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].
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 phosphate derivative**

Kabachnike Fields reaction and Pudovik reaction are the most versatile methods for preparing α-amino phosphate 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 (1H, 13C, and 31P) spectroscopy.

IR spectra (KBr pellets) were obtained with a Shimadzu, IR-60 model spectrometer. 1H, 13C, and 31P NMR spectra were recorded on a Bruker (Avance DRS) 500 MHz spectrometer and chemical shifts were determined relative to TMS and 85% H3PO4, 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. 1H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.25 (m, 6H, CH₃), 4.10 (m, 4H, CH₂), 6.96–8.44 (m, 8H, Ph), 8.23 (m, 1 H, CH-P), 10.40 (N–H). 13C NMR (125.77 MHz, d-DMSO, ppm): δ = 29.7 (s, 2 C, CH₃), 46.4 (d, 2 C, CH₂), 61.5 (s, 1C, CH-P), 113–135.1 (CPh), 152.7 (Cipso-CH), 158.6 (Cipso-NH), 189. (CCl). 31P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.29 ppm. IR data (KBr, cm⁻¹): 3437 (υ N–H); 3059 w (CHAr); 2922 s (CHAliph); 1615 s (υ Ar); 1459 s (υAr); 1266 s (υP=O); 1043 s (υP–O). Anal. calcd. for C₁₇H₂₀Cl₂NO₃P: 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. 1H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.24 (m, 6H, CH₃), 4.01 (m, 4H, CH₂), 6.59–7.78 (m, 6H, Ph), 8.13 (m, 1 H, CH-P), 10.40 (N–H). 13C NMR (125.77 MHz, d-DMSO, ppm): δ = 29.7 (s, 2 C, CH₃), 46.4 (d, 2 C, CH₂), 61.5 (s, 1C, CH-P), 113–135.1 (CPh), 152.7 (Cipso-CH), 158.6 (Cipso-NH), 189. (CCl). 31P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.29 ppm. IR data (KBr, cm⁻¹): 3437 (υ N–H); 3059 w (CHAr); 2922 s (CHAliph); 1615 s (υ Ar); 1459 s (υAr); 1266 s (υP=O); 1043 s (υP–O). Anal. calcd. for C₂₁H₁₆Cl₄NO₃P: C, 52.59; H, 5.19; N, 3.61%. Found: C, 52.83; H, 5.42; N, 3.37%.

**Fig. 1** The synthesis process of target compounds M1–M6
M3; Diethyl(4-chlorophenyl)(2-hydroxyphenylamino) methylphosphonate

Mp: 102–106 °C. Brownish-yellow powder. 1H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.38 (m, 6H, CH3), 4.14 (m, 4H, CH2), 4.30 (s, 2H, CH2), 6.43–7.46 (s, 1H, OH), 8.21 (1H, CH-P), 7.21–7.88 (m, 7H, ph), 9.10 (N-H), 11.63–138.1 (CPh), 158.6 (Cipso-CH), 156.1 (Cipso-CH), 173.3 (COH), 190.9 (CCl). 31P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.136 s (d, JP-H = 11.8 Hz), ppm. IR data (KBr, cm−1): 3376b (υO-H); 2978 w (CHAr); 2930 b (CHAliph); 1491 m–1626 m (υAr); 1245 s (υP-O) 1205 s (υP-O). Anal. calcd. for C17H20Cl2NO4P: C, 50.51; H, 4.99; N, 3.47%. Found: C, 57.59; H, 5.85; N, 3.98%

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].

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

Mp: 115–117 °C. Yellow powder. 1H NMR (500.13 MHz, d-DMSO, ppm): δ = 1.38 (m, 6H, CH3), 4.14 (m, 4H, CH2), 6.92 (s, 1H, OH), 8.21 (1H, CH-P), 7.21–7.88 (m, 7H, ph), 9.10 (N-H), 11.63–138.1 (CPh), 158.6 (Cipso-CH), 156.1 (Cipso-CH), 173.3 (COH), 190.9 (CCl). 31P NMR (202.46 MHz, d-DMSO, ppm): δ = 0.092 s (d, JP-H = 9.8 Hz), ppm. IR data (KBr, cm−1): 3397b (υO-H); 3304 m (υO-H); 3042 w (CH2); 2925 w (CH2); 2975 w (CHAliph); 1623 s;1582 s (υAr); 1491 s; 1205 s (υP-O). Anal. calcd. for C17H21ClNO3P: C, 55.22; H, 5.72; N, 3.79%. Found: C, 55.34; H, 5.80; N, 3.88%

Fungicidal activity study
Mythic 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 mycicidal growth inhibition was calculated using Eq. (1) [21, 22].

Mythic growth inhibition (MGI)% = \left[\frac{C - T}{C}\right] \times 100

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
20 µL of the tested compound were added to each well of distilled water, 20 µL PBS buffer, 20 µL of the substrate, substrate-based assay carried out in a microplate, 100 µL using a L-3,4-dihydroxyphenylalanine (L-DOPA) substrate, 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.

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 15,000g 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 15,000g 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).
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 pathogens compared to other compounds (Table 1). 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 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 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

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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±1.5 | 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                   | –  | –  | –  | 25±1.81 | –  | –  |
|                 | 25                   | –  | –  | –  | 20±1.52 | –  | –  |

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

–: The compound was tested at this concentration
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
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

![DFT comparison of M1–M4 and M6 and L-DOPA.](image)

**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 Klopmann-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 (R2 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 R2 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.

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**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 M8 provided the DFT study. MA and NS were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

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**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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References
1. Oliver RF, Hewitt HG. Fungicides in crop protection. 2nd ed. Cabi: Wallingford; 2014.
2. Walter H. New fungicides and new modes of action. In: Modern fungicides and antifungal compounds VI. 16th International Reinhardsrubn Symposio- num, Friedrichroda, Germany, 2014; April 25–29, (2010), 47–54. Deutsche Phytophymedizinische Gesellschaft eV Selbstverlag.
3. Morton V, Staib T. A short history of fungicides. APS Net Features. 2008. https://doi.org/10.1094/APSNetFeature-2008-0308.
4. Fest C, Schmidt KJ. The chemistry of organophosphorus pesticides. Berlin: Springer; 2012.
5. Gonzalez-Santoyo J, Cordoba-Aguilar A. Phenoloxidase: a key component of the insect immune system. Entom Exp Appl. 2012;142(1):1–16.
6. Cerenius L, Söderhäll K. The prophenoloxidase-activating system in inver- tebrates. Immunol Rev. 2004;189(1):116–26. https://doi.org/10.1111/j.1600-065X.2004.00116.x.
7. Ashida M, Brey PT. Role of the integument in insect defense: pro-phenoloxidase cascade in the cuticular matrix. Natl Acad Sci. 1991;99(23):10698–702. https://doi.org/10.1073/pnas.92.23.10698.
8. Lai SC, Chen CC, Hou RF. Immunolocalization of prophenoloxidase in the process of wound healing in the mosquito Armigeres subalbatus (Diptera: Culicidae). J Med Entomol. 2002;39(2):266–74. https://doi.org/10.1016/S0022-2585(01)00226-4.
9. Lu A, Zhang Q, Zhang J, Yang B, Wu K, Xie W, Luan YX, Ling E. Insect prophenoloxidase: the view beyond immunity. Front Physiol. 2014;5:252. https://doi.org/10.3389/physiol.2014.00252.
10. Gholami T, Ghadamyari M, Oliaee AO, Amjami FN. Effects of inhibitors on haemolymph phenoloxidase from rosaceous branch borer, Osphera- antera coerulensis (Coleoptera: cerambycidae). J Plant Prot Res. 2013;1:18–24.
11. Allen JG, Atherton FR, Hall MJ, Hassall CH, Holmes SW, Lambert RW, Nisbet LJ, Ringrose PS. Phosphonopeptides, a new class of synthetic anti- bacterial agents. Nature. 1978;272(5648):56–8. https://doi.org/10.1038/272056a0.
12. Abdou WM, Barghash RH, Bekheit MS. Carbodiimides in the synthesis of enam-ino- and a-amino phosphonates as peptidomimetics of analgesic/ antiinflammatory and anticancer agents. J Arch Pharm Chem Life Sci. 2012;345(1):884–95. https://doi.org/10.1002/adpc.201200142.
13. Devi Ne, Dodgga S, Donka R, Chamarthi NR. CeCl3. 7H2O-SiO2: an effec- tiveds mioccular inhibit of the rice plant infected with Xanthornan titan. Ann Phytopath Soc Japan. 1981;47:555–61. https://doi.org/10.3186/jjphytopath.47.555.
14. Wu T, Zivanovic S, Draughon PA, Conway WS, Sams CE. Physiochemical properties and bioactivity of fungal chinin and chitotan. J Agric Food Chem. 2005;53(10):3888–94. https://doi.org/10.1021/jf048202s.
15. Hatada Y, Ohta Y, Horikoshi K. Hyperproduction and application of alphaa- garase to enzymatic enhancement of antioxidant activity of porphyrin. J Agric Food Chem. 2006;54(26):9895–900. https://doi.org/10.1021/jf0613684.
16. Ullah I, Khan A, Ali L, Khan A, Waqas M, Lee JJ, Shin JH. An insecticidal compound produced by an insect-pathogenic bacterium sup- presses host defenses through phenoloxidase inhibition. Molecules. 2014;19(12):20913–28. https://doi.org/10.3390/molecules191220913.
17. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Scalmani G, Barone V, Mennucci B, Petersson GA, Nakatsuji H, Caricato M, Li X, Hratchian HP, Izmaylov AF, Bloino J, Zheng G, Sonnenberg JL, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Vreven T, Montgomery JA, Jr., Peralta J, Ogliaro F, Bearpark M, Heyd JJ, Brothers E, Kudin KN, Staroverov VN, Keith T, Kobayashi R, Normand J, Raghavachari K, Rendell A, Baurant JC, Jeng SS, Tomasi J, Cossi M, Rega N, Millam JM, Klene M, Knox JE, Cross JB, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Martin RL, Morokuma K, Zakrzewski VG, Voth GA, Salvador, Dannenberg JJ, Dapprich S, Daniels AD, Farkas O, Foresman JB, Ortiz JV, Cioslowski J, Fox DJ, Gaussian 09, Revision D01, Gaussian, Inc., Wallingford CT. (2013).
18. Newton MD. Applications of electronic structure theory. Modern Theor Chem. 1977:4246–8.
19. Hay PJ, Wadt WR. Ab initio effective core potentials for molecular calculations. Potentials for K to Au including the outermost core orbitals. J Chem Phys. 1985;82(1):299–310. https://doi.org/10.1063/1.448975.
20. Wadt WR, Hay PJ. Ab initio effective core potentials for molecular calculations. Potentials for main group elements Na to Bi. J Chem Phys. 1985;82(1):284–98. https://doi.org/10.1063/1.448800.
21. Glendening ED, Reed AE, Carpenter JE, Weinhold F. NBO Version 3.1, Tci. Madison: University of Wisconsin; 1998.
22. SAS 9.3. SAS Institute Inc. Cary, NC, USA. 2011.
23. Wang BL, Zhu HW, Ma Y, Xiong LX, Li YQ, Zhao Y, Zhang JF, Chen YW, Zhou S, Li ZM. Synthesis, insecticidal activities, and SAR studies of novel pyridylpyrazole acid derivatives based on amide bridge modification of antranilic diamide insecticides. J Agric Food Chem. 2013;61(23):5483–93. https://doi.org/10.1021/jf4012467.
24. Liu XH, Chen PQ, Wang BL, Li YH, Wang SH, Li ZM. Synthesis, bioactivity, theoretical and molecular docking study of 1-cyano-N-substituted-cyclo- propanecarboxamide as ketol-acid reductoisomerase inhibitor. Bioorg Med Chem. 2007;15(13):3784–88. https://doi.org/10.1016/j.bmc.2007.04.003.
25. Sun NB, Fu JQ, Weng JQ, Jin JZ, Tan CX, Liu YH. Microwave assisted syn- thesis, antifungal activity and DFT theoretical study of some novel 1, 2, 4-triazole derivatives containing the 1, 2, 3-thiadiazole moiety. Molecules. 2013;18(10):12725–39. https://doi.org/10.3390/molecules181012725.
26. Dannenberg JJ. Using perturbation and frontier molecular orbital theory to predict diastereofacial selectivity. Chem Rev. 1999;99(4):1225–42. https://doi.org/10.1021/cr980328f.
27. Cho JK, Shaik S. Electron transfer vs polar mechanisms. Transition-state structures and properties for reactions of a cation radical and a nucleo- phile. J Am Chem Soc. 1991;113:9890–1.
38. Klopman G. Chemical reactivity and the concept of charge-and frontier-controlled reactions. J Am Chem Soc. 1968;90(2):223–34. https://doi.org/10.1021/ja01004a002.

39. Klopman G. The control of chemical reactivity. J Mol Struct. 1983;103:121–9. https://doi.org/10.1016/0166-1280(83)85013-1.

40. Salem L. Intermolecular orbital theory of the interaction between conjugated systems. I. General theory. J Am Chem Soc. 1968;90(3):543–52. https://doi.org/10.1021/ja01005a001.

41. Chase MR, Raina K, Bruno J, Sugumaran M. Purification, characterization and molecular cloning of prophenoloxidases from Sarcophaga bullata. Insect Biochem Mol Bio. 2000;30(10):953–67. https://doi.org/10.1016/S0965-1748(00)00068-0.

42. Sugumaran M. Unified mechanism for sclerotization of insect cuticle. Adv Insect Physiol. 1998;27:229–334. https://doi.org/10.1016/S0065-2806(08)60014-4.

43. Sugumaran M. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res. 2002;15(1):2–9. https://doi.org/10.1034/j.1600-0749.2002.00056.x.

44. Li Y, Wang Y, Jiang H, Deng J. Crystal structure of Manduca sexta prophenoloxidase provides insights into the mechanism of type 3 copper enzymes. Proc Natl Acad Sci. 2009;106(40):17002–6. https://doi.org/10.1073/pnas.0906095106.

45. Decker H, Schweikardt T, Tuczek F. The first crystal structure of tyrosinase: all questions answered? Angw Chem Int Ed. 2006;45(28):4546–50. https://doi.org/10.1002/anie.200601255.

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