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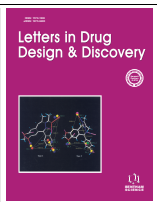
RESEARCH ARTICLE



A Facile Synthesis and Drug Design of Some New Heterocyclic Compounds Incorporating Pyridine Moiety and Their Antimicrobial Evaluation

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Abstract: ***Background*:** An efficient synthesis of *hitherto* unreported 4-heteroarylpyridines was described *via* regioselective 1,3-dipolar cycloaddition reactions of 3-(dimethylamino)-1-(pyridin-4-yl)prop-2-en-1-one (**2**) with nitrilimines **4a-h** to afford the corresponding pyrazole derivatives **6a-h**. Hydrazinolysis of **6a-c,e-f** yielded the respective pyrazolopyridazines **7a-f**. The enaminone **2** reacts also with 6-aminothiouracil (**8**) to yielded thione **9**. The reaction of **9** with hydrazonoyl chlorides **3** yielded products **13a-h**. Pyridine analogs substituted in the 4-position with a pyridinones **18**, **20**, **22** or naphthofuran **24** were also synthesized. The structures of the newly synthesized compounds were confirmed by spectral data and elemental analyses.

***Method*:** The newly synthesized products were evaluated for their antimicrobial activities.

***Results*:** The results revealed that compounds **18** and **24** have good activities compared with Cefepime reference drug. Moreover, the computational studies using MOE 2014.09 software are confirming the results in biological activity.

Keywords: Acetylpyridine, antimicrobial activity, enaminone, hydrazonyl halides, molecular docking.

1. INTRODUCTION

Functionalized pyridine derivatives have received much attention due to their diverse biological activities such as antimicrobial [1-4], anticonvulsant [5], antiviral [6], anti-HIV [7], and antimycobacterial [8], anticancer and anti-inflammatory [9, 10] activities. Also some pyridine derivatives emerged as integral backbones of over 7000 existing drugs [11, 12]. Furthermore some fused pyridines have considerable interest due to several biological activities [13]. In the last two decades we have been involved in a program aiming to synthesize functionally substituted heterocyclic compounds with anticipated biological activities that can be used as biodegradable agrochemicals from cheap laboratory available starting materials [14-22]. In the frame of this program, some new functionally substituted azoles, azines and / or azoloazines carrying a pyridyl moiety were synthesized and screened for their antimicrobial activity using 4-acetylpyridine **1** as starting material to fulfill this objective (Scheme **1**). Enaminones are versatile poly-dentate reagents that have been explored extensively in the last decade as building blocks in organic synthesis [23-28]. Thus, compound **1** was transformed into the enaminone **2** which was used as our actual starting compound to synthesize our target compounds.

2. RESULTS AND DISCUSSION

2.1. Chemistry

3-(Dimethylamino)-1-(pyridin-4-yl)prop-2-en-1-one (**2**) was prepared according to literature method [29] from the reaction of 4-acetylpyridine **1** with dimethylformamide dimethylacetal (DMF-DMA) (Scheme **1**). Treatment of the enaminone **2** with nitrilimines **4a-h**, [liberated in situ from the corresponding hydrazonoyl halides **3a-h**, respectively, with triethylamine in refluxing toluene], it afforded the 3,4-disubstituted-1*H*-pyrazoles **6a-h**, respectively (Schemes **1**). The latter reaction products were assumed to be formed *via* initial 1,3-dipolar cycloaddition of the nitrilimines **4a-h** to the activated double bond in compound **2** to afford the non-isolable cycloadducts **5a-h** which undergo loss of dimethylamine yielding the final pyrazole derivatives **6a-h** [30-32].

The structures of the products **6a-h** were in agreement with their spectral and analytical data. For example, the ^{1}H

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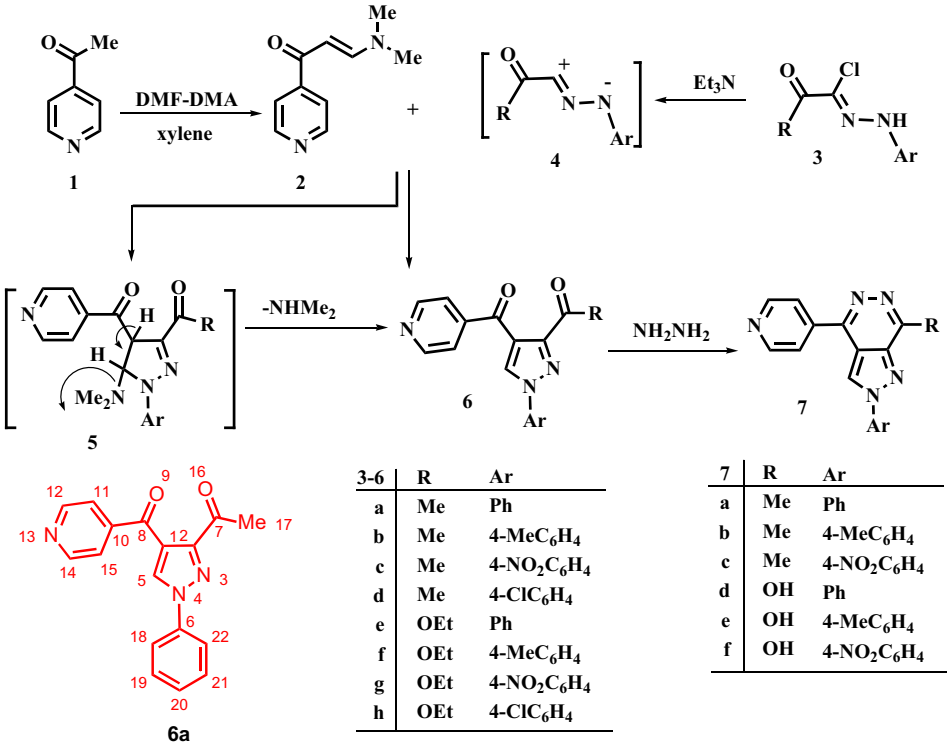


3-6	R	Ar
a	Me	Ph
b	Me	4-MeC_6H_4
c	Me	4-$NO_2C_6H_4$
d	Me	4-ClC_6H_4
e	OEt	Ph
f	OEt	4-MeC_6H_4
g	OEt	4-$NO_2C_6H_4$
h	OEt	4-ClC_6H_4

7	R	Ar
a	Me	Ph
b	Me	4-MeC_6H_4
c	Me	4-$NO_2C_6H_4$
d	OH	Ph
e	OH	4-MeC_6H_4
f	OH	4-$NO_2C_6H_4$

Scheme 1. Synthesis of pyrazoles **6a-h** and pyrazolopyridazines **7a-f**.

NMR spectra of the isolated products **6a-h** revealed in each case a singlet signal in the region of 8.45-8.79 which indicates the presence of the pyrazole H-5 rather than H-4 in addition to the characteristic signals assignable for CH_3CO, CH_3CH_2COO and PhNHCO groups. The mass spectra of all products **6a-h** exhibited in each case, a molecular ion peak at the correct molecular weight for the respective compound (see Experimental).

The structures of pyrazoles **6a-c,e-f** were further confirmed chemically *via* their reaction with hydrazine hydrate to afford pyrazolo[3,4-*d*]pyridazines **7a-f**, respectively (Scheme **1**). The structures of products **7a-f** were elucidated by elemental and spectral (IR, ^{1}H NMR, mass) data. The IR spectra of the isolated products revealed, in each case, the absence of the two bands corresponding to two carbonyl groups in pyrazole **6**. Their mass spectra showed, in each case, a peak corresponding to the molecular ion.

The reaction of enaminone **2** with 6-amino-2-thioxo-(1*H*)-pyrimidin-4-one (**8**) was also investigated and can produce 5-(pyridin-4-yl)-2-thioxo-2,3-dihydropyrido[2,3-*d*]pyrimidin-4(1*H*)-one (**9**) or the isomeric structure **10** (Scheme **2**). The ^{1}H NMR spectrum of the latter reaction product revealed a doublet signal at δ 8.26 ppm assignable to the pyridine H-2 proton rather than the pyridine H-4 proton [33] which is consistent with isomeric structure **9**. Furthermore, the literature reports support that the reaction of heterocyclic amines to the double bond of the enaminone occurs with concurrent elimination of dimethylamine [28]. On the basis of these findings, structure of **10** was discarded and the isolated product from the studied reaction was assigned structure **9**.

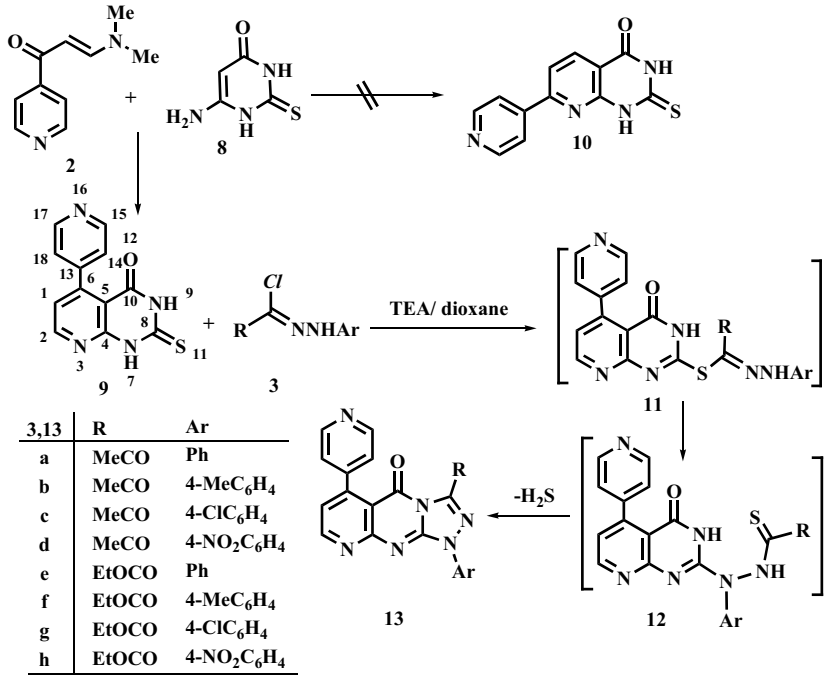
Treatment of thione **9** with hydrazonoyl halides **3a-h** in dioxane, in the presence of triethylamine under reflux gave in each case a single product consistent with structure **13** (Scheme **2**) based on spectroscopic data (IR, ^{1}H NMR and MS) and elemental analyses (see Experimental).

As depicted in Scheme **2**, the reaction proceeded through S-alkylation to give the non-isolable S-alkylated intermediates **11** followed by Smiles rearrangement to afford the thiohydrazide intermediates **12** which underwent *in situ* cyclization via hydrogen sulfide elimination to give the final products **13.**

Treatment of enaminone **2** with hippuric acid **14** in refluxing acetic anhydride led to the formation of a product that was assigned as the *N*-(2-oxo-6-(pyridin-4-yl)-2*H*-pyran-3-yl)benzamide **17**. Structure **17** was confirmed on the basis of its elemental analysis and spectral data (see Experimental).

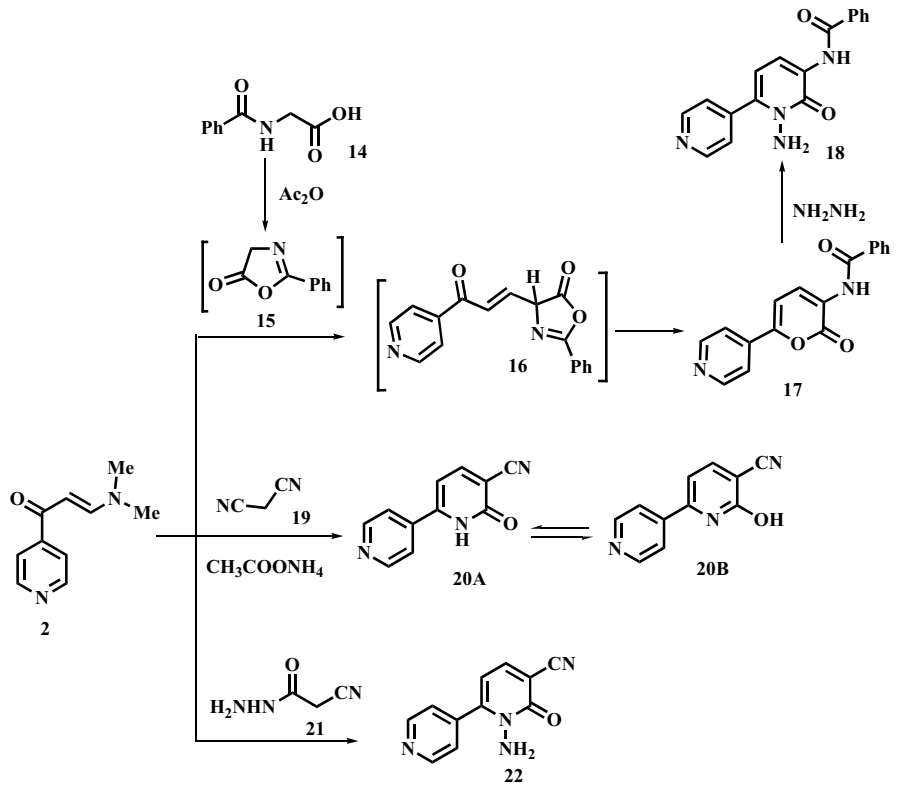
Compound **17** is assumed to be formed *via* the reaction of the intermediate oxazolone **15** which is formed in *situ* with the enaminone **2**, yielding the non-isolable intermediate **16**, that further rearranges into the pyranone **17** (Scheme **4**). Hydrazinolysis of **17**, yielded *N*-aminopyridinone derivative **18** as the final product based on the ^{1}H NMR spectral data in which a abroad signal appeared at δ 3.70 assignable to NH_2 protons (disappear after addition D_2O).

Treatment of the enaminone **2** with malononitrile in acetic acid in the presence of ammonium acetate under reflux afforded the pyridinone derivative **20** (Scheme **3**). The structure of **20** was substantiated by the IR spectra which displayed a characteristic band at υ = 2186 cm^{-1} assignable for CN group (*cf.* experimental part).



3,13	R	Ar
a	MeCO	Ph
b	MeCO	4-MeC_6H_4
c	MeCO	4-ClC_6H_4
d	MeCO	4-$NO_2C_6H_4$
e	EtOCO	Ph
f	EtOCO	4-MeC_6H_4
g	EtOCO	4-ClC_6H_4
h	EtOCO	4-$NO_2C_6H_4$

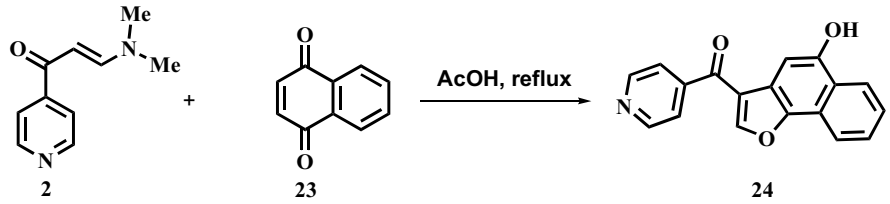
Scheme 2. Synthesis of triazolopyridopyrimidines **13a-h.**



Scheme 3. Synthesis of pyridinones **18, 20** and **22**.

Moreover, enaminone **2** reacted with 2-cyanoacetohydrazide to yield N-aminopyridinone derivative **22** (Scheme **3**). The structure of compound **22** was deduced from its spectral data (IR, ^{1}H NMR, and ESI-MS) and elemental analyses (*cf.* experimental part).

The enaminone **2** was also allowed to react with 1,4-naphthoquinone in acetic acid at room temperature, to yield a single product assigned as (5-hydroxynaphtho[1,2-*b*]furan-3-yl)(pyridin-4-yl)-methanone **24** which was assumed to be formed *via* an initial addition of the electron-rich moiety C2 in the enaminone **2** to the activated electron-poor double bond system in the quinone to afford the product **24** (Scheme **4**). Its ^{1}H NMR spectrum displayed singlets at δ 4.25 ppm attributable to the NH2 protons. Its IR spectrum revealed the appearance of an absorption band at 3434 cm^{-1} due to the OH



Scheme 4. Synthesis of naphthofuran derivative **24.**

group, in addition to the carbonyl absorption band at 1661 cm^{-1}. Its mass spectrum showed a peak corresponding to its molecular ion at *m/z* = 289 [M^+] (see Experimental).

2.2. Antimicrobial Activity

Synthesis of chemical modifications of existing antibacterial agents in order to generate novel molecules with better therapeutic properties is necessary because of the emergence of multidrug resistant bacteria [34]. The 2-pyridone derivatives have proven to be effective antimicrobial agents [35-38]. The clinical candidate, CG400549 [39] and the promising lead compound, PT171 [40] were rationally designed as broad spectrum antibacterial agents. Moreover, the 2-pyridone CG400549 (Chart **1**) was identified as a potent antibacterial against multidrug-resistant staphylococci strains [41]. Based on these considerations, we report herein the synthesis, characterization, antibacterial, antifungal studies of a novel series of 4-heteroarylpyridines, aiming to obtain new potent antibacterial and antifungal agents.

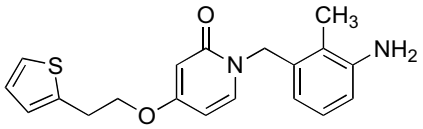


Chart 1. The 2-pyridone based clinical candidate CG400549.

In vitro antimicrobial screening of the synthesized 4-heteroarylpyridines **6a-h** , **7a-f**, **18**, **20** and **24** was evaluated against two antibacterial species, namely, *Staphylococcus aureus* (*SA*) as example of Gram-positive bacteria and *Escherichia coli (EC)* as example of Gram-negative bacteria and two fungal strains namely, *Aspergillus fumigates* (*AF*) and *Aspergillus niger* (*AN*) using *Cefepime* as a standard antimicrobial agent.

In testing the antimicrobial activity of these compounds, more than one test organism was used to increase the chance of detecting antibiotic principles in the tested materials. The sensitivity of a microorganism to antibiotics and other antimicrobial agents was determined by the assay plates.

The results of testing for antibacterial and antifungal effects are summarized in Tables **1** and **2**, respectively. As shown by these results, the new pyridine derivatives tested displayed variable *in vitro* antibacterial and antifungal actions. In general, the chemical structure of the whole molecule, comprising the nature of the heterocyclic system as well as the type of the substituted function present in the heterocyclic ring structure, has a pronounced effect.

From the screening results, it can be seen that:

For antibacterial activities.

- Maximum inhibition for *S. aureus* was recorded in case of compound **18**, followed by compound **24**, compound **6b** and **7d**. Low inhibition was recorded in case of compounds **6a, 6h, 6e, 7a** and **69** respectively.
- Maximum inhibition for *E. coli* was recorded in case of compound **6f** and **7b**, followed by compound **7f**. Low inhibition was recorded in case of compounds **6d**, **7e, 20** and **7c** respectively.

Table 1. Antibacterial assay of the tested compounds.

Compounds	*Staphylococcus aureus*	*Escherichia coli*
	Inhibition Zone Diameter (mm)	Inhibition Zone Diameter (mm)
6a	3	0
6b	5	0
6c	0	1
6d	0	2
6e	2	0
6f	0	5
7a	2	0
7b	0	5
7c	0	1
7d	4	0
7e	0	2
7f	0	4
6g	1	0
6h	3	0
18	7	0
20	0	2
24	6	0
Cefepime	8	10

For antifungal activities.

- Most of the tested compounds virtually lack any noticeable antifungal activities except only four compounds.
- The two tested fungal species were inhibited identically by the 4 active compounds.
- Compound **24** showed the most inhibition effect among all tested compounds where 6 mm inhibition zone was detected, followed by compounds **6a** (5 mm) and **6g** (5 mm) then compound **6b** (4 mm).

Table 2. Antifungal assay of the tested compounds.

Compounds	*Aspergillus flavus*	*Aspergillus niger*
	Inhibition Zone Diameter (mm)	Inhibition Zone Diameter (mm)
6a	5	5
6b	4	4
6g	3	3
24	6	6
Cefepime	12	10

2.3. Molecular Docking

2.3.1. Antibacterial

The crystal structure of the Enoyl-[acyl-carrier-protein] reductase [NADH] (InhA) which involved in the pathway fatty acid biosynthesis, is part of Lipid metabolism and was retrieved from the Protein Data Bank (PDB ID: 3FNE) complexed with native inhibitor 2-(2,4-dichlorophenoxy)-5-(pyridin-2-ylmethyl) phenol (PDB ID: 8PC) (Fig. **1**). MOE 2014.09 package software was used for the preparation of protein and ligands and performing docking process. The target protein was taken, the ligand was extracted, hydrogens were added and their positions were optimized. The minimized protein was defined as the receptor using the binding site module. The binding site was defined from the cavity finding method which was modified to accommodate all the important interacting residues in the active site of the enzyme. Conformational searches of the ligands were carried out using MOE 2014.09 package.

Catalytic activity of enoyl-[acyl-carrier-protein] reductase [NADH] (InhA).

An acyl-[acyl-carrier protein] + NAD^+ = a trans-2, 3-dehydroacyl-[acyl-carrier protein] + NADH.

2.3.2. Molecular Docking Studies

Docking of native ligand of the Enoyl-[acyl-carrier-protein] reductase [NADH] (InhA) protein on its active binding site reveled many interactions with total binding energy -3.3 (kcal/mol) (Fig. **2**).

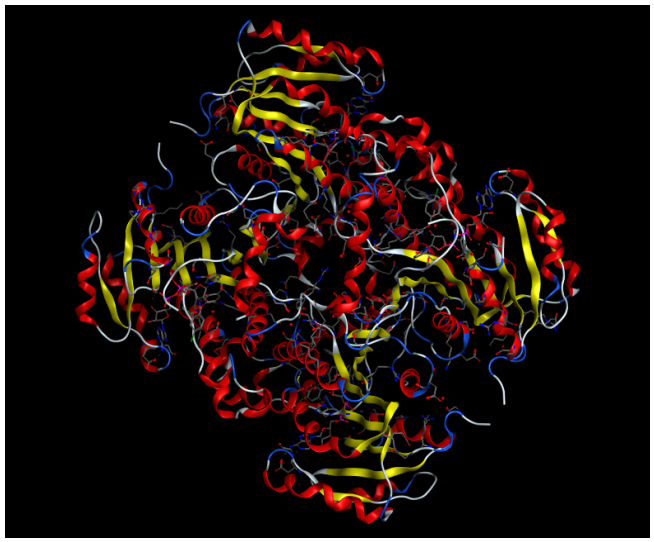


Fig. (1). The crystal structure of the enoyl-[acyl-carrier-protein] reductase [NADH] (InhA).

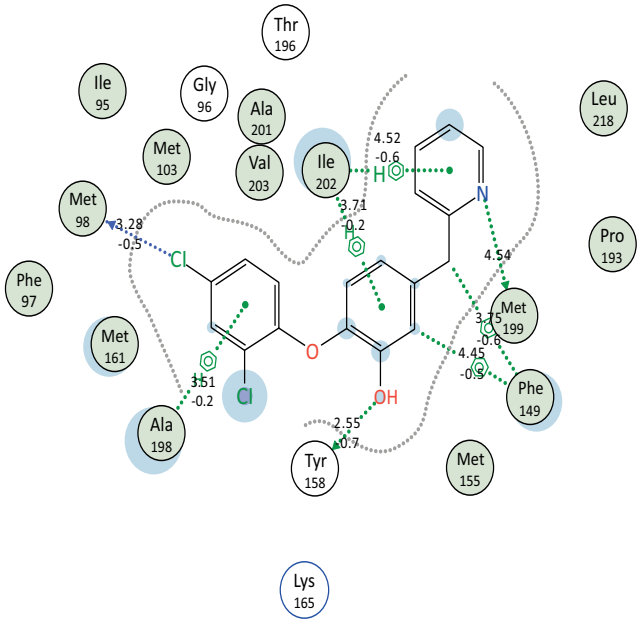


Fig. (2). 2D representation showing the interaction of the native ligand with the pocket amino acids residues on the active binding site of InhA protein.

For compound **24** with the most antimycobacterial activity docking on the active binding site of InhA protein revealing many interactions with total binding energy -4.7 (kcal/mol) which gives good indications about the affinity of the compound as inhibitor to the InhA protein. The most active pharmacophore of the compound **24** is the hydroxyl group which interact with the Ile 194 amino acid residue by hydrogen donor interaction with energy of binding -3.4 (kcal/mol) then the carbonyl group which interact with Met 199 by hydrogen donor interaction with binding energy -0.8 (kcal/mol). Met 199 interact also with the compound 26 by hydrogen donor interaction with the benzene ring with binding energy -0.3 (kcal/mol) finally pi-hydrogen interaction with binding energy -0.2 (kcal/mol) between pro 193 and compound **24** (Fig. **3a,b,c**).

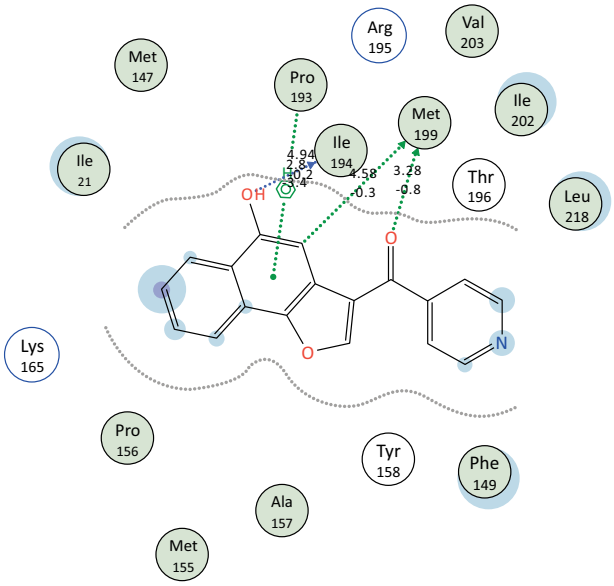


Fig. (3a). 2D representation showing the interaction of the compound **24** with the pocket amino acids residues on the active binding site of InhA protein.

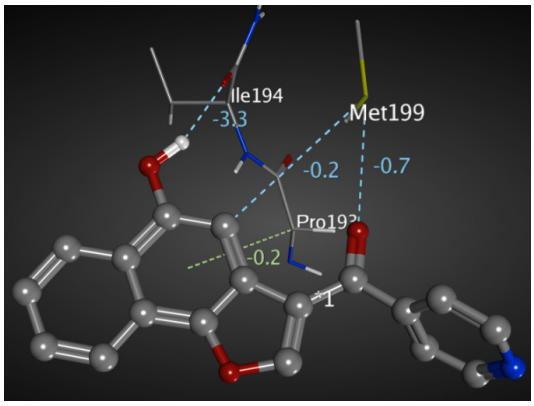


Fig. (3b). 3D representation showing the interaction of the compound **24** with the pocket amino acids residues on the active binding site of InhA protein.

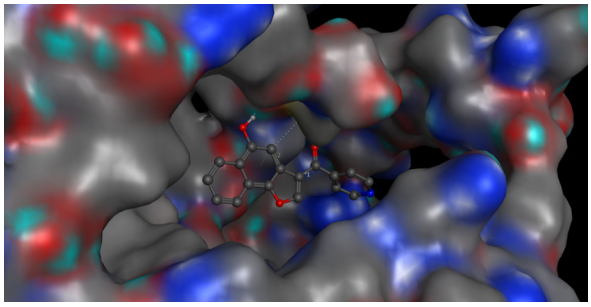


Fig. (3c). 3D representation of the hydrophobicity surface InhA protein showing the compound **26** on the active binding site.

2.3.3. Antifungal

The crystal structure of the Lanosterol 14-alpha demethylase catalyzes C14-demethylation of lanosterol, 24, 25-dihydro-lanosterol and obtusifoliol which is critical for ergosterol biosynthesis. It transforms lanosterol into 4,4'-dimethyl cholesta-8,14,24-triene-3-beta-ol and was retrieved from the Protein Data Bank (PDB ID: 2CIB) complexed with native inhibitor (2S)-2-[(2,1,3-Benzothiadiazol-4-ylsulfonyl) amino]-2-phenyl-n-pyridin-4-ylacetamide (PDB ID: CM6) (Fig. **4**).

MOE 2014.09 package software was used for preparation of protein and ligands and performing docking process. The minimized protein was defined as the receptor using the binding site module. The binding site was defined from the cavity finding method which was modified to accommodate all the important interacting residues in the active site of the enzyme. Conformational searches of the ligands were carried out using MOE 2014.09 package.

Fig. (4). The crystal structure of the Lanosterol 14-alpha demethylase enzyme.

2.3.4. Catalytic Activity

A 14-alpha-methylsteroid + 3 O_2 + 3 NADPH = a Delta(14)-steroid + formate + 3 $NADP^+$ + $4H_2O$.

2.3.5. Molecular Docking Studies

Docking of native ligand of the Lanosterol 14-alpha demethylase enzyme on its active binding site revealed pi-hydrogen interaction with the Phe 78 amino acid residue with total binding energy -0.7 (kcal/mol) (Fig. **5**).

For compound **24** with the most antimycobacterial activity docking on the active binding site of Lanosterol 14-alpha demethylase enzyme revealing many interactions with total binding energy -1.8 (kcal/mol) which gives good indications about the affinity of the compound as inhibitor to the interested enzyme. The most active pharmacophores of the compound **24** are the cyclic rings which interact by pi-hydrogen interactions with phe 78, Met 79 and Phe 83 respectively, with total binding energies -0.3 (kcal/mol) with Phe 78, -0.8 (kcal/mol) with Met 79 while Phe interact with the compound 26 by two pi-hydrogen interactions with binding energies -0.3 (kcal/mol) and -0.4 (kcal/mol) (Fig. **6a,b,c**).

3. CONCLUSIONS

In this study, 3-(dimethylamino)-1-(pyridin-4-yl)prop-2-en-1-one (**2**) was synthesized and used as a key intermediate for the synthesis of a new series of pyrazole derivatives *via* its 1,3-dipolar cycloaddition reactions with nitrilimines. Hydrazinolysis of the latter pyrazoles yielded the respective

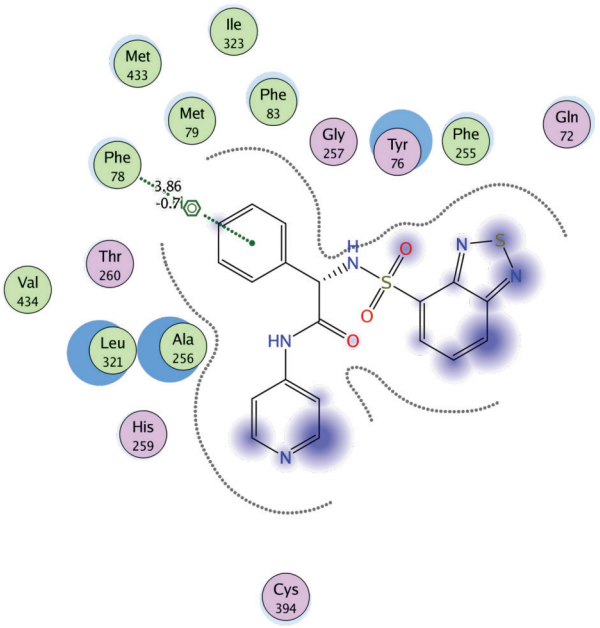


Fig. (5). 2D representation showing the interaction of the native ligand with the pocket amino acids residues on the active binding site of Lanosterol 14-alpha demethylase enzyme.

pyrazolopyridazines. Furthermore, the enaminone **2** was reacted with 6-aminothiouracil, hippuric acid, 2-cyanoacetohydrazide and 1,4-naphthoquinone to yielded the respective products. The mechanism that account for formation of products was discussed. The structures of the newly synthesized products were elucidated based on elemental analysis, spectral data and by alternative methods. The antimicrobial activities of the synthesized compounds were screened and the results showed that compounds **18** and **24** have good activities compared with Cefepime reference drug. Moreover, molecular docking study predicted the best binding mode between compound **24** and the Enoyl-[acyl-carrier-protein] reductase [NADH] (InhA).

4. EXPERIMENTAL

4.1. Chemistry

All melting points were determined on an electrothermal Gallenkamp apparatus and are uncorrected. Solvents were generally distilled and dried by standard literature procedures prior to use. The IR spectra were measured on a Pye-Unicam SP300 instrument in potassium bromide discs. The ^{1}H-NMR and ^{13}CNMR spectra were recorded on a Varian Mercury VXR-300 spectrometer (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR) and the chemical shifts were related to that of the solvent DMSO-*d6*. The mass spectra were recorded on a GCMSQ1000-EX Shimadzu and GCMS 5988-A HP spectrometers at 70 eV ionizing potential. Elemental analyses and spectral measurements were carried out at both the microanalytical center at Cairo University and the analytical laboratory of the institute of organic chemistry, Technical University of Dresden, Germany. The biological activity studies were carried out in the Botany & Microbiology Department, Faculty of Science, Cairo University. Hydrazonoyl halides [42] were prepared as previously reported in the respective literature.

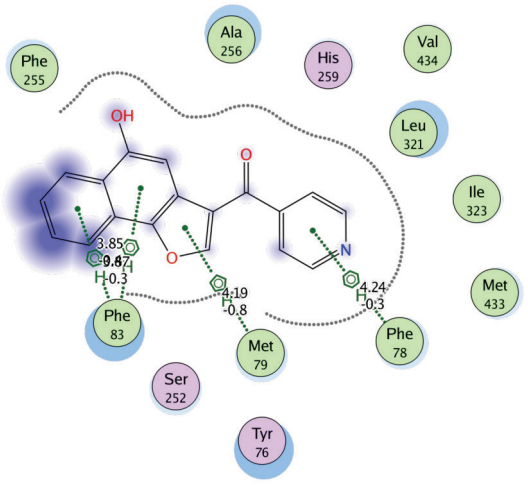


Fig. (6a). 2D representation showing the interaction of the compound **24** with the pocket amino acids residues on the active binding site of Lanosterol 14-alpha demethylase enzyme.

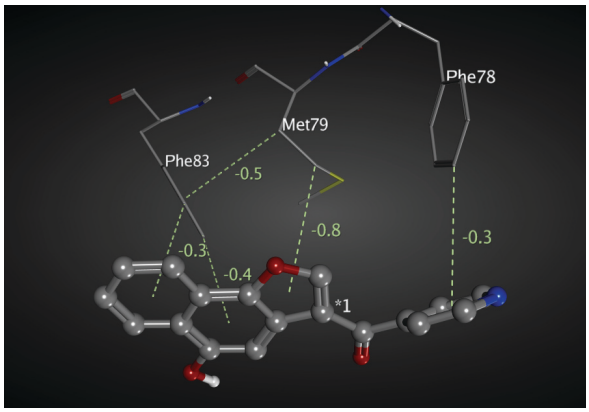


Fig. (6b). 3D representation showing the interaction of the compound **24** with the pocket amino acids residues on the active binding site of Lanosterol 14-alpha demethylase enzyme.

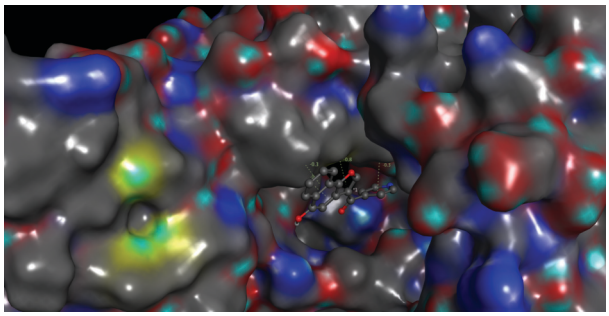


Fig. (6c). 3D representation of the hydrophobicity surface Lanosterol 14-alpha demethylase enzyme showing the compound **24** on the active binding site.

*Synthesis of 4-((1-aryl-3-substituted-1*H*-pyrazol-4-yl)carbonyl) pyridine derivatives (6a-h)*

To a stirred solution of 3-(dimethylamino)-1-(pyridin-4-yl)prop-2-en-1-one (**2**) (0.176 g, 1 mmol) and the appropriate hydrazonoyl halides **3a-h** (1 mmol) in toluene (15 mL), an equivalent amount of triethylamine (0.1 mL) was added. The reaction mixture was heated under reflux for 8 h. The precipitated triethylamine hydrochloride was filtered off,

and the filtrate was evaporated under reduced pressure. The residue was triturated with MeOH. The solid product, so formed in each case, was collected by filtration, washed with water, dried, and crystallized from proper solvent to afford the corresponding pyrazole derivatives **6a-h**. The products **6a-h** together with their physical constants are listed below.

1-(4-Isonicotinoyl-1-phenyl-1*H*-pyrazol-3-yl)ethanone (6a)

Light green solid, yield 77%; mp 95-97 ^{o}C (EtOH); IR (KBr) ν cm^{-1}: 3062, 2924 (C-H), 1710, 1680 (2C=O), 1597 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.61 (s, 3H, CH_3), 6.90-7.96 (m, 9H, Ar-H's), 8.61 (s,1H, pyrazole-H5); ^{13}C-NMR (DMSO-d_6) δ 23.8, 119.2, 121.8, 123.8, 125.4, 129.1, 133.5, 139.3, 140.3, 146,3, 149.4, 192.7, 194.3; MS *m/z* (%): 291 (M^+). Anal. Calcd for $C_{17}H_{13}N_3O_2$ (291.10): C, 70.09; H, 4.50; N, 14.42. Found: C, 70.01; H, 4.38; N, 14.36%.

1-(4-Isonicotinoyl-1-(p-tolyl)-1*H*-pyrazol-3-yl)ethanone (6b)

Light green solid, yield 75%; mp 143-145 ^{o}C (EtOH); IR (KBr) ν cm^{-1}: 3032, 2921 (C-H), 1710, 1678 (2C=O), 1595 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.23 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 7.08-7.84 (m, 8H, Ar-H's), 8.55 (s,1H, pyrazole-H5); MS *m/z* (%): 305 (M^+). Anal. Calcd for $C_{18}H_{15}N_3O_2$ (305.12) : C, 70.81; H, 4.95; N, 13.76 . Found: C, 70.64; H, 4.88; N, 13.69%.

1-(4-Isonicotinoyl-1-(4-nitrophenyl)-1*H*-pyrazol-3-yl) ethanone (6c)

Orange solid, yield 72%; mp 230-232 ^{o}C (Dioxane); IR (KBr) ν cm^{-1}: 3098, 2924 (C-H), 1713, 1684 (2C=O), 1594 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.64 (s, 3H, CH_3), 7.31-8.44 (m, 8H, Ar-H's), 8.79 (s,1H, pyrazole-H5); MS *m/z* (%): 336 (M^+). Anal. Calcd for $C_{17}H_{12}N_4O_4$ (336.09): C, 60.71; H, 3.60; N, 16.66. Found: C, 60.65; H, 3.48; N, 16.51%.

1-(1-(4-Chlorophenyl)-4-isonicotinoyl-1*H*-pyrazol-3-yl) ethanone (6d)

Light green solid, yield 78%; mp 120-122 ^{o}C (EtOH); IR (KBr) ν cm^{-1}: 3098, 2924 (C-H), 1713, 1684 (2C=O), 1594 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.61 (s, 3H, CH_3), 7.06-8.00 (m, 8H, Ar-H's), 8.56 (s,1H, pyrazole-H5); MS *m/z* (%): 325 (M^+). Anal. Calcd for $C_{17}H_{12}ClN_3O_2$ (325.06): C, 62.68; H, 3.71; N, 12.90. Found: C, 62.61; H, 3.59; N, 12.82%.

Ethyl 4-isonicotinoyl-1-phenyl-1*H*-pyrazole-3-carboxylate (6e)

Brown solid, yield 79%; mp 135-137 ^{o}C (EtOH); IR (KBr) ν cm^{-1}: 3037, 2925 (C-H), 1730, 1636 (2C=O), 1598 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.22 (t, 3H, CH_3, J = 7.2 Hz), 2.47 (s, 3H, CH_3), 4.20 (q, 2H, CH_2, J = 7.2 Hz), 7.22-7.90 (m, 9H, Ar-H's), 8.59 (s, 1H, pyrazole-H5); MS *m/z* (%): 321 (M^+). Anal. Calcd for $C_{18}H_{15}N_3O_3$ (321.11): C, 67.28; H, 4.71; N, 13.08. Found: C, 67.21; H, 4.65; N, 12.97%.

Ethyl 4-isonicotinoyl-1-(p-tolyl)-1*H*-pyrazole-3-carboxylate (6f)

Brown solid, yield 77%; mp 180-182 ^{o}C (Dioxane);IR (KBr) ν cm^{-1}: 3037, 2979 (C-H), 1728, 1636 (2C=O), 1596 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.18 (t, 3H, CH_3, J = 7.2 Hz), 2.17 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 4.23 (q, 2H, CH_2, J = 7.2 Hz), 6.92-7.80 (m, 8H, Ar-H's), 8.57 (s, 1H, pyrazole-H5); MS *m/z* (%): 335 (M^+). Anal. Calcd for $C_{19}H_{17}N_3O_3$ (335.13): C, 68.05; H, 5.11; N, 12.53%. Found: C, 67.90; H, 5.04; N, 12.44%.

Ethyl 4-isonicotinoyl-1-(4-nitrophenyl)-1*H*-pyrazole-3-carboxylate (6g)

Dark brown solid, yield 75%; mp 165-167 ^{o}C (Dioxane); IR (KBr) ν cm^{-1}: 3032, 2978 (C-H), 1734, 1639 (2C=O), 1594 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.21 (t, 3H, CH_3, J = 7.2 Hz), 2.52 (s, 3H, CH_3), 4.16 (q, 2H, CH_2, J = 7.2 Hz), 7.06-8.31 (m, 8H, Ar-H's), 8.53 (s, 1H, pyrazole-H5); MS *m/z* (%): 366 (M^+). Anal. Calcd for $C_{18}H_{14}N_4O_5$ (366.10): C, 59.02; H, 3.85; N, 15.29. Found: C, 58.93; H, 3.77; N, 15.16%.

Ethyl 1-(4-chlorophenyl)-4-isonicotinoyl-1*H*-pyrazole-3-carboxylate (6h)

Brown solid, yield 77%; mp 125-127 ^{o}C (EtOH); IR (KBr) ν cm^{-1}: 3034, 2923 (C-H), 1729, 1633 (2C=O), 1597 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.25 (t, 3H, CH_3, J = 7.2 Hz), 2.53 (s, 3H, CH_3), 4.20 (q, 2H, CH_2, J = 7.2 Hz), 7.04-7.99 (m, 8H, Ar-H's), 8.45 (s, 1H, pyrazole-H5); MS *m/z* (%): 355 (M^+). Anal. Calcd for $C_{18}H_{14}cClN_3O_3$ (355.07): C, 60.77; H, 3.97; N, 11.81. Found: C, 60.58; H, 3.92; N, 11.69%.

Synthesis of 7-substituted-2-phenyl-4-(pyridin-4-yl)-2*H*-pyrazolo[3,4-d]pyridazine derivatives 7a-f

Hydrazine hydrate (80%, 2 mL) was added to a solution of the appropriate pyrazole **6a-c, e-f** (1 mmol) in EtOH (10 mL). The reaction mixture was heated under reflux for 1 h, concentrated in vacuum, and diluted with water. The precipitate obtained was filtered off, washed with ice-cold water, dried and crystallized from EtOH. The synthesized pyrazolo[3,4-*d*]pyridazines **7a-f** together with their physical and spectral data are listed below.

7-Methyl-2-phenyl-4-(pyridin-4-yl)-2*H*-pyrazolo[3,4-d]pyridazine (7a)

Green solid, yield 70%; mp 175-177 ^{o}C (Dioxane); IR (KBr) ν cm^{-1}: 3048, 2923 (C-H), 1596 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.91 (s, 3H, CH_3), 7.11-8.24 (m, 9H, Ar-H's), 8.81 (s,1H, pyrazole-H5); ^{13}C NMR (DMSO-d_6): δ 17.9 (CH_3), 121.5, 123.0, 124.6, 124.9, 125.5, 129.5, 130.0, 131.6, 138.4, 143.1, 148.36, 153.3 (Ar-C); MS *m/z* (%): 287 (M^+). Anal. Calcd for $C_{17}H_{13}N_5$ (287.12): C, 71.06; H, 4.56; N, 24.37. Found: C, 70.93; H, 4.51; N, 24.29%.

7-Methyl-4-(pyridin-4-yl)-2-(p-tolyl)-2*H*-pyrazolo[3,4-d] pyridazine (7b)

Green solid, yield 75%; mp 195-197 ^{o}C (Dioxane); IR (KBr) ν cm^{-1}: 3034, 2920 (C-H), 1611 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.25 (s, 3H, CH_3), 2.88 (s, 3H, CH_3), 7.03-8.11 (m, 8H, Ar-H's), 8.80 (s,1H, pyrazole-H5); MS *m/z* (%): 301 (M^+). Anal. Calcd for $C_{18}H_{15}N_5$ (301.13): C, 71.74; H, 5.02; N, 23.24. Found: C, 71.69; H, 5.06; N, 23.17%.

7-Methyl-2-(4-nitrophenyl)-4-(pyridin-4-yl)-2*H*-pyrazolo [3,4-d]pyridazine (7c)

Brown solid, yield 71%; mp 167-169 ^{o}C (Dioxane); IR (KBr) ν cm^{-1}: 3030, 2924 (C-H), 1594 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.92 (s, 3H, CH_3), 7.87-8.54 (m, 8H, Ar-H's), 8.83 (s,1H, pyrazole-H5); MS *m/z* (%): 332 (M^+). Anal. Calcd for $C_{17}H_{12}N_6O_2$ (332.10): C, 61.44; H, 3.64; N, 25.29. Found: C, 61.37; H, 3.57; N, 25.20%.

2-Phenyl-4-(pyridin-4-yl)-2H-pyrazolo[3,4-d]pyridazin-7-ol (7d)

Light green solid, yield 72%; mp 205-207 °C (Dioxane); IR (KBr) ν cm^{-1}: 3416 (OH), 3063, 2924 (C-H), 1674 (C=O), 1599 (C=N); ^{1}H NMR (DMSO-d_6) δ: 6.73-8.15 (m, 9H, Ar-H's), 8.73 (s,1H, pyrazole-H5), 12.79 (br., s, 1H, OH, D_2O-exchangeable); MS *m/z* (%): 289 (M^+). Anal. Calcd for $C_{16}H_{11}N_5O$ (289.10): C, 66.43; H, 3.83; N, 24.21. Found: C, 66.35; H, 3.75; N, 24.17%.

4-(Pyridin-4-yl)-2-(p-tolyl)-2H-pyrazolo[3,4-d]pyridazin-7-ol (7e)

Light green solid, yield 71%; mp 194-196 °C (Dioxane); IR (KBr) ν cm^{-1}: 3424 (OH), 3043, 2921 (C-H), 1674 (C=O), 1597 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.22 (s, 3H, CH_3), 6.70-8.02 (m, 8H, Ar-H's), 8.83 (s,1H, pyrazole-H5), 12.79 (br., s, 1H, OH, D_2O-exchangeable); MS *m/z* (%): 289 (M^+). Anal. Calcd for $C_{17}H_{13}N_5O$ (303.11): C, 67.32; H, 4.32; N, 23.09. Found: C, 67.25; H, 4.26; N, 23.01%.

2-(4-Nitrophenyl)-4-(pyridin-4-yl)-2H-pyrazolo[3,4-d] pyridazin-7-ol (7f)

Red solid, yield 75%; mp 235-237 °C (Dioxane); IR (KBr) ν cm^{-1}: 3425 (OH), 3049, 2924 (C-H), 1675 (C=O), 1595 (C=N); ^{1}H NMR (DMSO-d_6) δ: 7.90-8.47 (m, 8H, Ar-H's), 8.76 (s,1H, pyrazole-H5), 12.84 (br., s, 1H, OH, D_2O-exchangeable); MS *m/z* (%): 289 (M^+). Anal. Calcd for $C_{16}H_{10}N_6O_3$ (334.08): C, 57.49; H, 3.02; N, 25.14. Found: C, 57.26; H, 3.04; N, 25.11%.

Synthesis of 5-(pyridin-4-yl)-2-thioxo-2,3-dihydropyrido [2,3-d]pyrimidin-4(1H)-one (9)

A mixture of enaminone **2** (0.176 g, 1 mmol) and 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (**8**) (0.143 g, 1 mmol) in acetic acid (10 mL) was refluxed for 6 h. The reaction mixture was cooled and diluted with MeOH and the solid product was collected by filtration and recrystallized from DMF to give thione **9** as brown solid in yield 76%, mp 352-354 °C; IR (KBr) υ: 3362, 3229 (2 NH), 3048, 2921 (C-H), 1671 (CO)cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 8.06 (s, 1H, J = 8.1 Hz, pyridine-H), 8.13 (d, 2H, J = 5.0 Hz, pyridine-H), 8.44 (s, 1H, J = 8.1 Hz, pyridine-H), 8.80 (d, 2H, J = 5.0 Hz, pyridine-H), 12.73 (s, 1H, D_2O-exchangeable, NH), 13.26 (s, 1H, D_2O-exchangeable, NH) ppm; MS *m/z* (%): 256 (M^+). Anal. Calcd for $C_{12}H_8N_4OS$ (256.04): C, 56.24; H, 3.15; N, 21.86. Found: C, 56.17; H, 3.09; N, 21.77%.

Synthesis of 1-aryl-3-substituted-6-(pyridin-4-yl)pyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5(1H)-ones (13a-h).

General procedure: TEA (1.4 mL, 10 mmol) was added to a mixture of equimolar amounts of thione 9 (0.256 g, 1 mmol) and the appropriate hydrazonoyl halides 3a-h (1 mmol) in dioxane (10 mL) at room temperature. The reaction mixture was refluxed till all of the starting materials have been disappeared and hydrogen sulfide gas ceased to evolve (6-10 h, monitored by TLC). The solvent was evaporated and the residue was triturated with MeOH. The solid formed was filtered and recrystallized from the proper solvent to give the products 13a-h, respectively. The products 13a-h together with their physical constants are listed below.

3-Acetyl-1-phenyl-6-(pyridin-4-yl)pyrido[2,3-d][1,2,4] triazolo[4,3-a]pyrimidin-5(1H)-one (13a)

Light brown solid, yield 69%; mp 280-282 °C (DMF); IR (KBr) ν cm^{-1}: 3061, 2923 (C-H), 1627, 1664 (2C=O), 1595 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.65 (s, 3H, CH_3), 6.93-8.26 (m, 7H, Ar-H's), 8.74-8.81 (m, 4H, Ar-H's); MS *m/z* (%): 382 (M^+). Anal. Calcd. for $C_{21}H_{14}N_6O_2$ (382.12): C, 65.96; H, 3.69; N, 21.98. Found: C, 65.89; H, 3.60; N, 21.87%.

3-Acetyl-6-(pyridin-4-yl)-1-(p-tolyl)pyrido[2,3-d][1,2,4] triazolo[4,3-a]pyrimidin-5(1H)-one (13b)

Light brown solid, yield 72%; mp 190-192 °C (Dioxane); IR (KBr) ν cm^{-1}: 3061, 2923 (C-H), 1672, 1647 (2C=O), 1597 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.35 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 6.89-8.78 (m, 10H, Ar-H's); MS *m/z* (%): 396 (M^+). Anal. Calcd. for $C_{22}H_{16}N_6O_2$ (396.13): C, 66.66; H, 4.07; N, 21.20. Found: C, 66.53; H, 4.01; N, 21.13%.

3-Acetyl-1-(4-chlorophenyl)-6-(pyridin-4-yl)pyrido[2,3-d] [1,2,4]triazolo[4,3-a]pyrimidin-5(1H)-one (13c)

Light brown solid, yield 73%; mp 178-180 °C (Dioxane); IR (KBr) ν cm^{-1}: 3048, 2925 (C-H), 1668, 1626 (2C=O), 1599 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.67 (s, 3H, CH_3), 6.97-8.78 (m, 10H, Ar-H's); MS *m/z* (%): 416 (M^+). Anal. Calcd. for $C_{21}H_{13}ClN_6O_2$ (416.08): C, 60.51; H, 3.14; N, 20.16. Found: C, 60.45; H, 3.08; N, 20.11%.

3-Acetyl-1-(4-nitrophenyl)-6-(pyridin-4-yl)pyrido[2,3-d][1, 2,4]triazolo[4,3-a]pyrimidin-5(1H)-one (13d)

Light orange solid, yield 70%; mp 170-172 °C (Dioxane); IR (KBr) ν cm^{-1}: 3048, 2924 (C-H), 1672, 1630 (2C=O), 1595 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.65 (s, 3H, CH_3), 7.06-8.99 (m, 10H, Ar-H's); MS *m/z* (%): 427 (M^+). Anal. Calcd. for $C_{21}H_{13}N_7O_4$ (427.10): C, 59.02; H, 3.07; N, 22.94. Found: C, 58.89; H, 3.01; N, 22.86%.

Ethyl 5-oxo-1-phenyl-6-(pyridin-4-yl)-1,5-dihydropyrido[2, 3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (13e)

Light yellow solid, yield 69%; mp 200-202 °C (Dioxane); IR (KBr) ν cm^{-1}: 3044, 2962 (C-H), 1751, 1669 (2C=O), 1585 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.29 (t, 3H, CH_3, J = 7.2 Hz), 4.31 (q, 2H, CH_2, J = 7.2 Hz), 6.85-8.27 (m, 11H, Ar-H's); MS *m/z* (%): 412 (M^+). Anal. Calcd. for $C_{22}H_{16}N_6O_3$ (412.13): C, 64.07; H, 3.91; N, 20.38. Found: C, 63.92; H, 3.85; N, 20.30%.

Ethyl 5-oxo-6-(pyridin-4-yl)-1-(p-tolyl)-1,5-dihydropyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (13f)

Light yellow solid, yield 71%; mp 215-217 °C (Dioxane); IR (KBr) ν cm^{-1}: 3031, 2983 (C-H), 1750, 1671 (2C=O), 1584 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.33 (t, 3H, CH_3, J = 7.2 Hz), 2.36 (s, 3H, CH_3), 4.42 (q, 2H, CH_2, J = 7.2 Hz), 6.93-8.21 (m, 10H, Ar-H's); MS *m/z* (%): 426 (M^+). Anal. Calcd. for $C_{23}H_{18}N_6O_3$ (426.14): C, 64.78; H, 4.25; N, 19.71. Found: C, 64.66; H, 4.21; N, 19.64%.

Ethyl 1-(4-chlorophenyl)-5-oxo-6-(pyridin-4-yl)-1,5-dihydropyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (13g)

Light yellow solid, yield 72%; mp 185-187 °C (DMF); IR (KBr) ν cm^{-1}: 3042, 2924 (C-H), 1750, 1667 (2C=O),

1586 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.27 (t, 3H, CH_3, J = 7.2 Hz), 4.40 (q, 2H, CH_2, J = 7.2 Hz), 6.97-8.64 (m, 10H, Ar-H's); MS *m/z* (%): 446 (M^+). Anal. Calcd. for $C_{22}H_{15}ClN_6O_3$ (446.09): C, 59.13; H, 3.38; N, 18.81. Found: C, 59.13; H, 3.38; N, 18.81%.

Ethyl 1-(4-nitrophenyl)-5-oxo-6-(pyridin-4-yl)-1,5-dihydro-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidine-3-carboxylate (13h)

Dark yellow solid, yield 67%; mp 244-246 ^{o}C (DMF); IR (KBr) ν cm^{-1}: 3030, 2923 (C-H), 1752, 1669 (2C=O), 1586 (C=N); ^{1}H NMR ($CDCl_3$) δ: 1.30 (t, 3H, CH_3, J = 7.2 Hz), 4.30 (q, 2H, CH_2, J = 7.2 Hz), 7.41-8.49 (m, 10H, Ar-H's); MS *m/z* (%): 457 (M^+). Anal. Calcd. for $C_{22}H_{15}N_7O_5$ (457.11): C, 57.77; H, 3.31; N, 21.44. Found: C, 57.68; H, 3.27; N, 21.35%.

Reactions of Enaminone 2 with Hippuric Acid (14)

A solution of enaminone **2** (0.176 g, 1 mmol) and hippuric acid (**19**) (0.17 g, 1 mmol) in acetic anhydride (10 mL) was heated under reflux for 2 h. The reaction mixture was concentrated *in vacuum*. The solid product obtained upon cooling was filtered off and recrystallized from DMF to yield pyranone derivative **17** as light rose solid, yield 69%; mp 283-285 ^{o}C; IR (KBr) υ = 3435 (NH), 3076, 2924 (C-H), 1708, 1664 (2 C=O), 1596 (C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 6.81 (d, 1H, J = 7.6 Hz, pyran-H5), 6.93 (d, 1H, J = 7.6 Hz, pyran-H4), 7.41-8.26 (m, 7H, Ar-H), 7.68-8.70 (m, 2H, Ar-H), 9.67 (s, 1H, NH) ppm; MS, *m/z* (%): 292 (M^+). Anal. Calcd for $C_{17}H_{12}N_2O_3$ (292.08): C, 69.86; H, 4.14; N, 9.58. Found: C, 69.79; H, 4.10; N, 9.51%.

Reaction of 17 With Hydrazine

To a solution of the pyranone **17** (0.292 g, 1 mmol) in acetic acid (10 mL) hydrazine hydrate (1 mL) was added and the mixture was heated under reflux for 4 h. The reaction mixture was acidified by HCl / ice mixture and the formed product was filtered and crystallized from EtOH to give pyidinone derivative **18n** as white solid, yield 649%; mp 100-102 ^{o}C; IR (KBr) υ = 3424, 3240 (NH_2), 3052, 2929 (C-H), 1679, 1660 (2 C=O), 1597 (C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 3.72 (s, br, 2H, NH_2, D_2O exchangable), 7.21-8.39 (m, 12H, Ar-H), 9.61 (s, 1H, NH) ppm; MS *m/z* (%): 306 (M^+). Anal. Calcd for $C_{17}H_{14}N_4O_2$ (306.11): C, 66.66; H, 4.61; N, 18.29. Found: C, 66.47; H, 4.53; N, 18.20%.

Reaction of Enaminone 9 with Active Methylene Compounds

To a solution of enaminone **2** (0.176 g, 1 mmol) in acetic acid (10 mL) malononitrile or 2-cyanoacetohydrazide (1 mmol) was added in the presence of ammonium acetate anhydrous (0.3 g). The mixture was heated under reflux for 10 hr. After concentration and cooling to room temperature, the precipitated product was collected by filtration, washed well with EtOH, dried and recrystallized from DMF to give the pyridine derivatives **20** and **22,** respectively.

6-Hydroxy-[2,4'-bipyridine]-5-carbonitrile (20)

Dark violet crystals, yield 67%; mp 300-302 ^{o}C; IR (KBr) υ 3353 (OH), 3070, 3005, 2920 (C-H), 2186 (CN), 1642 (C=O), 1589 (C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 4.41 (s, 1H, OH), 6.09 (d, 1H, J = 7.6 Hz, pyridinone-H5), 7.41-7.83 (m, 4H, Ar-H), 8.92 (d, 1H, J = 7.6 Hz, pyidinone-H4) ppm; MS, *m/z* (%) 197 (M^+). Anal. calcd for $C_{11}H_7N_3O$ (197.06): C, 67.00; H, 3.58; N, 21.31; found: C, 66.94; H, 3.49; N, 21.27%.

1-Amino-6-oxo-1,6-dihydro-[2,4'-bipyridine]-5-carbonitrile (22)

Yellow solid, yield 69%; mp 350-352 ^{o}C; IR (KBr) υ 3434, 3290 (NH_2), 3070, 3005, 2920 (C-H), 2186 (CN), 1651 (C=O), 1608 (C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 3.73 (s, 1H, NH_2), 5.98 (d, 1H, J = 7.1 Hz, pyridine-H), 7.73 (d, 2H, pyridine-H), 8.20 (d, 1H, J = 7.1 Hz, pyridine-H), 8.56 (d, 2H, pyridine-H) ppm; MS, *m/z* (%) 197 (M^+). Anal. calcd for $C_{11}H_8N_4O$ (212.07): C, 62.26; H, 3.80; N, 26.40; found: C, 62.20; H, 3.72; N, 26.33%.

Synthesis of (5-hydroxynaphtho[1,2-b]furan-3-yl)(pyridin-4-yl)methanone (24)

To a stirred solution of enaminone **2** (0.176 g, 1 mmol) in glacial acetic acid (10 mL), 1,4-naphthoquinone (**23**) (0.158 g, 1 mmol) was added, then the resulting mixture was stirred for 6 h at room temperature. The solvent was evaporated under reduced pressure, and the solid product obtained was filtered off and recrystallized from DMF to afford a pure solid of compounds **24** as dark brown solid, yield 65%; mp 150-152 ^{o}C; IR (KBr) ν cm^{-1}: 3434 (OH), 3069, 2924 (C-H), 1661 (C=O), 1590 (C=N); ^{1}H NMR (DMSO-d_6) δ: 7.09-7.53 (m, 9H, ArH's), 8.82 (s, 1H, furan-H-2), 10.28 (br., s, 1H, OH, D_2O exchangeable); MS *m/z* (%): 289 (M^+). Anal. Calcd. for $C_{18}H_{11}NO_3$ (289.07): C, 74.73; H, 3.83; N, 4.84%. Found: C, 74.68; H, 3.74; N, 4.75%.

4.2. Biological Activity

The antimicrobial activity evaluation of the synthesized compounds was carried out at the Botany & Microbiology Department, Faculty of Science, Cairo University, Giza, Egypt according to the reported method [43].

4.3. Molecular Modeling

Docking Study was performed using the MOE 2014.09 software. Regularization and optimization for protein and ligand were performed. Each docked compound was assigned a score according to its fit in the ligand binding pocket (LBP) and its binding mod.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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