than the control in the first 100 min; however, it was found to be lower than control with a longer treatment time. In contrast, when fungus treated with **M4**, the relative permeability rate of the cell membrane was always lower than the control; moreover, within 300 min, it was less than **M2**-treated fungus and differed after that. The relative permeability rate of thiophanate methyl-treated fungus was always higher than control and other treatments (Fig. 2). It seems that the cell membrane was not affected by **M2** and **M4** compounds, which may decrease the ability of these molecules to inhibit the fungus growth by interfering with the cell membrane.

The effect on mycelial soluble protein content

The soluble protein content was detected in the presence and absence of the **M4** compound at 100 µg/mL. Figure 3 displayed that within an hour, the soluble protein content was higher than control, then it dropped in a time-dependent manner. It can be seen that the soluble protein content was 19.7 ± 0.68% and 25.91% ± 1.89 lower than the control in 12 and 24 h, respectively; moreover, it was lower than thiophanate methyl-treated fungus after

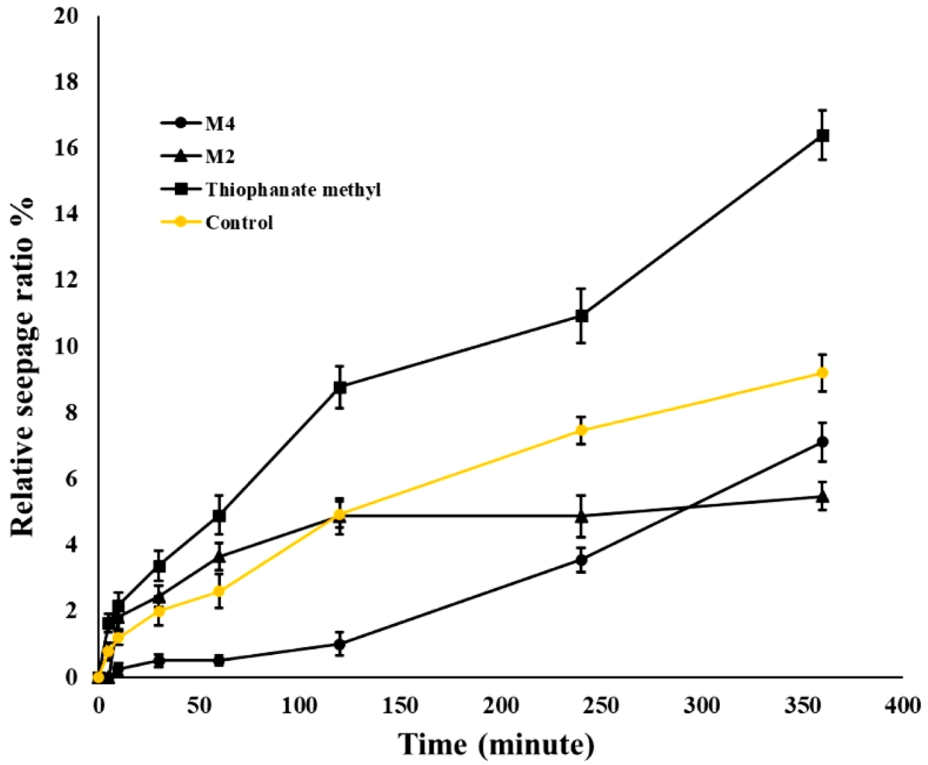

Fig. 2 The effect of compounds **M2** and **M4** on membrane permeability of *M. phaseolina* at 150 µg/mL. The results were compared with control (without compound) at the corresponding period. The error bars represent the mean ± SE of the three repeats ($P < 0.05$)

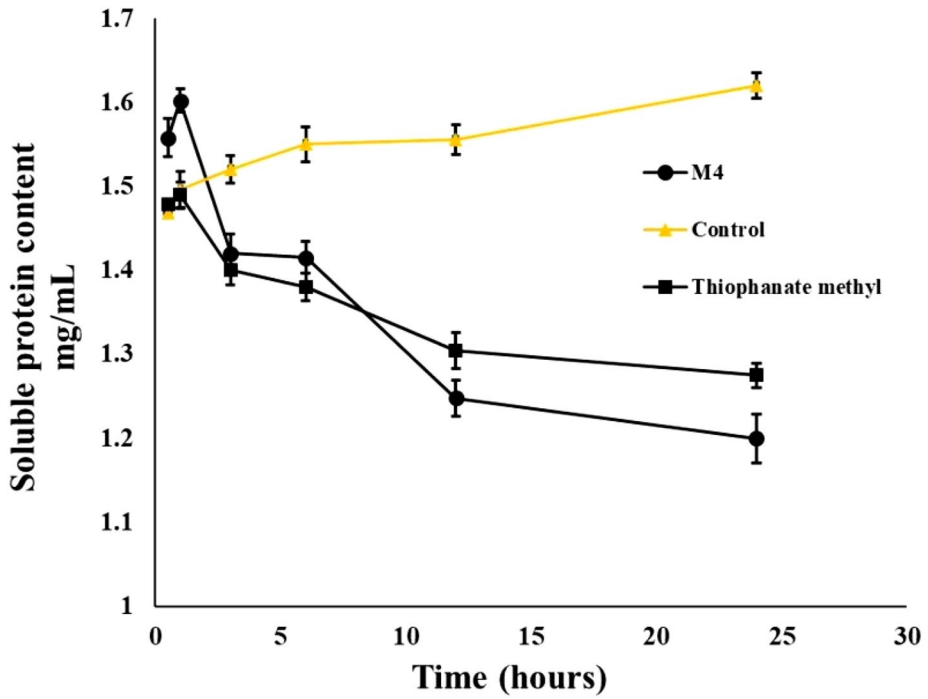

Fig. 3 The effect of compound **M4** on the mycelial soluble protein content of *M. phaseolina* at 100 µg/mL as compared to control (without compound) at the corresponding period. Thiophanate-methyl 70% WP was used as positive control. The error bars represent the mean ± SE of the three repeats ($P < 0.05$)

8 h. These results suggest that **M4** may reduce pathogen growth by inhibiting protein synthesis.

Anti-phenol oxidase activity

The activity of synthesized compounds on the insects immune-associated characteristics was tested on the *G. mellonella* phenol oxidase enzyme. Both **M4** and **M3** revealed phenol oxidase inhibitory activity more than kojic acid (Figs. 4, 5). **M4** inhibited the enzyme activity by 100% at 1000 µg/mL; also, it showed 86% ± 1.73 and 73.67% ± 2.02 of enzyme inhibition at 500 and 250 µg/mL, respectively. **M3** displayed 34.3% ± 1.2 to

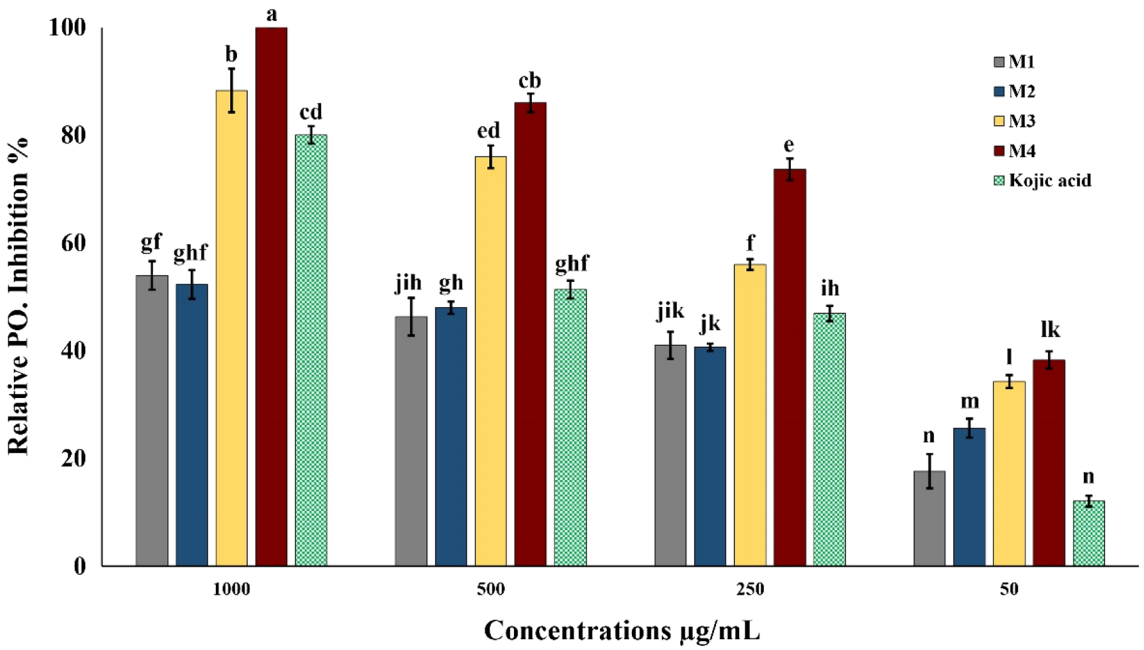

Fig. 4 Phenol oxidase inhibition by increasing concentrations of tested compounds. The treatments compression was done using LSD test based on completely random design. Values are averages of three replications ($n = 3$). The error bars represent the mean ± SE of the three repeats

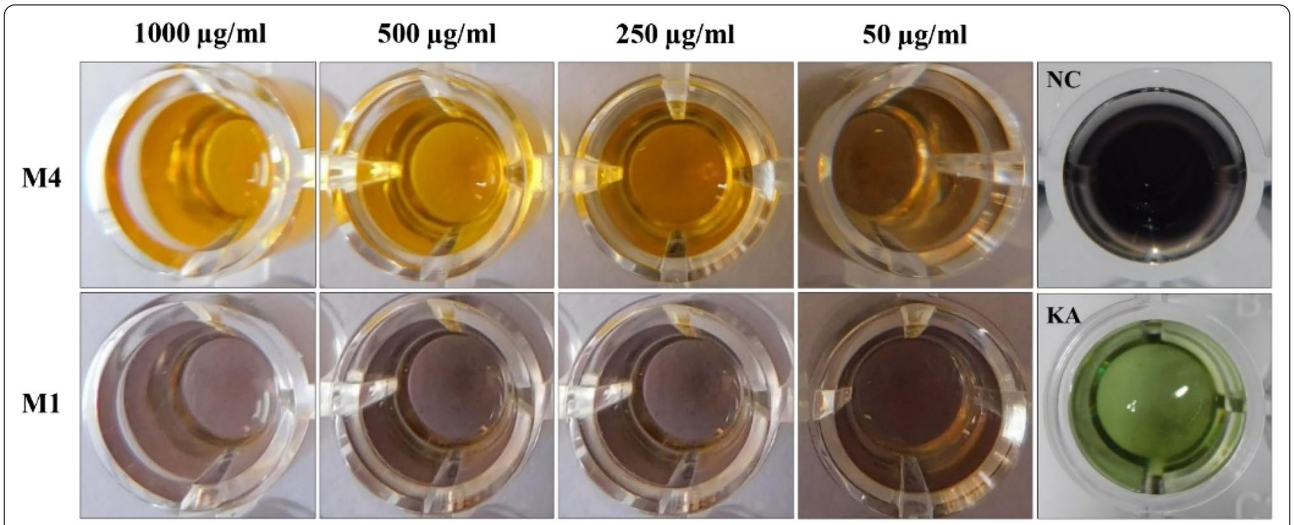

Fig. 5 Phenol oxidase inhibitory activity of **M1** and **M4**. The black color indicates the interaction between the enzyme and the substrate in **NC** (the negative control without compound). **M4** inhibited the enzyme activity in a concentration-dependent manner, **M1** also showed moderate inhibitory activity, (**KA** = kojic acid at 1000 µg/mL)

88.3% ± 4.05 of enzyme inhibition at 50 to 1000 µg/mL. **M1** and **M2** without OH-substituent on the aniline moiety were less capable than **M3** and **M4**. **M1** exhibited 54% ± 2.65 to 17.67% ± 3.17 inhibition at 1000 to 50 µg/mL, respectively, while **M2** did not exceed 52.3% ± 2.67 at the highest dose (Fig. 4). The anti-phenol oxidase activity was not significantly affected whether the molecule has one or two Cl-substituents on the phenyl ring; however, it increased considerably by replacing the Cl-substituent with OH-substituent on the aniline moiety. These findings indicate the crucial role of the hydroxyl group in the phenol oxidase inhibitory activity of tested compounds. Phenol oxidase interferes with several biological pathways in insects like melanization and sclerotization cascades [5–9]. By the inhibition of phenol oxidase, our compounds may interfere with the insects immune system and block the melanization process which leads to the weakness of insects defense [5–9].

DFT calculation

We performed DFT calculations **M1–M4**, **M6**, and the phenol oxidase substrate L-DOPA. The plots of the frontier orbitals, their calculated energy, and their natural atomic charge distribution are mentioned in Fig. 6 and Table 2. The Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) are helpful tools for evaluating the molecules' bioactivity [32–36]. They refer to the parts of the molecules that can interact with the receptors [37]. Figure 6 showed that the HOMO orbital delocalized on the

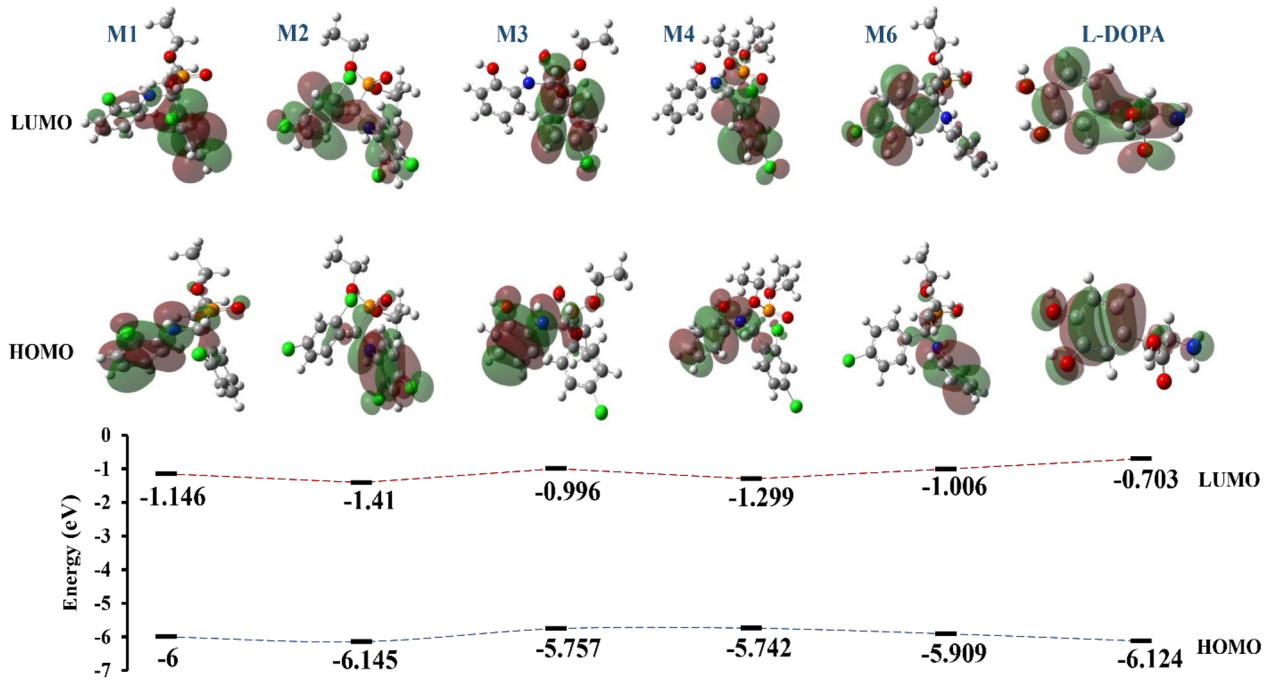

Fig. 6 DFT comparison of **M1–M4** and **M6** and L-DOPA. The DFT calculations were carried out with Gaussian 09 software, HOMO and LUMO maps were extracted from GuassView 6.0 program based on the optimized structures

Table 2 Natural atomic charges of the compounds **M1–M4** calculated by NBO analysis

Name	P	O1	O2	O3	N	R1	R2	R3	R4
M1	2.336	− 1.104	− 0.852	− 0.860	− 0.631	–	Cl (− 0.025)	–	Cl (− 0.008)
M2	2.348	− 1.098	− 0.850	− 0.860	− 0.641	Cl (–0.015)	Cl (− 0.005)	Cl (0.007)	Cl (0.023)
M3	2.318	− 1.101	− 0.853	− 0.865	− 0.639	–	O (− 0.711)	Cl (− 0.018)	–
M4	2.339	− 1.098	− 0.845	− 0.862	− 0.648	–	O (− 0.707)	Cl (0.005)	Cl (0.005)
L-DOPA	–	**− 0.694***	**− 0.715**	–	**− 0.851**	–	–	–	–

*The natural atomic charges of L-DOPA refers to the nitrogen and oxygen atoms of hydroxyls bonded to the ring

aniline moiety and the P–C–N bridge in all the compounds (**M1**–**M4**, and **M6**), which refer to the impact of the aniline moiety on their bioactivity. These findings are in agreement with the experimental results that indicated the importance of the aniline moiety substituents on the fungicidal activity. According to the Klopman-Salem equation [37–40], the interaction between two systems (e.g., enzyme and substrate) can be controlled by two major factors, the charges of the interacted atoms and the delocalization position of the frontier molecular orbitals. Thus, we have studied the delocalization of the frontier molecular orbitals of **M1**–**M4** compounds and compared them with the enzyme–substrate L-DOPA. L-3,4-dihydroxyphenylalanine or L-DOPA acts as a substrate for phenol oxidase enzyme [5, 41, 42]. This molecule interacts with the copper-binding region within the enzyme [42–45]. We have investigated the inhibitory activity of our molecules based on their similarity with L-DOPA in the delocalization of the frontier molecular orbitals and charge distribution. As can be seen in Fig. 6, the HOMO orbital delocalized in a suitable section on the aniline moiety and the hydroxyl groups of **M3** and **M4**. However, a tiny section of the HOMO orbital delocalized on the phosphorus and phosphorus-related oxygen atoms in **M1** and **M2** compounds, which reduces their impact on the compounds' reactivity. Also, compared to L-DOPA (−6.124 eV), the energy of HOMO in **M3** and **M4** (−5.757 eV and −5.742 eV) have more differences than the energy of HOMO in **M1** and **M2** (−6 eV, −6.145 eV). It could be said that the bioactivity is not only orbital-controlled in the compounds having phenolic oxygen (**M3** and **M4**) but also is controlled by the atomic charge, as can be indicated in NBO analysis.

Natural population analysis (NPA)

As can be noted from Table 2, the atomic charges of the phosphorus and oxygen atoms bonded to phosphorus are similar in all molecules (**M1**–**M4**), which may indicate that this part is not the primary factor affecting their bioactivity. Both **M3** and **M4** have OH-substituent on the aniline moiety (R_2 substituent); this makes their structure similar to the enzyme–substrate L-DOPA, which has two hydroxyl groups interact with the copper-binding region within the enzyme [42–45]. When Cl-substituent replaces the OH-substituent in R_2 in **M1** and **M2**, the anti-phenol oxidase activity significantly dropped down. Table 2 displays the small negative charge of the hydroxyl oxygen atoms of both **M3** (−0.711) and **M4** (−0.707). These charges are almost equal to the charge of hydroxyl-oxygen atoms in L-DOPA (−0.715 and −0.694) (Table 2). It can be concluded that the negative charge of the oxygen atom related to the aniline-hydroxyl group may control the anti-phenol oxidase activity of both **M3** and **M4**. Phenol oxidase is an essential protein for an insect's immunity and defense, and it is involved in the encapsulation and melanization process as a defense reaction [5, 7–9]. These hydroxyl-related oxygen of **M3** and **M4** may interact with the copper-binding region within the enzyme active site [42–45] and prevent the melanization process. These theoretical studies displayed the importance of the aniline moiety on the fungicidal activity of our compounds; besides, it showed the impact of charge distribution in their anti-phenol oxidase activity.

Conclusions

This paper has investigated the dual bioactivity of some α-amino phosphonate derivatives. Most of the synthesized compounds revealed a level of fungicidal activity against both *Macrophomina phaseolina*, and *Pythium aphanidermatum* especially **M4**. Both **M3** and **M4** displayed a good anti-phenol oxidase activity, which may imply their capacity to interfere with the insects immune system. The theoretical study pointed out the role of charge distribution on the phenol oxidase inhibitory activity. It indicated the impact of the aniline moiety substituents on the fungicidal activity. We hope that our compounds will serve as a base for the future to develop novel organophosphorus fungicides with additional insects phenol oxidase inhibitory activity, also; the authors suggest further assays on both target and non-target organisms.

Acknowledgements

We are grateful to Ali Mokhtassi-Bidgoli from Department of Agronomy (Tarbiat Modares University) for his support in statistical analysis of this work.

Authors' contributions

MA, KG, and AAEV participated in the synthesis and characterization of the compounds; MA, NS and MM provided the antifungal and anti-phenol oxidase assays; MA and MB provided the DFT study. MA and NS were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by Tarbiat Modares University, Tehran, Iran.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Received: 29 March 2021 Accepted: 23 July 2021
Published online: 27 September 2021

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