Synthesis, characterization (IR, $^1$H, $^{13}$C & $^{31}$P NMR), fungicidal, herbicidal and molecular docking evaluation of steroid phosphorus compounds

Abstract: Phosphorus containing steroidal derivatives such as 3β-oxo-[diazaphosphalidine-2'-one] stigmast-5-ene and 3β-oxo-[diazaphosphalidine-2'-one] stigmast-5,22-diene were designed, synthesized and characterized using spectroscopic techniques (IR, $^1$H, $^{13}$C & $^{31}$P NMR, HRMS) and elemental analysis. The fungicidal and herbicidal studies of the compounds were performed and the experimental outcomes showed that compound 4 showed a good fungicidal activity against mycelium growth of fungi, while in the case of herbicidal activity, both compounds show a moderate activity compared to the commercial drug; Atrazine. The binding free energy of active compound 4 to the receptor named 4-Hydroxyphenylpyruvate dioxygenase (HPPD) was calculated using the molecular docking study. The HPPD is one of the most effective targets of plants for the herbicide study.

Keywords: Steroidal derivatives; characterization; fungicide, molecular docking; NMR.

1 Introduction

Organophosphorus [1] chemistry is of paramount importance for a wide range of biological, chemical and industrial processes. Compounds containing phosphorus [2-4] first reported in certain countries were found useful for pest control. Phosphorus and its compounds are versatile and important to living organisms in everyday life. All living organisms rely on the compound having a phosphorus atom as an integral part of the system to properly manage the existence of life. For example, the genetic code required for every form of life is preserved in DNA, a phosphate ester. The main link between producing energy and requiring energy in living cells is instituted by another phosphate ester, adenosine triphosphate (ATP). Leading bone material is calcium phosphate, and bones were used as the main source for the production of elemental phosphorus historically. Although phosphorus compounds are essential for life, they can also be very dangerous. The toxic nature of some phosphorus compounds has been used in the development of nerve gases and insecticides. The blooming of these harmful phosphoric compounds began after the First World War and culminated during the Second World War. Germany and England have established research programs to develop and design powerful chemical warfare agents [5]. The finding of the biological activities of the organophosphorus derivatives was revealed by a fortuitous study of Lange and Kreuger [6] according to which the temporary exposure to the diethyl phosphorofluoridated vapors would have powerful cholinergic effects in humans. Synthesis of organophosphorus compounds was reported [7], and it was estimated that more than fifty thousand such compounds were prepared and screened for pesticide activity, of which over five dozen were commercially produced to date [8-14]. Of all the heterocyclic molecules containing phosphorus, the system of five- and six-membered rings with two heteroatoms directly connected to a four-coordinate phosphorus atom is the most common. The five-member phosphorus containing heterocycles with heteroatoms directly attached to phosphorus are also easy to synthesize. The synthesis of such compounds with N- and pentavalent P atoms (phosholidines) including carbonyl groups were managed by Becke-Goehring and Wolf [15] reacting oxamide with phosphorus pentachloride. The reactivity of the heteroatom to the nucleophiles is greatly improved
because of the strain of the ring; the ring opening of a five-membered heterocyclic phosphorus compound after the nucleophilic attack at the phosphoryl center is favored due to a relief of the ring-strain [16]. On the other hand, the work reported by Oertel and colleagues [17] sustained the presence of phosphorus containing steroids in the blood and the phosphatidyl steroids, linked to α-globulins. Moreover, steroid monoesters of orthophosphoric acid have also been reported and prepared by direct phosphorylation with phosphorus pentachloride or phosphorus oxychloride and pyridine, followed by hydrolysis [18]. Cyclic or heterocyclic compounds attached directly to steroidal skeletons have also been used as pesticides and herbicides to protect crops from pests that have infected the proper growth of food production plants by absorbing nutrients and carbohydrates from host plants [19-20].

1.5.1 Synthesis of phospholidinones (3 and 4) in stigmastane series.

2. General procedure

2.1 3β-oxo-[diazaphosphalidine-2'-one] stigmast-5-ene (3)

The 3β-Stigmast-5-en-3-ol (Δ5-stigmastene-3β-ol) (1) (1 mmol, 1 equiv) was mixed to a solution of phosphorous oxychloride (2.6 mmol), triethyl amine (1.2 mmol) in 25ml dry ether at 25°C under nitrogen. The reaction mixture was stirred for 30 minutes and cooled to 0°C. To this, triethylamine (2 equiv) and ethylene diamine (2 equiv) were added and stirred for 5 hrs at room temperature. Its completion and formation of the products was checked by TLC, the reaction mixture was filtered. The filtrate was washed with water and dried over anhydrous sodium sulphate. Purification of the crude product was accomplished by column chromatography using silica gel with petroleum ether (85:15) as eluent. The product was further purified by repeated crystallization from methanol but failed to crystallize to give a semi solid product (3) (Scheme 1).

Scheme 1: Synthesis of phospholidinones (3 and 4) in stigmastane series.
2.1.2 3β-oxo-[diazaphosphalidine-2′-one] stigmast-5,22-diene (4)

Under similar reaction conditions (3β)-stigmast-5,22-diene-3-ol (Δ5,22-stigmastadien-3β-ol) (2) with ethylene diamine furnished the compound 4 after treatment in the same way as compound 3, was named as 3β-oxo-[diazaphosphalidine-2′-one] stigmast-5,22-diene (4) (Scheme 1).

Anal. Calc. for C31H53N2O2P: C 70.83; H 10.65; N 5.82. Found: C 70.83; H 10.65; N 5.81. IR, νmax: 3411 (NH), 2937, 2867 (ω), 1639 (C≡N), 1271 (NH-CH), 1284 (P=O), 1193 cm⁻¹ (P-O). 1H NMR (CDCl₃, 300 MHz) δ: 5.4 (br, 1H, Cα-vinyl proton), 5.2 (br, m, 2H, NH), 5.15 (d, 1H, C22-H), 5.02 (d, 1H, C23-H), 4.6 (m, Cβ-α-H (W1/2 = 15 Hz axional)), 3.1 (four protons of NH₂-CH₂-CH₂-NH₂), and other methyl protons gave signals at 1.04, 1.01, 0.92, 0.83, 0.81 and 0.70 (Me); 31P NMR (CDCl₃, 120 MHz): 17.4 (s); δC: 37.9 (1), 32.5 (C2), 75.9 (C3), 40.7 (C4), 141.1 (C5), 119.6 (C6), 31.9 (C7), 32.1 (C8), 50.7 (C9), 36.3 (C10), 23.1 (C11), 39.1 (C12), 41.8 (C13), 57.1 (C14), 24.3 (C15), 28.5 (C16), 56.2 (C17), 11.7 (C18), 19.5 (C19), 35.9 (C20), 21.3 (C21), 138.9 (C22), 130.1 (C23), 51.1 (C24), 32.1 (C25), 21.4 (C26), 20.1 (C27), 23.9 (C28), 12.4 (29), 42.1 ppm (2C′& C′′); HRMS: m/z: calc for C₃₁H₅₃N₂O₂P (M+H)⁺ 516.38, found 516.49.

2.2 Laboratory Bioassay

2.2.1 Fungicidal Activity

Phosphorus containing compound 3 and 4 were evaluated for their in vitro fungicidal studies against pathogenic fungi observing mycelium growth rate protocol as mentioned in literature [21-24]. Fungi assayed in this work included Pythium aphanidermatum, Rhizoctonia solani, Botrytis cinerea, and Sclerotinia sclerotiorum. Under sterile conditions, 10 mg of the examined steroid derivatives were weighed, dissolved in 1 mL of dimethyl sulfoxide (DMSO) and prepared to 10mg/ml concentration solutions. 200 mL potato dextrose agar (PDA) was mixed with concentration of the tested compounds under 50°C. The final concentration of each steroidal derivative was of 50μg/mL. DMSO in sterile distilled water served as the control. The medium with the tested compounds at a final concentration of 50μg/mL for the introductory testing was poured into sterilized petri dishes. After allowing the petri dishes to cool, 7 mm diameter mycelium disks were placed in the center of the PDA dishes and incubated at 25°C for 2-3 days. Each experiment was performed in triplicate. The change in diameter of the mycelium circle (diameter of the hypha) was measured by cross bracketing method or by a ruler. Commercial fungicides; fluopicolide, and pyraclostrobin were used as positive controls. The inhibition or relative inhibition (equation 1) rate of phosphorus-containing steroids on fungi was calculated and compared to a blank experiment, respectively, using the following formula:

\[
\text{Inhibition rate (\%) = } \frac{C - T}{C} \times 100
\]

Where C is the diameter of hypha on blank test and T, change in diameter (in mm) of hypha after testing.

2.2.2 Herbicidal Activity

The herbicidal activities of the phosphorus containing steroids (3 and 4) were screened in vitro against two weeds such as Brassica campestris (Rape), and Echinochloa crusgalli (Barnyardgrass) according to the literature [25]. A suspension of 5g of agar powder was mixed in 1 L distilled water and heated to melt after then cooled to 40–50°C. The each synthesized sample was dissolved in DMSO to prepare stock solution of 10g/L. the 0.1 mL of stock solution was mixed to 50 mL of agar at 45°C to prepare the required concentrations (10 and 100 mg/L). Subsequently, 5 mL of the agar-containing steroids was poured to the beaker (10 ml) and cooled, and uniform germinating seeds of weed were placed on the surface of the agar mass. The beaker was sealed with a piece of plastic wrap which contained some small holes. Thereafter, cultivations were made in an incubator set at 28 ± 1°C and 50–55% relative humidity for 12 h in the light and 12 h in the dark alternatively for 3 days. DMSO was taken as blank control while Acetochlor as a positive control. Each experiment was carried out three times with 3 replicates. After 3 days of cultivation, weed lengths are measured and the rate of growth inhibition is determined with respect to the untreated control. The study focused on the percentage of germination, the measurement of the length of roots and stems. The comparative test was conducted in distilled water with DMSO. The inhibition rate was calculated from the growth of the plant (root and stem) using the following equation; Relative inhibition rate (\%) = [(AB-AT)/AB]×100, where AH is the average height of the plant during the growth inhibition or relative inhibition (equation 1) rate of phosphorus-containing steroids on fungi was calculated and compared to a blank experiment, respectively, using the following formula:

\[
\text{Relative growth inhibition (\%) = } \frac{A_B - A_T}{A_B} \times 100
\]

2.3 Molecular Docking

Compound 4 used in the molecular docking study was designed using ChemDraw Pro 12v and Chem3D pro and...
the constructed structure was optimized using Gaussian 03W program package [26] with DFT /B3LYP / 6-31G (d, p) basis set to provide a correct alignment of the geometry of the compound. The crystalline structure of 4-hydroxyphenylpyruvate dioxygenases was taken from the protein database (PDB ID 1TFZ). Prior to molecular docking simulation, the 3D structure of protein function as a receptor was prepared using molecular docking software such as Discover Studio 4.0v [27], and Molegro Virtual Viewer [28]. All molecules such as heteroatoms, water, docked ligand and co-crystallized solvent were detached from the PDB file and missing assignments such as charges, hybridization, bond order and lengths were correctly attributed using the Molegro Virtual Viewer. It is important to mention that the active site of the receptor was clarified on the basis of the volume occupied by the docked ligand already placed in an active site of proteins. AutoDock4 [29, 30] was used to evaluate molecular docking, energy profile and Discovery Studio 4.0 used for visualization and interaction of compound interactions with amino acid residues in the active sites.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 Chemistry

The phosphorus containing steroidal products 3 and 4 were readily synthesized in good yields as shown in Scheme 1. The overall yield of compounds 3 and 4 were 70 and 65%, respectively. Semi-solid products were obtained after column chromatography purification. The semi-solid compound 3 was analyzed and found to be C_{31}H_{55}N_{2}O_{2}P. Products were characterized using physicochemical techniques. Presence of nitrogen and phosphorus in compound 3 were diagnosed by IR bands (Figure 1) that were showing at 3345 (NH stretching) [31], 1271 (NH-CH) [32], 1317 (P=O) and 1090 cm\(^{-1}\) (-P-O-) [33]. Other significant bands at 2937, 2867 and 1583 cm\(^{-1}\) were assigned to aliphatic C-H stretch and C = C as weakly intense band. Moreover, the band at 1401 cm\(^{-1}\) displayed asymmetric CH3 bending modes of steroidal skeleton in phospholidinones. These values are correlated with reported values in analogous compounds [3].

Compound 4 was identified in the same manner as compound 3 and the IR values of Compound 4 were given in the experimental section. \(^1\)H NMR spectrum represented important signals to support the formation the synthesized compounds. A broad multiplet of NH (D2O-exchangeable) at δ 5.1 was shown for two protons. Another sign as appeared as a singlet at δ3.3 attributed to two methylene functional groups that lay between two nitrogen atoms of the diazaphospholidine ring. Characteristic signals appeared at δ4.5 and at δ 5.4 attributed to the C3-H and C6 vinyl proton of the steroidal moiety, respectively. Two olefinic protons in the case of compound 4 resonated as characteristic downfield signals at 5.15 and 5.02 in the \(^1\)H NMR spectrum which are similar to C22-H and C23-H of stigmasterol, respectively [34]. The other protons resonated at the specific positions mentioned in the experimental section. \(^{13}\)C NMR of compound 3 gave recognizable signals at 140.9, 121.7 and 76.1 ppm, which are labeled with C5, C6 (double bond) and C3 respectively. While compound 4 showed important signals at 138.9, 130.1, 141.1, 119.6 and 75.9 ppm attributed to C22, C23, C5, C6 and C3, respectively. The downfield shift in case of C22, C23, and C5 and C6 atoms furnished information of unsaturated system. These assignments were found in good agreement with the values of the literature [35-36]. The upfield signals at 11.7, 19.3, 19.5, 19.9, 20.7 and 23.4 correspond to carbon atom C18, C21, C19, C26, C27

Figure 1: The IR spectrum of compound (3).

Figure 2: The DEPT of the product (3).
Distortionless Enhancement by Polarization Transfer (DEPT) NMR is a recommended technique to examine the occurrence of primary, secondary and tertiary carbon atoms and distinguish between them. The DEPT of compound 3 (Figure 2) showed the existence of 31 carbons, including the carbon of diazaphosphalidine. The carbons could be grouped as representative of CH₃, CH₂, CH or quaternary carbon (QC) by DEPT-135. The DEPT-135 of compound (3) (Figure 2) suggested the presence of 28 carbons: six peaks showed upward signals due to CH₃ groups, nine peaks also showed upward signals for CH groups and downward peaks directed the presence of thirteen CH₂ groups. The non-appearance of signals at 36.1, 41.9 and 140.9 ppm in the DEPT spectrum reaffirms the presence of three quaternary carbon atoms as expected in the structure of compound 3. In addition, in the ³¹P NMR spectrum of the compound (Figure 3), the appearance of a signal at 17.5 for compound 4 and 17.4 for compound 3 indicated the presence of a P atom in the synthesized compounds [37]. The molecular ion peaks in the high-resolution mass spectrometry (HRMS) of the compounds have provided additional assistance in the structural elucidation of the synthesized compounds given in the experimental section.

### 3.2 Fungicidal Activity

The synthesized compounds were evaluated for their fungicidal activity against four pathogenic fungi listed in Table 1 applying a concentration of 50 μg / ml and the mycelium growth rate method was used. Pyraclostrobin and Fluopicolide [24] were used as a positive control to compare the obtained screening results. The results of the bioassay reported in Table 1 showed that the steroid derivatives 3 and 4 showed evident fungicidal activity. Both compounds showed low to moderate activity against fungi when compared with values of references reported in literature. Compound; 3β-oxo-[diazaphosphalidine-2'-one] stigmast-5-ene (4) with two unsaturated center showed inhibitory activities in the range of 50-65% while compound 3 gave 25-50% inhibition effect. The inhibitory activity of the phosphorus containing heterocyclic at the 3-position of steroidal compounds can be good candidates for fungicidal activity after some additional modification, and can be applied to inhibit pathogenic fungi in the future.

### 3.3 Herbicidal Activity

Relative inhibition activity of phosphorus containing compounds 3 and 4 against monocotyledons and dicotyledonous plants such as *Echinochloa crusgalli* and *Brassica campestris*, respectively, called by the common name barnyard grass and tape plants, were screened using reported protocol [25]. Reference drug, Atrazine was used to compare the results obtained from the biological assay. The percentile growth inhibitions of the seedling of two plants are shown in Table 2. As can be seen from Table 2, compound 4 shows a better inhibition for both plants at a concentration of 100 mg /L compared to the compound

### Table 1: Fungicidal activities of steroidal derivatives 3 and 4 against four types of pathogenic fungi.

| Compound | Pathogenic strains (Fungicidal activity (%)/50 μg/mL) | P. aphanidermatum | R. solani | B. cinerea | S. sclerotiorum |
|----------|----------------------------------------------------------|------------------|-----------|------------|----------------|
| 3        |                                                          | 25               | 30        | 50         | 40             |
| 4        |                                                          | 50               | 60        | 55         | 65             |
| Fluopicolide |                                                  | 99               | 45        | 45         | 80             |
| Pyraclostrobin |                                                  | 48               | 100       | 85         | 100            |

![Figure 3: ³¹P NMR spectrum of 3β-oxo-[diazaphosphalidine-2'-one] stigmast-5-ene.](image)
3. Preliminary evidence showed that compound 4 presented much better herbicidal activities against the dicotyledonous plant than against the monocotyledonous plant. Compound 4 with two unsaturated centers in the skeleton offers better candidates for inhibition than that of an unsaturated center of compound 3. In addition, an active steroid compound 4 was used for molecular docking in order to know the enzymatic target and the interaction with active sites of the enzyme to inhibit the proper orientation of amines in proteins as well as the synthesis of HPPD in plants. The HPPD is important for normal growth of plants [38].

Table 2: Herbicidal activity (%) of steroids (3 and 4) against seedling growth of two weeds.

| Compound | Echinocloa crusgalli | Brassica campestris |
|----------|----------------------|---------------------|
|          | 10 mg/L | 100 mg/L | 10 mg/L | 100 mg/L |
| 3        | 21      | 40       | 22      | 47       |
| 4        | 25      | 59       | 30      | 63       |
| Atrazine | 85      | 75       | 90      | 95       |

3.4 Analysis of molecular docking results

Steroid (4), with diazaphosphalidine ring, was chosen for the docking with 3D-cristal of HPPD (Figure 4a) because it showed the best herbicidal activity between two compounds. The important interactions of compound 4 with the active Arabidopsis thaliana HPPD (AtHPPD) site were shown in Figure 4. 4-Hydroxyphenylpyruvate (HPPA) and homogenate (HGA) are part of 4-hydroxyphenylpyruvate dioxygenase (HPPD) before conversion into an active precursor. In plants, HGA is an important precursor for plant survival and for the biosynthesis of tocopherol and plastachinone. Both are essential for normal plant growth [39-42]. When the HPPD is blocked by any means, sunlight will damage the proper growth of the plants. The plants become cloudy and eventually they are subject to necrosis and death, therefore, any inhibitor of HPPD is given the name bleaching herbicides [43-45]. In order to verify the inhibitory behavior of compound 4 with active receptor sites, the molecular docking study was performed to ascertain the deeply binding nature of compounds with the receptor through interaction. Steroidal compound 4 (Figure 4b) was found completely inserted into active pocket of the receptor (Figure 4c) and...
interacted with amino acids Gln272 and Pro368 through hydrogen bonding including groups of amino acids such Tyr276, Thr275, Ser242, Asn402, Gln286 and Thr275 which surrounded compound 4 in active sites via van der Waals interactions (Figure 4d,4f and 4e). In addition to the polar interactions, compound 4 showed hydrophobic interactions with Phe407, Leu406 and Met314 including pi-sigma interaction with phenylalanine (Phe403 and 371) of the receptor.

All these interactions with phosphorus containing steroidal molecule provide the best binding pose of the docked ligand (compound 4) resulting in a binding energy of -9.33 kcal/mol. Ongoing interpretation about interactions of ligand and HPPD receptor provided the positive views of inhibitor nature of compound 4 and it interacted with Gln272 [46] amino acid through hydrogen bonding leading to the suppression of the proper functioning of the HPPD.

4 Conclusion

The biologically active compounds 3 and 4 of steroids were synthesized and fully characterized using physicochemical techniques. The products were evaluated for their in vitro agricultural bioassay. The molecular docking study of the compound was conducted to elucidate the mechanisms of steroidal phospholidinones as an effective biologically active agent with AtHPPD acting as a receptor that binds to 4-hydroxyphenylpyruvate dioxygenase to hinder proper weed growth. Therefore, the docked compound 4 can be considered as a potential herbicide for further examination.

Conflict of interest: Authors declare no conflict of interest.

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