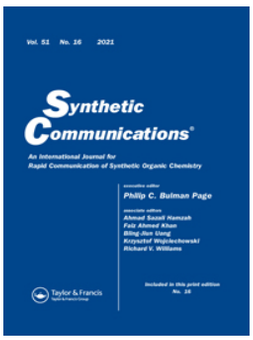

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Synthesis and *in vitro* antimicrobial, antioxidant, and antiproliferative activities of some new pyrano[2,3-c]pyrazoles containing 1,2-azaphospholes, 1,3,2-diazaphosphinines and phosphonate moieties

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Synthesis and *in vitro* antimicrobial, antioxidant, and antiproliferative activities of some new pyrano[2,3-c]pyrazoles containing 1,2-azaphospholes, 1,3,2-diazaphosphinines and phosphonate moieties

Tarik E. Ali[a,b], Mohammed A. Assiri[a], Hafez M. El-Shaaer[b], Somaia M. Abdel-Kariem[b], Wafaa R. Abdel-Monem[b], Somaya M. El-Edfawy[b], Noha M. Hassanin[b], Ali A. Shati[c], Mohammad Y. Alfaifi[c], and Serag Eldin I. Elbehairi[c,d]

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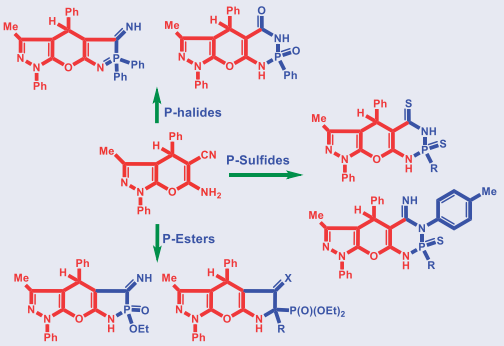
ABSTRACT

New 1,2-azaphosphole, 1,3,2-diazaphosphinine, and phosphonate derivatives containing pyrano[2,3-*c*]pyrazole moiety were achieved. The synthetic pathways depended on the reaction of 6-amino-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile with some phosphorus reagents such as diethyl phosphite, phosphorus sulfides, and phenyl phosphorus halides under different conditions. Compounds **6** and **7** recorded potent antibacterial and antifungal activities with lower toxicity, while compounds **4** and **6** displayed promising antioxidative properties. Further, compounds **4** and **9** exhibited potent cytotoxic effects against MCF-7, HepG-2, and HCT-116 cancer cells. The early apoptotic cell death was observed by the compounds in all types of the treated cells. Compounds **3**, **5**, **7**, **10**, and **14** recorded low to moderate percentages of necrosis and late apoptosis toward all treated cells.

GRAPHICAL ABSTRACT



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Introduction

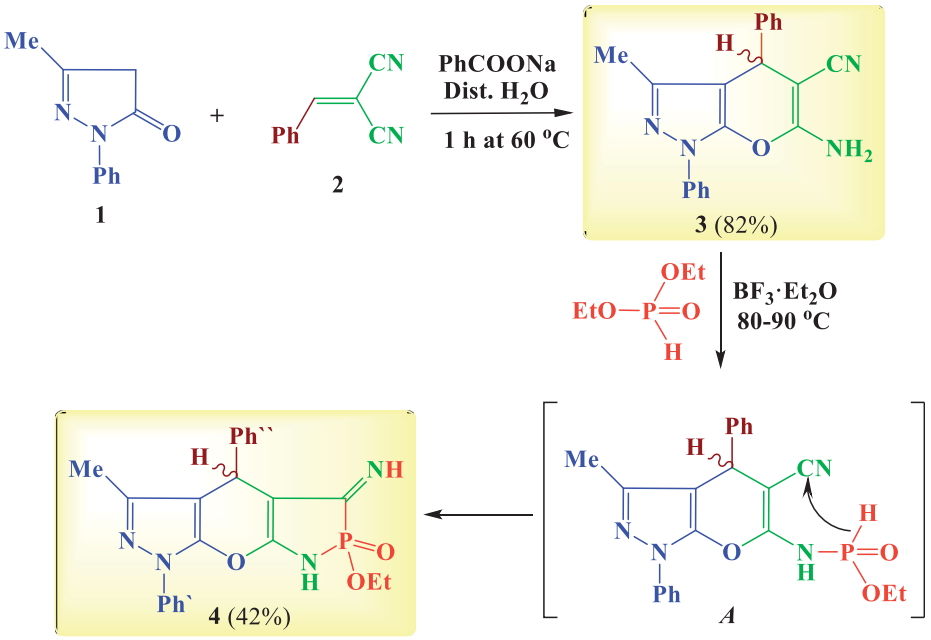
Organophosphorus compounds are important intermediates in organic synthesis and have been widely used as a pharmaceutical,[1] agricultural,[2] and chemical agents.[3,4] For example, phosphorus heterocycles[5] have received considerable interest because of their unique biological activities as antimicrobial[6] and their anticancer effects.[7,8] Ifosfamide and cyclophosphamide are two important examples of phosphorus heterocycles that were launched on the market more than 30 years ago and are still used in the treatment of cancer.[9,10] Also, phosphonate groups are chemically stable analogs of phosphates and play numerous vital roles in the biochemistry of living organisms. Phosphonates constitute an important class of bioisosteres for medicinal chemists and chemical biologists.[11] The usefulness of this class of molecules has been highlighted by the COVID-19 pandemic. Phosphonate nucleotide analogs, such as tenofovir and cidofovir, can inhibit SARS-CoV-2 RNA polymerase[12] and may thus represent a much-needed therapeutic weapon against this viral threat. On the other hand, pyrano[2,3-c]pyrazole compounds are the major structural motif in several natural products and synthetic compounds of widely recognized pharmacological properties.[13] In this context, pyranopyrazole systems have well been recognized for their potent biological activities including antimicrobial,[14] antioxidant,[15] antiviral[16], and antitumor.[17]

In view of the aforementioned details, and in extension to our efforts concerning the design of new polyheterocycles containing phosphorus heterocycles or phosphonate groups with possible biological properties,[18–22] we herein display the synthesis of some novel bioactive polycyclic compounds composed of pyrano[2,3-*c*]pyrazole skeleton containing phosphorus heterocycles or phosphonate groups in one molecular frame which might show enhanced biological activity. The suggested synthetic pathways depend on the reaction of the 6-amino-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazole-5-carbonitrile with some phosphorus reagents such as diethyl phosphite (in the presence or absence of electrophilic reagents), phosphorus sulfides, and phosphorus halides under different reaction conditions. In addition, the isolated products were screened for their antimicrobial, antioxidant, and antiproliferative activities.

Results and discussion

The known starting material, 6-amino-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*] pyrazole-5-carbonitrile (**3**) was synthesized in good yield as reported in the literature[23] through the reaction of 3-methyl-1-phenyl-5-pyrazolone (**1**) with 2-benzylidenemalononitrile (**2**) in distilled water containing a catalytic amount of sodium benzoate (Scheme 1). Substrate **3** possesses one reactive electrophilic center (C≡N) and another nucleophilic center (NH_2) which can be attacked by electrophilic and nucleophilic phosphorus reagents. This provides a wide opportunity for the construction of phosphorus heterocycles fused with pyranopyrazole system.

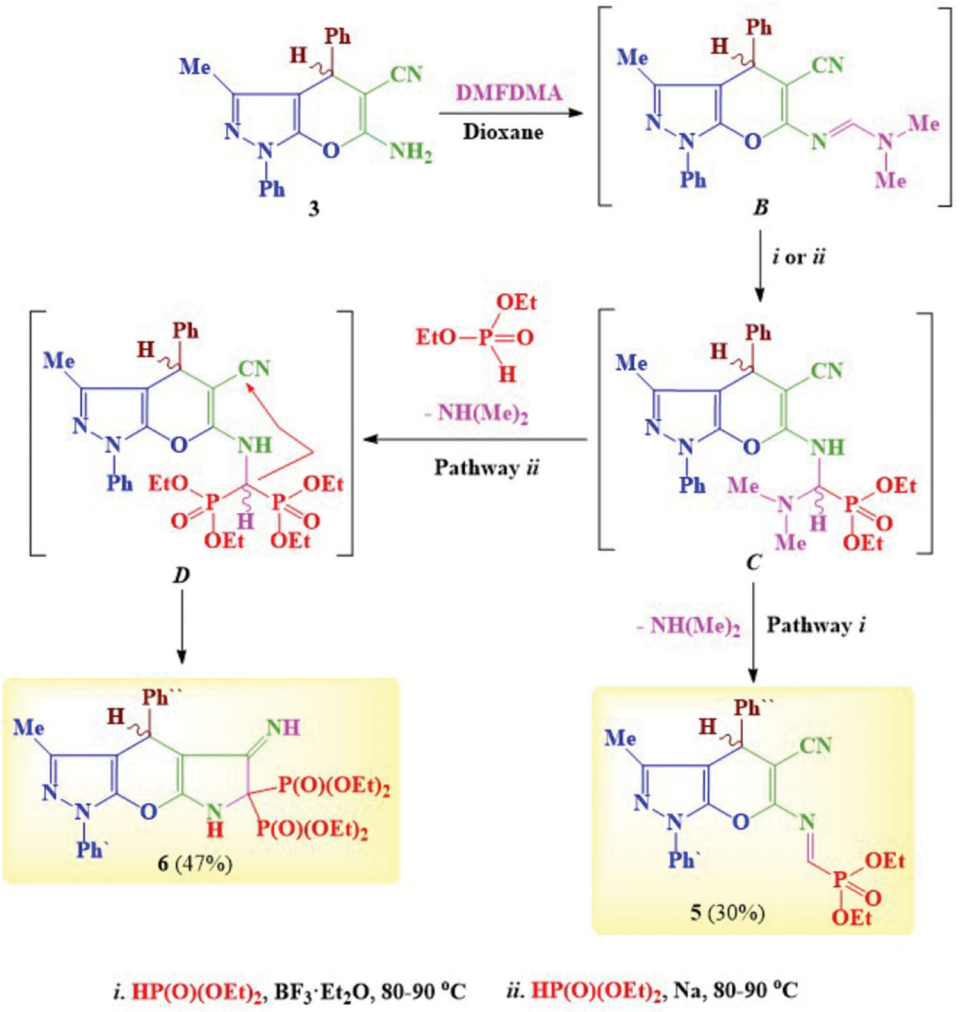
Phosphorus esters have gained significant interest as versatile reagents in the construction of various novel phosphorus heterocycles.[19,22] Thus, diethyl *H*-phosphonate reacted with substrate **3** at 80–90 °C in the presence of $BF_3{\cdot}Et_2O$ as a catalyst to yield the nonisolable intermediate **A** via removal of the ethanol molecule. The latter



Scheme 1. The reaction of substrate **3** with diethyl *H*-phosphonate.

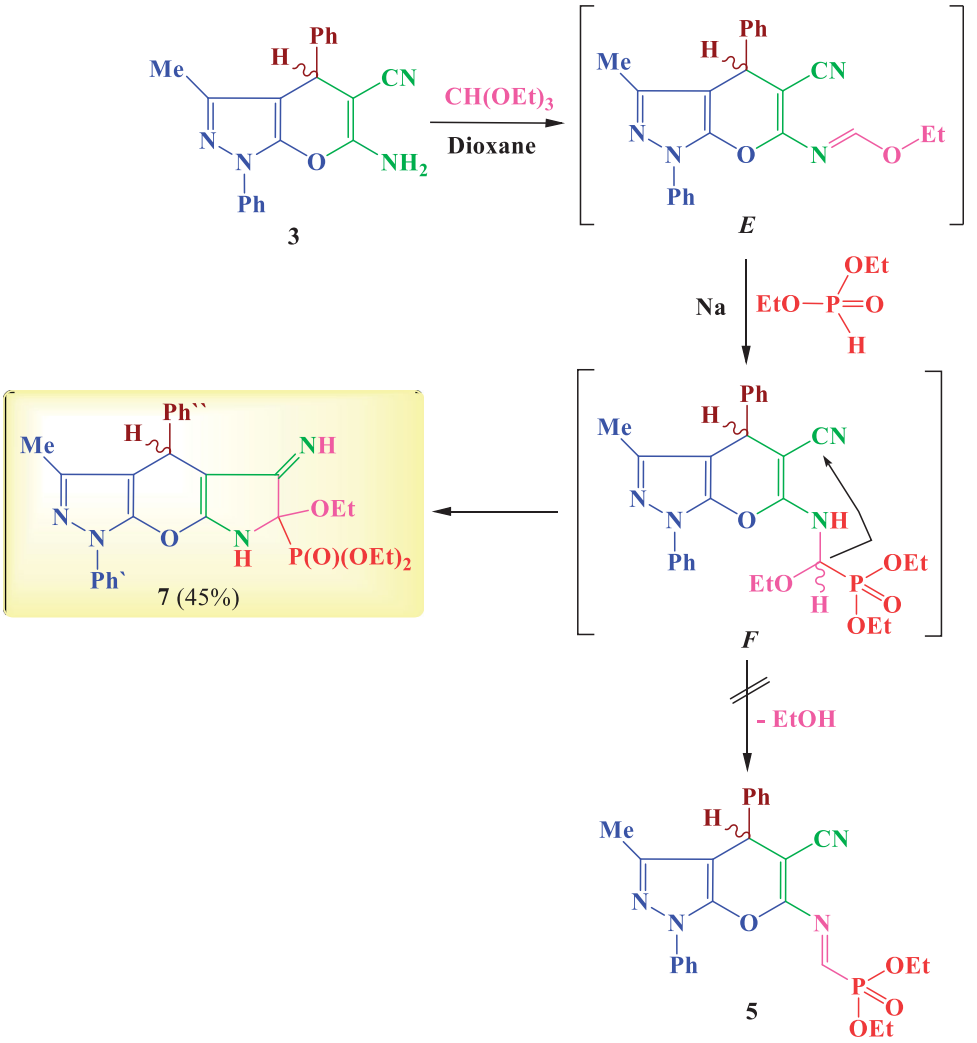
intermediate underwent an intramolecular heterocyclization through addition of *H*-phosphorus atom on the nitrile group to give 6-ethoxy- 5-imino-3-methyl-6-oxido-1,4-diphenyl-1,4,5,7-tetrahydropyrazolo[4′,3′:5,6]pyrano[3,2-*d*][1,2]azaphosphole (**4**) (Scheme 1). The M^+ peak for product **4** was recorded at *m/z* 420 in its mass spectrum, while its ^{31}P-NMR spectrum recorded a singlet at δ 16.2 ppm. Moreover, the ^{1}H-NMR spectrum of compound **4** revealed specific one ethoxy group at δ 1.03 (t, CH_3) and 3.79–3.87 (m, OCH_2) as well as the NH protons resonated as two singlets at δ 9.94 and 11.52 ppm. Furthermore, its ^{13}C-NMR spectrum exhibited some specific carbon atoms at δ 16.1 (CH_3), 60.2 (OCH_2), and a doublet signal at δ 142.6 ppm (J_{PC} = 150.9 Hz) for P–C=NH.[24]

Dimethylformamide dimethylacetal (DMFDMA) is considered one of the very important precursors in the construction of novel bioactive compounds.[25] Thus, heating of substrate **3** with DMFDMA in dry dioxane under reflux for 6 h, followed by addition of diethyl phosphite and BF_3·Et_2O then further heated under reflux for 10 h afforded diethyl{[(5-cyano-3-methyl-1,4-diphenyl-1,4-dihydropyrano[2,3-*c*]pyrazol-6-yl)imino]methyl}phosphonate (**5**) in 30% yield (Scheme 2). When the previous reaction was carried out using a two-fold excess of diethyl phosphite and a catalytic amount of sodium metal, it furnished the interesting novel pyrrolo[3′,2′: 5,6]pyrano[2,3-*c*]pyrazolyl bis(phosphonate) **6** as a sole product in 47% yield (Scheme 2).[26] The formation of products **5** and **6** was explained through condensation of NH_2 group of substrate **3** with the acetal carbon of the DMFDMA reagent giving the nonisolable *N*,*N*-dimethylformimidamide intermediate **B**. The latter intermediate reacted with an equimolar amount of diethyl phosphite in the presence of BF_3·Et_2O or Na metal via a nucleophilic addition of *H*-phosphorus atom at azomethine group to give the nonisolable



Scheme 2. The reaction of substrate **3** with DMfDMA followed by diethyl phosphite.

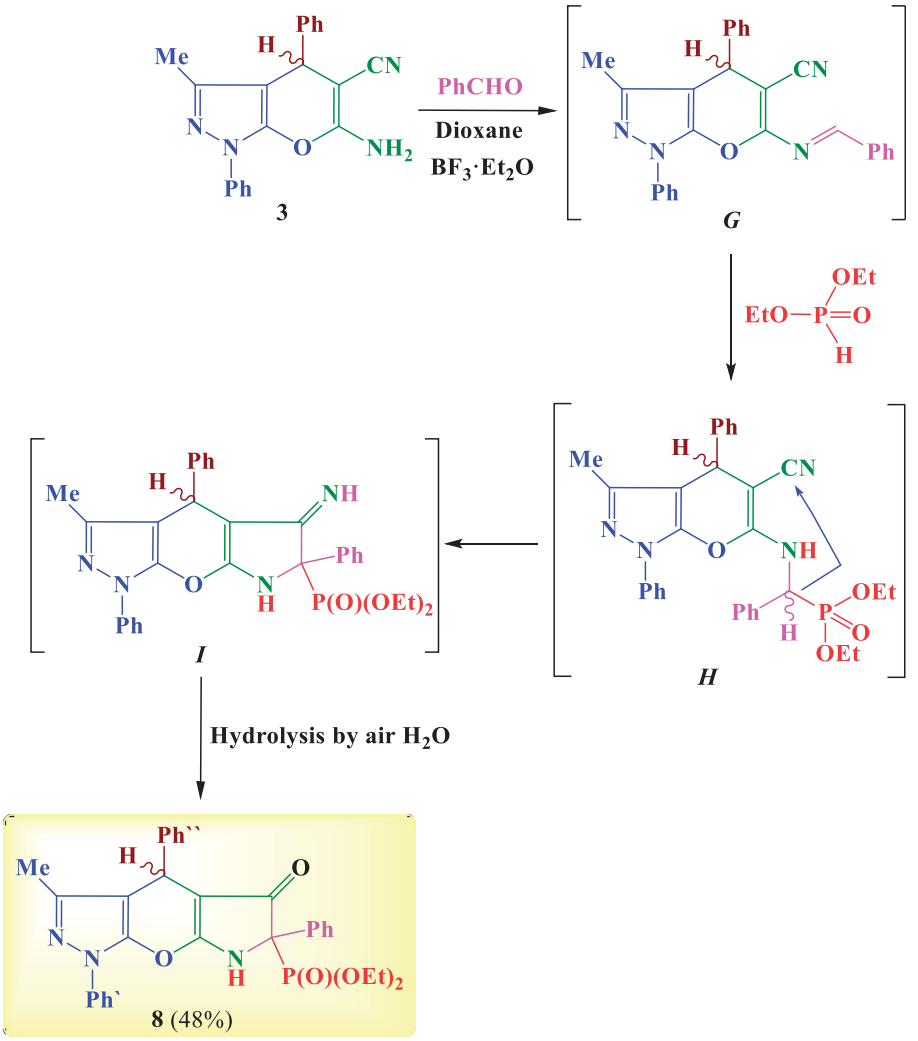
α-aminophosphonate **C** (Scheme 2). In the case of pathway *i*, the intermediate **C** lost one molecule of dimethylamine to yield the isolated product **5**, while in case of pathway *ii*, condensation of the second molecule of diethyl phosphite with the intermediate **C** gave the bis(phosphonate) intermediate **D** with the elimination of dimethylamine molecule (Scheme 2). This intermediate **D** underwent ring closure via the addition of the active CH–P group at the electrophilic C≡N function to isolate the final product **6** (Scheme 2). The IR spectrum of product **5** displayed important absorption bands at 2195 (C≡ N) and 1200 (P=O) cm^{-1}, while its mass spectrum exhibited the M^+ peak at *m/z* 476 (27%). The ^{13}C-NMR spectrum of product **5** exhibited a specific signal relevant to C≡N at δ 111.8 ppm and a doublet signal at δ 143.9 ppm (J=140.5 Hz) attributable to CH–P. Also, its ^{1}H-NMR spectrum affirmed the proposed structure by recording two ethoxy groups and the specific N=CH–P proton as a doublet at δ 8.58 with coupling



Scheme 3. The reaction of substrate **3** with triethyl orthoformate followed by diethyl phosphite.

constant (J=22 Hz). On the other hand, the ^{1}H-NMR spectrum of compound **6** exhibited four ethoxy groups as two multiplets at δ 0.86–1.31 (4 CH_3) and 3.87–4.15 (4 OCH_2) ppm, besides two broad signals at δ 11.85 and 13.97 (2 NH) ppm. Also, its mass spectrum displayed the expected M^+ peak at m/z 614 (24%). Additionally, its ^{13}C-NMR spectrum revealed the specific carbon atom of C–P at δ 53.6 ppm as a doublet signal (J_{PC}=142.6 Hz) and another signal relative to C=NH at δ 144.8 ppm.

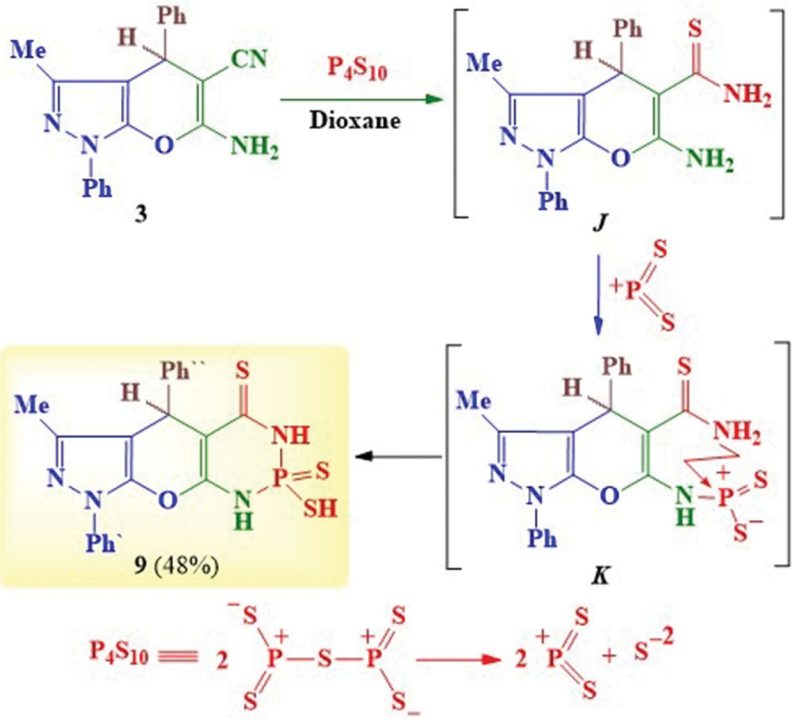
Likewise, treatment of substrate **3** with triethyl orthoformate in dry dioxane followed by addition of a solution of diethyl phosphite containing a catalytic amount of sodium metal yielded diethyl {6-ethoxy-5-imino-3-methyl-1,4-diphenyl-4,5,6,7-tetrahydro-1*H*-pyrrolo[3′,2′:5,6]pyrano[2,3-*c*]pyrazol-6-yl}phosphonate (**7**) and not the expected product **5** (Scheme 3).[27] The formation of compound **7** is similar to the formation of compound **6**. The IR spectrum of product **7** recorded the presence of the important bands of two NH and exocyclic C=N functions at 3425, 3363, and 1624 cm^{-1}, respectively.



Scheme 4. The reaction of substrate **3** with benzaldehyde followed by diethyl phosphite.

Moreover, its ^{1}H-NMR spectrum recorded the chemical shifts of two NH protons as two broad signals at δ 11.82 and 13.98 ppm. In addition, its ^{13}C-NMR spectrum exhibited a doublet signal of C–P at δ 67.0 ppm (J_{PC} = 144.6 Hz), while the carbon atom of C = NH was resonated at δ 146.3 ppm.

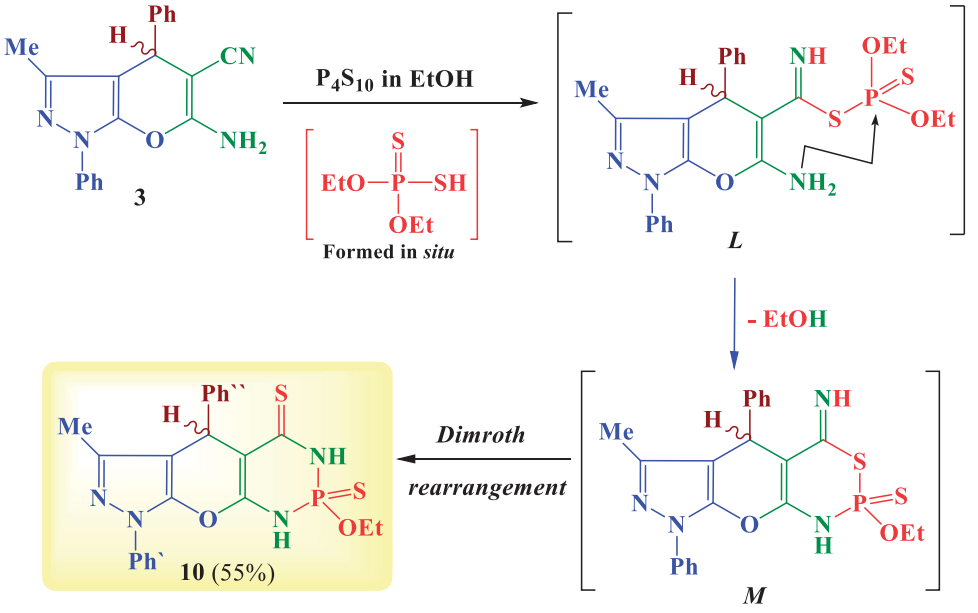
In the same manner, treatment of substrate **3** with benzaldehyde and diethyl phosphite in the presence of $BF_3{\cdot}Et_2O$ as a catalyst in dry dioxane under the Kabachnik-Field reaction[28] afforded the nonisolable α-amniophosphonate **H**. The latter intermediate underwent an intramolecular cyclization via addition of the active hydrogen of CH–P group at the nitrile function followed by auto-hydrolysis to isolate diethyl {3-methyl-5-oxo-1,4,6-triphenyl-4,5,6,7-tetrahydro-1*H*-pyrrolo[3′,2′:5,6]pyrano[2,3-*c*]pyrazol-6-yl}phosphonate (**8**) (Scheme 4). The IR spectrum of product **8** showed absorption bands for NH and C = O functions at 3422 and 1691 cm^{-1}, respectively while its ^{31}P-NMR spectrum recorded a singlet at δ 22.0 ppm. Furthermore, its M^+ peak was



Scheme 5. The reaction of substrate **3** with P_4S_{10} in dioxane.

recorded at *m/z* 555 (12%) in its mass spectrum. The ^{1}H-NMR spectrum of the cyclic aminophosphonate **8** recorded additional multiplets for aromatic protons produced from benzaldehyde reagent when compared with the starting material **3**, besides a singlet relevant to NH proton at δ 11.25 ppm. In its ^{13}C-NMR spectrum, the carbon atom of C–P moiety resonated as a doublet at δ 68.4 ppm with a coupling constant 143.4 Hz while the C = O resonated as a singlet at δ 165.4 ppm.

Lawesson's reagent and phosphorus decasulfide are convenient thionating agents and powerful platforms to construct novel compounds including bioactive phosphorus and sulfur-based heterocycles. Thus, treatment of substrate **3** with phosphorus decasulfide in dry dioxane afforded 2-sulfanyl-6-methyl-5,8-diphenyl-2-sulfido-4-thioxo-3,5,8-trihydropyrazolo[4′,3′:5,6]pyrano[2,3-*d*][1,3,2]diazaphosphinine (**9**) (Scheme 5). The isolated product **9** was formed through the addition of P_4S_{10} reagent on the C≡N group to give the nonisolable thioamide intermediate **J**.[29] The latter intermediate underwent nucleophilic attack of the neighboring NH_2 group at the electrophilic phosphorus atom of the PS_2 fragment, followed by an intramolecular cyclization (Scheme 5).[30] The IR spectrum of compound **9** showed the disappearance of C≡N group and displayed broad absorption bands at 3442, 3349 (two NH), 2664 (SH), and 1195 (C=S) cm^{-1}. Moreover, its ^{1}H-NMR spectrum showed a singlet for SH proton at δ 2.40 ppm and two broad signals at δ 10.02 and 11.86 ppm for two NH protons. Further, the ^{13}C-NMR spectrum of product **9** displayed the carbon atom of C = S at δ 178.4 ppm. In addition, its mass

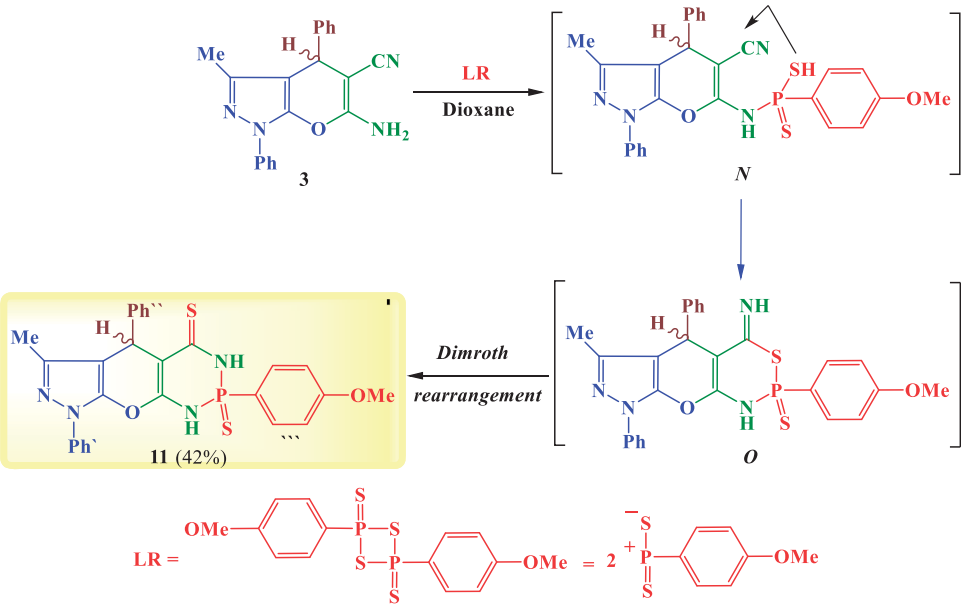


Scheme 6. The reaction of substrate **3** with P_4S_{10} in ethanol.

spectrum recorded the expected M^+ at *m/z* 456 which confirmed the suggested structure.

In the same manner, when compound **3** was reacted with P_4S_{10} in absolute ethanol, it furnished the fused triheterocyclic system **10** in 55% yield (Scheme 6).[31] The mass spectrum was great evidence for the formation of compound **10** which recorded its M^+ at *m/z* 468. Its ^{1}H-NMR spectrum revealed one ethoxy group at δ1.01 (t, CH_3) and 3.41 (q, OCH_2) ppm. Furthermore, its ^{13}C-NMR spectrum displayed the specific carbon atoms of CH_3, OCH_2, and C=S at δ 17.2, 62.2, and 177.1 ppm, respectively. Besides, the ^{31}P-NMR spectrum recorded a singlet at δ 45.2 ppm. The proposed pathway suggested that the chemically active *O,O*-diethyl dithiophosphoric acid intermediate (formed in *situ* by refluxing P_4S_{10} in absolute ethanol) underwent a nucleophilic attack of SH group at the C≡N group of substrate **3** forming the intermediate **L**. The latter intermediate removed ethanol molecule through an intramolecular cyclization followed by *Dimroth* rearrangement to produce the desired product **10** (Scheme 6).

On the other hand, treatment of compound **3** with Lawesson's reagent (LR) in dry dioxane gave the nonisolable intermediate **N** which underwent ring closure through a nucleophilic attack of SH group at phosphorus atom of -SP(S)Ar fragment to form the nonisolable intermediate **O**. *Dimroth* rearrangement of the latter intermediate led to the formation of 2-(4-methoxyphenyl)-6-methyl-5,8-diphenyl-2-sulfido-4-thioxo-3,5,8-trihydropyrazolo[4′,3′:5,6]pyrano[2,3-*d*][1,3,2]diazaphosphinine (**11**) (Scheme 7).[32,33] The ^{31}P-NMR spectrum of product **11** displayed a signal at δ 50.1 ppm. Also, its ^{13}C-NMR spectrum showed specific signals at δ 54.4 and 175.9 ppm relative to MeO and C=S groups, respectively. In addition, the ^{1}H-NMR spectrum recorded a singlet at δ 3.86 ppm relevant to MeO protons and two broad signals at δ 11.56 and 13.16 ppm for two NH protons. The mass and IR spectra of product **11** were in accordance with the suggested structure (see experimental section).

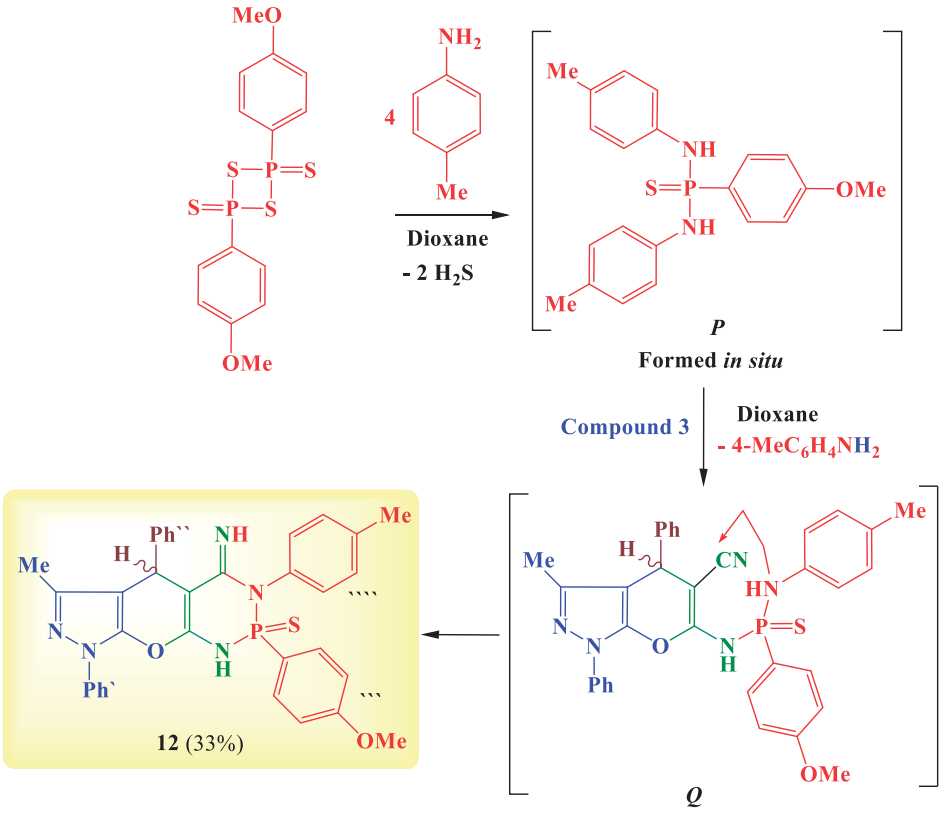


Scheme 7. The reaction of substrate **3** with LR in dioxane.

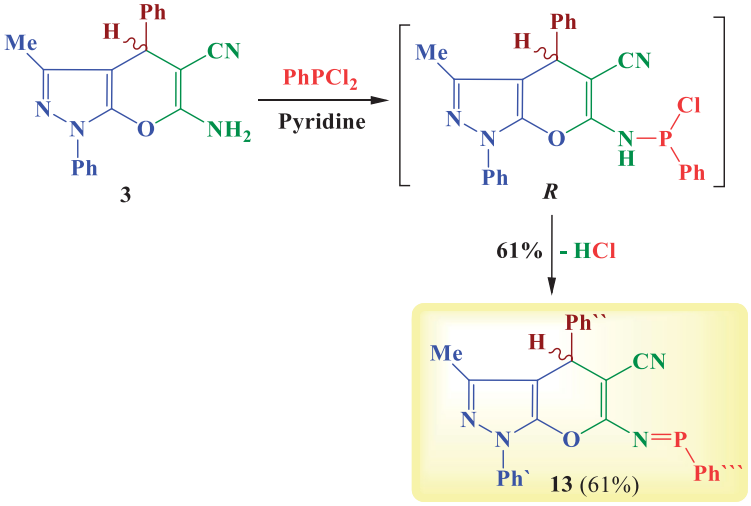
Furthermore, compound **3** reacted with *P*-(4-methoxyphenyl)-*N*,*N*′-bis(4-methylphenyl) phosphonothioic diamide **P** (formed by the reaction of LR with *p*-toluidine in molar ratio 1:4)[34] to produce the nonisolable intermediate **Q** with the removal of *p*-toluidine molecule. Ring closure of the latter intermediate by attacking of NH group at the C≡N group led to the sole product **12** in 33% yield (Scheme 8). The IR spectrum of product **12** showed characteristic absorption bands due to two NH and P = S functions at 3289, 3191, and 798 cm^{-1}, respectively. A strong confirmation of the structure **12** was its ^{1}H-NMR spectrum which exhibited three singlets at δ 2.02, 2.31, and 3.89 ppm relevant to two Me and one MeO groups. Also, other signals appeared at δ 10.36 and 11.19 ppm for the two NH protons. In addition, its ^{13}C-NMR spectrum displayed characteristic signals for carbon atoms of 2 Me, MeO, and C = NH at δ 12.1, 13.9, 55.0, and 154.9 ppm, respectively. The mass spectrum of product **12** recorded *m/z* 496 (M^{+}–$MeOC_4H_6$, 12%).

Phenyl phosphorus halides play vital roles in the construction of bioactive organophosphorus compounds.[35] This encouraged us to study the reactivity of substrate **3** toward some phenyl phosphorus halides. Thus, compound **3** condensed with P,P-dichloro(phenyl)phosphine in dry pyridine to furnish 3-methyl-1,4-diphenyl-6-[(phenylphosphanylidene)amino]-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**13**) (Scheme 9). The IR spectrum of the latter product showed a characteristic band at 2223 cm^{-1} relevant to the C≡N function confirming the suggested structure. In addition, the carbon atom of C≡N resonated at δ 107.7 in its ^{13}C-NMR spectrum. The mass spectrum of compound **13** exhibited its M^{+} peak at *m/z* 434 (6%).

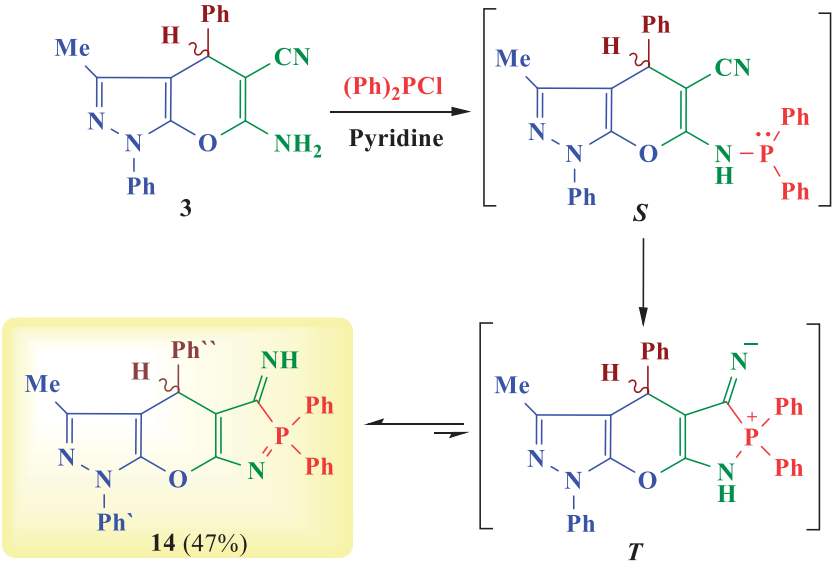
Next, P-chlorodiphenylphosphine condensed with compound **3** in dry pyridine to give the nonisolable intermediate **S**. The latter intermediate underwent ring closure through the addition of the nucleophilic phosphorus atom at the electrophilic carbon atom of C≡N group giving the intermediate **T** which was rearranged into 5-imino-3-



Scheme 8. The reaction of substrate **3** with the intermediate *P* (formed *in situ* from LR and *p*-toluidine).



Scheme 9. The reaction of substrate **3** with $PhPCl_2$ in pyridine.



Scheme 10. The reaction of substrate **3** with $(Ph)_2PCl$ in pyridine.

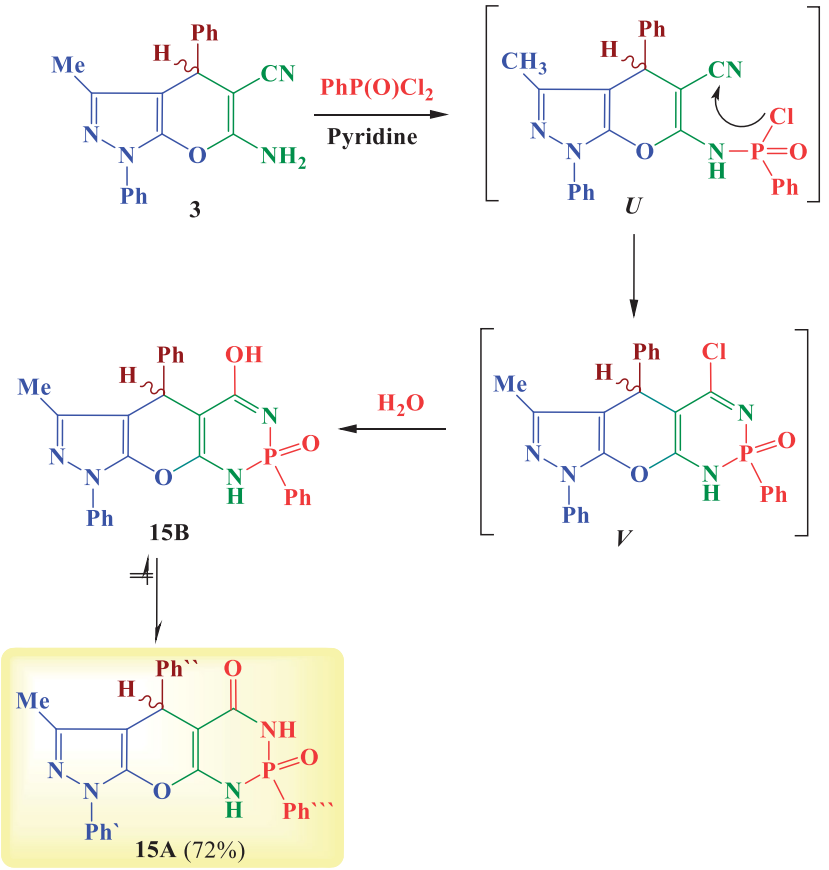
methyl-1,4,6,6-tetraphenyl-1,4-dihydro-5*H*-6λ^5-pyrazolo[4′,3′:5,6]pyrano[3,2-*d*][1,2]aza-phosphole (**14**) (Scheme 10). The IR spectrum of product **14** displayed absorption bands for NH and exocyclic C=N functions at 3354 and 1603 cm^{-1}, respectively. Its ^{1}H-NMR spectrum displayed a singlet relevant to NH proton at δ 13.01 ppm, beside multiplet signals for twenty aromatic protons at δ 6.98–7.62 ppm. In addition, the ^{13}C-NMR spectrum revealed the existence of a doublet at δ 150.5 ppm for C=NH due to coupling with phosphorus atom with J_{PC}=60 Hz.

Finally, addition of phenyl phosphonic dichloride to substrate **3** in dry pyridine furnished 2,5,8-triphenyl-3,5,8-trihydropyrazolo[4′,3′:5,6]pyrano[2,3-*d*][1,3,2]diazaphosphinine **15** in 72% yield (Scheme 11). The suggested pathway for this product based on condensation between compound **3** and phosphorus reagent to give the nonisolable intermediate **U**. The nucleophilic addition of chlorine atom at C≡N group in the latter intermediate afforded the 4-chloro-1,3,2-diazaphosphinine intermediate **V**. When the intermediate **V** was treated with ice-water for 30 minutes, it gave the final product **15** in the form of **15A** and not the other tautomeric form **15B** according to its spectroscopic data (Scheme 11). The IR spectrum confirmed the suggested structure and showed characteristic absorption bands at 3425, 3363, and 1679 cm^{-1} for two NH and C=O functions, respectively. The expected M^+ peak of compound **15** was recorded at *m/z* 468 in its mass spectrum while its ^{31}P-NMR spectrum showed a singlet at δ 34.31 ppm. The two NH protons resonated as two singlets at δ 12.68 and 14.63 ppm in its ^{1}H-NMR spectrum. Also, the ^{13}C-NMR spectrum exhibited the important signal of C=O at δ 164.8 ppm.

Biological evaluations

Antimicrobial activity

The in *vitro* antimicrobial activities of the synthesized products were screened toward three bacterial strains, namely *Streptococcus pyogenes, Staphylococcus aureus*, and



Scheme 11. The reaction of substrate 3 with $PhP(O)Cl_2$ in pyridine.

Escherichia coli, and three fungal strains, namely *Aspergillus niger*, *Aspergillus clavatus*, and *Candida albicans*.[36,37] Minimum inhibitory concentration (MIC) of all synthesized compounds was determined and given in Table 1. Ketoconazole and Ciprofloxacin as standard drugs were used for the antifungal and antibacterial activities, respectively. Variable antimicrobial activities toward the used microorganisms were recorded for the synthesized compounds. Compounds **5**, **9**, and **13** did not record any acceptable inhibitory effects toward all bacteria and fungi organisms in comparison with substrate **3** and standard drugs. Compound **12** exhibited relatively moderate effects against all organisms. On the other hand, products **10**, **11**, **14**, and **15** showed good antibacterial and antifungal effects except compound **11** that recorded excellent antifungal activities equal to the standard drug. Furthermore, compounds **4**, **6**, **7**, and **8** displayed excellent antimicrobial activities toward most of the microorganisms except compound **8** that showed moderate to good effects against the fungi organisms. Generally, the relationship between the structure and activity indicated that the introduction of phosphorus heterocycles and phosphonate groups to pyranopyrazole system can increase the antimicrobial properties. Therefore, the presence of 1,2-azaphosphole ring fused with the pyranopyrazole system in compounds **4** and **14** exhibited extremely good to excellent antibacterial and antifungal activities. In addition, the existence of 1,3,2-diazphosphinine rings

Table 1. The in *vitro* antimicrobial activities as minimum inhibitory concentration (MIC, μg/mL)[a] for the synthesized compounds.

	Bacterial strains			Fungal strains		
Compound	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Escherichia coli*	*Aspergillus niger*	*Candida albicans*	*Aspergillus clavatus*
3	250	250	250	250	250	500
4	31.25	31.25	31.25	31.25	31.25	31.25
5	250	250	250	125	125	125
6	31.25	31.25	31.25	31.25	31.25	31.25
7	31.25	31.25	31.25	31.25	31.25	31.25
8	31.25	31.25	31.25	62.5	62.5	62.5
9	250	250	250	250	250	250
10	62.5	62.5	62.5	62.5	125	62.5
11	62.5	62.5	62.5	31.25	31.25	31.25
12	125	125	125	125	125	125
13	250	250	250	500	250	250
14	62.5	62.5	62.5	62.5	62.5	62.5
15	62.5	62.5	62.5	62.5	62.5	62.5
Ciprofloxacin	31.25	31.25	31.25			
Ketoconazole				31.25	31.25	31.25

[a]All results are expressed as mean ± SD from three experiments ($n = 3$).

Table 2. The cytotoxcity of the products **4**, **6**, **7**, and **8** using the Brine Shrimp bioassay.

Compound	Lethal Concentration (LC_{50}, μg/mL)[a]
4	1.65
6	7.32
7	5.67
8	2.36
Gallic acid	4.51

[a]All results are expressed as mean ± SD from three experiments ($n = 3$).

possess P=S and P=O groups as in products **10**, **11**, and **15** exhibited significant improvements in the antimicrobial effects in comparison with the starting material **3**. Interestingly, the design of skeleton pyrrolo[3′,2′:5,6]pyrano[2,3-*c*]pyrazole bearing diethyl phosphonate groups in compounds **6**, **7**, and **8** caused excellent inhibitory effects nearly to the standard drugs toward all the microorganisms except compound **8** which recorded good inhibitory effects against only fungal strains.

The cytotoxicity activity

The LC_{50} values of the most bioactive synthesized compounds **4**, **6**, **7**, and **8** against brine shrimp death were evaluated by the reported method using Gallic acid as a standard.[38,39] The results are summarized in Table 2. The lowest LC_{50} values were found in the case of compounds **4** and **8** at 1.65 and 2.36 μg/mL, respectively, indicating their higher cytotoxicity. On the other hand, compounds **6** and **7** displayed brine shrimp death at 7.32 and 5.67 μg/mL, respectively. Therefore, both compounds **6** and **7** can be active potent antibacterial and antifungal agents with lower cytotoxicity. So, we can conclude that skeleton pyrrolo[3′,2′:5,6]pyrano[2,3-*c*] pyrazole bearing diethyl phosphonate groups is the most active potent antimicrobial agent with lower toxicity in host cells.

Table 3. The *in vitro* antioxidant activities as inhibitory concentration (IC_{50}, μg/mL) for the synthesized compounds by using DPPH and H_2O_2 methods.

	Inhibitory concentration (IC_{50}, μg/mL)	
Compound	DPPH method	H_2O_2 method
3	16.21 ± 0.29	22.53 ± 0.51
4	11.86 ± 0.31	17.05 ± 0.51
5	35.12 ± 0.29	40.23 ± 0.54
6	10.12 ± 0.52	16.69 ± 0.31
7	13.25 ± 0.23	19.36 ± 0.62
8	14.67 ± 0.66	21.49 ± 0.34
9	25.87 ± 0.00	30.53 ± 0.00
10	18.38 ± 0.33	26.13 ± 0.26
11	15.63 ± 0.23	24.65 ± 0.29
12	24.36 ± 0.39	28.52 ± 0.27
13	19.35 ± 0.21	29.14 ± 0.50
14	12.43 ± 0.21	20.84 ± 0.33
15	36.42 ± 0.76	50.46 ± 0.25
Ascorbic acid	10.23 ± 0.23	18.62 ± 0.52

Antioxidant activity

The synthesized compounds were investigated for their in *vitro* antioxidant properties by DPPH[40,41] and H_2O_2[42] methods. The ascorbic acid was used as standard control. The lower IC_{50} values indicated to a higher antioxidant activity. The measured antioxidative properties of the synthesized compounds showed promising radical scavenging abilities (Table 3). The results revealed that compounds **5**, **9**, **12**, and **15** exhibited poor radical scavenging abilities in comparison with ascorbic acid. However, remarkable activities were observed with products **3**, **8**, **10**, **11**, and **13**. Interestingly, compounds **4** and **14** exhibited significant antioxidant activities due to the presence of 1,2-azaphosphole or 1,3,2-diazaphosphinine ring, two acidic NH groups, and extended conjugation. Besides, the connection of the pyrrole ring with NH and phosphonate group in compounds **6** and **7** caused promising antioxidative properties.

Antiproliferative activity

The *in vitro* antiproliferative activities of the synthesized compounds were determined by using the SRB assay[43] against three human cancer cell lines, mammary gland breast cancer (MCF-7), liver cancer (HepG-2), and human colon cancer (HCT-116) in comparison with doxorubicin as reference drug. The IC_{50} values of cytotoxic activities of the synthesized compounds **3**-**15** are presented in Table 4. It is obvious that the thirteen tested compounds displayed variable degrees of inhibitory effects on the tested human tumor cell lines. Compounds **3**, **5**, **12**, **13**, and **14** revealed no acceptable cytotoxic activities on all tumor cell lines. For action on MCF-7, the highest cytotoxic effects were demonstrated by compounds **4**, **6**, **8**, **9**, **10**, and **15** which showed the IC_{50} values in ranges from 3.7 to 8.4 μg/mL in comparison with doxorubicin (IC_{50} = 1.4 μg/mL). Moderate cytotoxic effects were also recorded by compounds **7** and **11** with values of IC_{50} = 16.9 and 14.5 μg/mL, respectively. The HepG-2 cell line was demonstrated to be

Table 4. The IC_{50} (μg/mL) of the synthesized compounds against different tumor cell lines.

	IC_{50} (μg/mL)		
Compound	MCF-7	HepG-2	HCT-116
3	23.6 ± 0.5	120.8 ± 6.6	46.8 ± 2.4
4	8.3 ± 0.4	10.5 ± 0.5	3.7 ± 0.7
5	73.5 ± 1.6	102.5 ± 4.1	27.9 ± 0.9
6	8.4 ± 0.3	18.8 ± 0.8	7.7 ± 0.6
7	16.9 ± 0.5	20.3 ± 0.3	10.7 ± 1.3
8	5.04 ± 0.3	14.5 ± 2.2	22.1 ± 1.5
9	3.7 ± 0.7	6.4 ± 0.2	10.6 ± 0.7
10	5.6 ± 1.03	34.4 ± 2.5	30.7 ± 1.5
11	14.5 ± 0.4	47.5 ± 2.1	7.6 ± 0.5
12	85.9 ± 3.1	45 ± 3.2	52.8 ± 1.5
13	46.3 ± 5.2	1732.3 ± 6.6	361.1 ± 5.9
14	388.4 ± 9.9	97.3 ± 5.2	536.1 ± 4.2
15	8.1 ± 0.9	87.1 ± 3.8	38.2 ± 3.6
Doxorubicin	1.4 ± 0.07	1.6 ± 0.04	2.0 ± 0.03

the least sensitive among the tested carcinoma cells. However, the highest sensitivity to compound **9** as its growth inhibitory effect was the displayed value of IC_{50} at 6.4 μg/mL. The moderate activity was shown by compounds **4**, **6**, **7**, and **8** which have IC_{50} values at 10.5, 18.8, 20.3, and 14.5 μg/mL, respectively. Compounds **10** and **11** revealed weak cytotoxicity properties. In addition, the HCT-116 cancer cell line was weakly affected by compounds **8**, **10**, and **15**. However, a significant growth inhibitory effect was noticed by compounds **4**, **6**, and **11** with IC_{50} values 3.7, 7.7, and 7.6 μg/mL, respectively, compared with doxorubicin (IC_{50}=2.0 μg/mL). The residual compounds **7** and **9** showed moderate antiproliferative activities on HCT-116 cell line with IC_{50} values 10.7 and 10.6 μg/mL, respectively. These results indicated a specific therapeutic index for those phosphorus compounds that have an auspicious perspective to be substances for the candidate for anticancer.

In this research, the following relationships of structure with activity were assumed by comparing the structures of the synthesized phosphorus compounds to their experimental cytotoxicity. In general, the study revealed that the new molecular frames exhibited cytotoxicity properties more slightly than the starting substrate **3**. The presence of phosphorus heterocycles or phosphonate groups combined with the pyranopyrazole moiety may be causing a broad spectrum of antiproliferative effects on various carcinoma cells (MCF-7, HepG-2, and HCT-116). The presence of phosphoryl moiety such as $P(O)(OEt)_2$ and N = P-Ph as directly attached on the pyranopyrazole system in compounds **5** and **13** reduced the cytotoxic effects in comparison with the substrate **3**. The construction of 1,3,2-diazaphosphinine ring fused with pyranopyrazole moiety in products **9**, **10**, **11**, **12**, and **15** exhibited acceptable antiproliferative properties. Remarkably, the existence of C=S and P=S group in the 1,3,2-diazaphosphinine unit as compounds **9**, **10** and **11**, enhanced the cytotoxic activity more than C=NH and C=O groups of 1,3,2-diazaphosphinine in compounds **12** and **15**. Interestingly, the design of 1,2-azaphosphole ring fused with pyranopyrazole core as in compounds **4** and **14** led to variable toxic activities. This indicated that the presence of cyclic NH-P(O)OEt system increased the cytotoxic activity more than $N=P(Ph)_2$ system. On the other hand, the introduction of pyrrole ring bearing diethyl phosphonate groups to pyranopyrazole core

as in compounds **6**, **7**, and **8**, increased the antiproliferative activities. Further, the presence of two diethyl phosphonate groups improved the antiproliferative potency of more than one diethyl phosphonate group.

Apoptotic effect

Apoptosis is a form of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay.

The treated tumor cell lines MCF-7, HepG-2, and HCT-116 were stained with acridine orange and ethidium bromide for 48 h. After, their examination under a fluorescent microscope, the cells appeared in the form of four colors as follows: living cells (normal green nucleus with normal, round, intact nuclei, and cytoplasm that indicates the viability of the cell control), early apoptotic (early-stage apoptosis is represented by changes and ultimate loss of the mitochondrial membrane potential, they appeared as a bright green nucleus with fragmented chromatin), necrotic cells (necrosis is caused by factors external to the cell or tissue, such as infection, or trauma which result in the unregulated digestion of cell components, they appeared as uniformly bright red-stained cell nuclei) and late apoptotic (late-stage apoptosis that can be identified by looking at the defragmentation of DNA, they appeared as red-stained nuclei with chromatin fragmentation) (Supplementary Figures 1–3).[44,45] The highly early apoptotic cell death was observed in all types of treated tumor cells in all compounds. The considerable necrotic activity compared with other compounds was displayed only in compound **10**, while the other compounds did not cause the death of the necrosis pathway in all cancer cells. The late apoptosis was obviously observed with compounds **3**, **5**, and **7** especially against MCF-7 and HepG-2 cell lines, while compounds **3**, **7**, and **14** were observed in the case of HCT-116 cell line (Supplementary Figures 4–6). The obtained results were referenced to the control that absence of any cell death manifestations.

Experimental

The melting points were determined in an open capillary tube on a digital Stuart SMP-3 apparatus. Infrared spectra were measured on FT-IR (Nicolet IS10) spectrophotometer using KBr disks and Perkin-Elmer 293 spectrophotometer using KBr disks. ^{1}H- and ^{13}C-NMR spectra were measured on Gemini-300BB spectrometer (400 and 100 MHz), using DMSO-d_6 as a solvent and TMS (δ) as an internal standard. ^{31}P-NMR spectra were measured on a Bruker (162 MHz) spectrophotometer using DMSO-d_6 as a solvent, TMS as an internal standard, and 85% H_3PO_4 as an external reference. Mass spectra were recorded on direct probe controller inlet part to single quadrupole mass analyzer in (Thermo Scientific GCMS). Elemental microanalysis was performed on Perkin-Elmer 2400II at the Chemical War department, Ministry of Defense. The purity of the synthesized compounds was checked by thin-layer chromatography (TLC) and elemental microanalysis. The biological evaluations were measured in the King Khalid University, Abha, KSA.

Synthesis of 6-ethoxy-5-imino-3-methyl-6-oxido-1,4-diphenyl-1,4,5,7-tetrahydropyrazolo [4′,3′:5,6]pyrano[3,2-d][1,2]azaphosphole (4)

A mixture of diethyl phosphite (1.5 mL, 10 mmol) and compound **3** (1.64 g, 5 mmol) in the presence of trifluoroboron etherate (0.1 mL) was fused on water bath at 80–90 °C for 6 h. The excess of reagent was evaporated under vacuum. The formed semi-solid was treated with *n*-hexane. The isolated solid was filtered off and crystallized from EtOH to give yellow solid in 42% yield (0.88 g); mp 166–168 °C. IR (KBr), (*ν* max, cm^{-1}): 3267 (br, 2NH), 3068 (C–H_{arom}), 2915, 2852 (C–H_{aliph}), 1658 (C = NH), 1604, 1581 (C = C), 1213 (P = O), 1081 (P–O–C). ^{1}H-NMR (400 MHz, DMSO-d_6): 1.03 (t, 3H, J = 6.8 Hz, CH_3), 1.97 (s, 3H, CH_3), 3.79–3.87 (m, 2H, OCH_2), 4.95 (s, 1H, H-4), 7.11–7.86 (m, 10H, Ph–H), 9.94 (s, 1H, NH), 11.52 (s, 1H, NH). ^{13}C-NMR (100 MHz, DMSO-d_6): 12.0 (CH_3), 16.1 (CH_3), 34.2 (C-4), 60.2 (OCH_2), 81.1 (C-4a), 117.7 (C-3a), 120.4 (C-2′,6′$_{phenyl}$), 124.5 (C-4″$_{phenyl}$), 126.0 (C-4′$_{phenyl}$), 127.5 (C-3″,5″$_{phenyl}$), 128.3 (C-2″,6″$_{phenyl}$), 129.3 (C-3′,5′$_{phenyl}$), 135.9 (C-1″$_{phenyl}$), 140.0 (C-1′$_{phenyl}$), 142.6 (d, J = 150.9 Hz, C-5), 144.9 (C-3), 151.2 (C-8a), 159.5 (C-7a). ^{31}P-NMR (162 MHz, DMSO-d_6): 16.2 ppm. MS (*m/z*, I %): 420 (M^+, 16%). Anal. Calcd. for $C_{22}H_{21}N_4O_3P$ (420.21): C, 62.85%; H, 5.03%; N, 13.33%. Found: C, 62.59%; H, 4.88%; N, 13.01%.

Conclusion

We suggested a simple, efficient, one-pot, three-component method for the construction of new functionalized pyrano[2,3-*c*]pyrazolyl and pyrrolo[3′,2′:5,6]pyrano[2,3-*c*]pyrazolyl phosphonates, 2-sulfido(oxido)-pyrazolo[4′,3′:5,6]pyrano[2,3-*d*][1,3,2]diazaphosphinines and pyrazolo[4′,3′:5,6]pyrano[3,2-*d*][1,2]azaphospholes. The suggested pathway depended on reaction substrate **3** with some phosphorus reagents under different conditions. The synthesized compounds were screened *in vitro* for their antimicrobial, antioxidant, and anticancer activities. Both compounds **6** and **7** recorded potent antibacterial and antifungal activities, while compounds **4** and **6** showed promising antioxidative properties. For antiproliferative activity, compounds **4** and **9** showed the most potent cytotoxic effects.

Full experimental details and spectral data of the synthesized compounds can be accessed on the publisher's website.

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