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SYNTHESIS, MOLECULAR DOCKING STUDIES AND IN VITRO ANTIMICROBIAL EVALUATION OF NOVEL PYRIMIDO[1,2-*a*]QUINOXALINE AND TRIAZINO[4,3-*a*]-QUINOXALINE DERIVATIVES

Rania B. Bakr,[a,b]* Nadia A. A. Elkanzi,[c,d] Amira A. Ghoneim,[c,e] and Shaima M. N. Moustafa[f,g]

[a]Department of Pharmaceutical Chemistry, College of Pharmacy, Jouf University, Sakaka, Aljouf 2014, Kingdom of Saudi Arabia. [b]Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt. [c]Chemistry Department, College of Science, Jouf University, P.O Box: 2014, Sakaka, Saudi Arabia. [d]Chemistry Department, Faculty of Science, Aswan University, P.O Box: 81528, Aswan, Egypt. [e]Chemistry Department, Faculty of Science, Zagazig University, Zagazig, Egypt. [f]Biology Department, College of Science, Jouf University, P.O Box: 2014, Sakaka, Saudi Arabia. [g]Department of Botany & Microbiology, Faculty of Science, Minia University, El-Minia, Egypt

Abstract – A series of 3-substituted-2,6-dihydro-1*H*-pyrimido[1,2-*a*]quinoxaline-5-carboxylates (**4a,b**), (**8c,d**) and (**13a,b**) were prepared by treating ethyl 3-amino-1,4-dihydroquinoxaline-2-carboxylate (**1**) with alkylidenemalononitrile derivatives. Moreover, 8*H*-benzo[5,6][1,2,4]triazino[4,3-*a*]quinoxaline-7-carboxylate derivatives (**18**, **20**, **22**, **24**) were synthesized from condensation reaction between diazonium salts **16** with resorcinol, 1-naphthol, malononitrile and ethyl acetoacetate respectively. Some of the newly synthesized compounds were screened for their antimicrobial activity studies. Furthermore, molecular docking were performed on the active compounds **1**, **4a** and **8c** to predict the mode of action of these novel compounds.

Quinoxalines are an important compounds due to their wide spectrum of biological activities such as antibacterial,[1-3] anticancer,[4,5] and activity as kinase inhibitors.[6] They are well known for their application in electroluminescent materials,[7] organic semiconductors[8] and DNA cleaving agents.[9] Considering the

significant applications in the fields of medicinal, industrial and synthetic organic chemistry, there has been tremendous interest in developing efficient methods for the synthesis of quinoxalines. In 2018, quinoxaline derivative (**A**) (Figure 1) was reported by El-Attar *et al.*[10] to exhibit significant antibacterial activities against *Pseudomones aeruginosa* as shown by the standard drug levofloxacin (MIC, 12.5 μg/mL). In addition, a new set of *N*-(substituted-phenyl)-2-[5-(quinoxalin-2-yloxymethyl)-[1,3,4]-oxadiazol-2-ylsulfanyl]acetamides had been synthesized and evaluated for their antimicrobial activity. From this set compound **B** (Figure 1) recorded comparable antimicrobial effect against *Pseudomones aeruginosa* (MICs of 50 μM) to that showed by chloramphenicol and ciprofloxacin as standards.[11] Moreover, 2,3-bis(bromomethyl)-6-(trifluoromethyl)quinoxaline (**C**) which has a strong electron-withdrawing and highly lipophilic trifluoromethyl group at the 6-position was prepared and found to show high activity against Gram-positive bacteria (MIC = 12.5 μg/mL).[12] Pyrimidine derivatives attracted much attention due to its importance in many biologically active compounds. This nucleus was reported to exhibit many biological activity anticancer,[13,14] anti-inflammatory,[15,16] antidiabetic[17] and antibacterial.[18,19] For example, pyrimidine derivative (**D**) showed higher inhibitory activity against *Neisseria gonorrhoeae* and *Staphylococcus aureus* than that of Spectinomycin. In addition to inhibitory activity against *Shigella flexneri* RCPM 1a8516, with a MIC value of 7.8 μg/mL.[20] Moreover, many triazine derivatives had been reported in literature to reveal antibacterial activity.[21,22] For example, Zhang *et al.*[23] synthesized triazine derivative (**E**) which was potent antimicrobial, with an MIC of 2.1 mmol/L

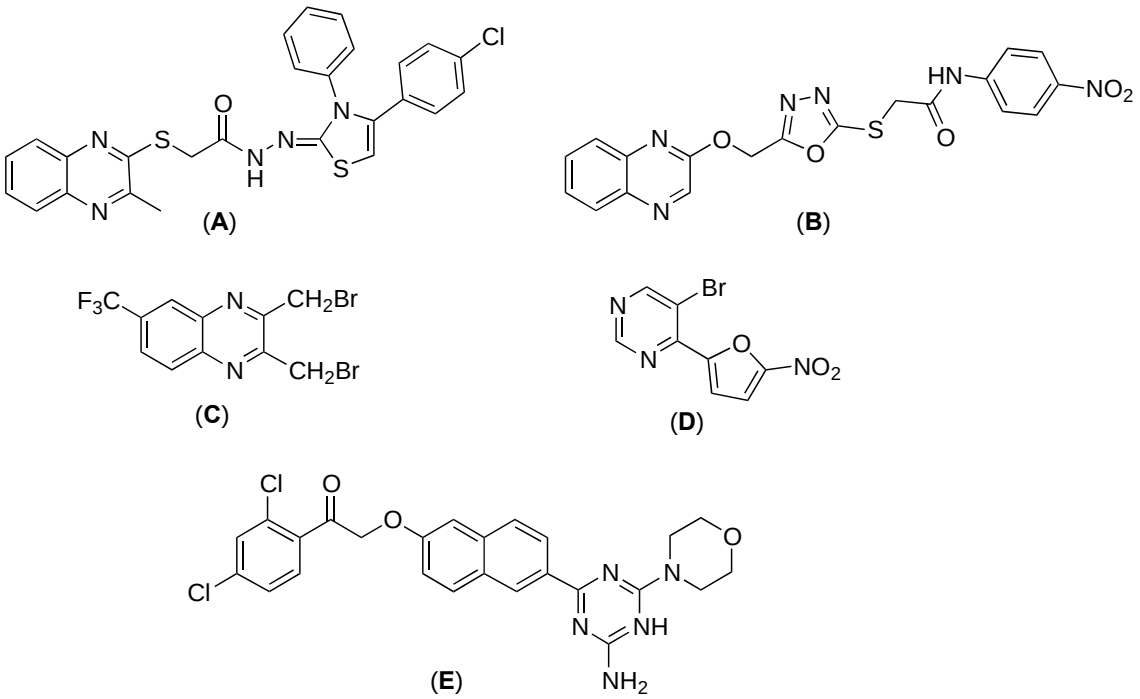
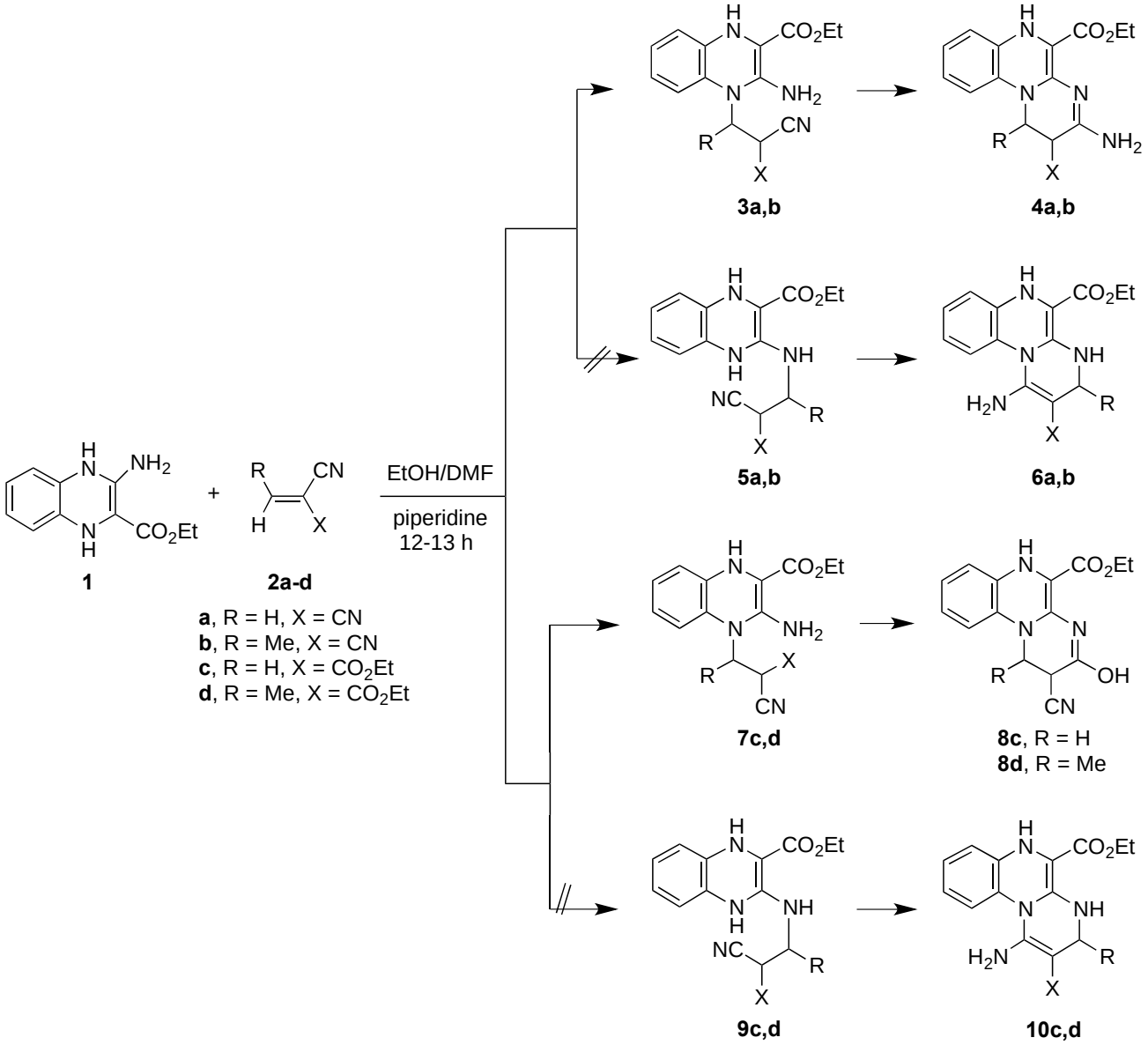


Figure 1. Chemical structures of some reported quinoxaline derivatives (**A**-**C**), pyrimidine derivative (**D**) and triazine derivative (**E**) as antimicrobial agents

against four multidrug-resistant, Gram-positive bacterial strains and also exhibited good activity, with an MIC of 16.5 mmol/L against *Candida albicans*. In the view of the aforesaid facts and in continuation of our previous work for synthesis of biologically active heterocycles,[16,24-26] we decided to synthesize novel derivatives of pyrimido[1,2-*a*]quinoxaline and triazino[4,3-*a*]quinoxaline of expected antimicrobial activity. Our design based upon mixing quinoxaline ring with pyrimidine scaffold and/or triazine heterocycle in one structure aiming at getting more selective antimicrobial agents with minimal side effects.

Chemistry

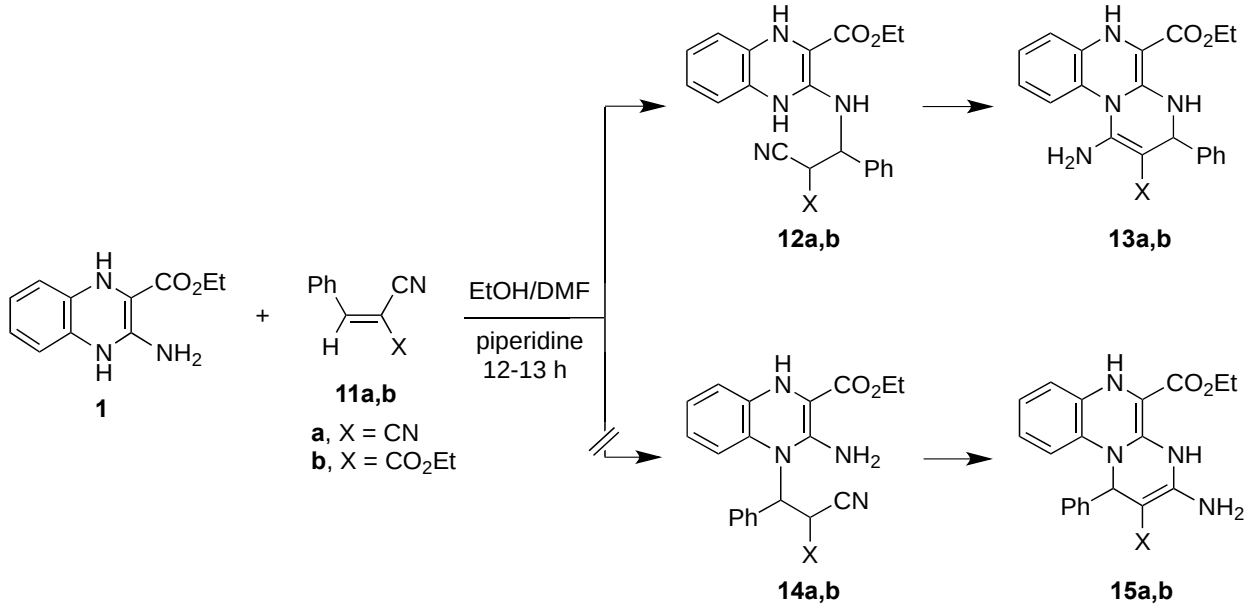
Alkylidene derivatives **2a-d** were obtained by condensation of the appropriate aldehydes with active methylene compounds.[27] The aminofunctionalized ethyl 3-amino-1,4-dihydroquinoxaline-2-carboxylate (**1**) underwent addition with alkylidenemalononitrile derivatives **2a** and **2b** upon refluxing in a mixture of ethanol and dimethyl formamide containing few drops of piperidine to afford the corresponding pyrimidoquinoxaline **4a** and **4b**, rather than compounds **6a** and **6b** as shown in Scheme 1. The reactions involving the addition of the NH group of the ethyl 3-amino-1,4-dihydroquinoxaline-2-carboxylate (**1**) to the alkylidenemalononitriles double bond through intermediates **3a** and **3b** followed by subsequent ring-closure step upon a nucleophilic attack of the amino functional group on the electrophilic cyano group. As similar, the addition of the ethyl 3-amino-1,4-dihydroquinoxaline-2-carboxylate (**1**) to the alkylidenecyanoacetate **2c** and **2d** were found to proceed *via* intermediates **7c** and **7d**. The cyclization reaction of intermediates **7c** and **7d** included an intramolecular nucleophilic attack of the amino groups of intermediates **7c** and **7d** on the ethyl carboxylate, rather than the cyano moiety to yield the fused heterocyclic compounds **8c** and **8d**. The chemical structures of compounds **4a**, **4b**, **8c**, and **8d** elucidated by using various spectroscopic techniques. The IR spectrum of compound **4a** showed the expected absorption bands at 2215 (CN), 3100–3400 (NHstr., NH_2str.) cm^{-1}. The ^1H NMR spectrum of **4a** showed a triplet-quartet signal of the ethyl carboxylate functionality at δ_H 1.23 and 4.17 ppm and the NH_2 signal appeared at 9.15 ppm as an a broad singlet. The EI-MS of **4a** showed the expected a molecular ion peak at *m*/*z* 297. On the other hand, the IR spectrum of compound **8c** showed the expected absorption bands of the OH, CN and CO moieties at ν_{max} 3430, 2216, 1735 cm^{-1}, respectively. The ^1H NMR spectrum of **8c** showed the triplet-quartet signal of the ethyl carboxylate functionality at δ_H 1.24 and 4.30 ppm. The OH signal appeared at 10.25 ppm as a broad singlet. The EI-MS of **8c** showed the expected molecular ion peak at *m*/*z* 298.



Scheme 1. Synthesis of pyrimido[1,2-*a*]quinoxaline derivatives **4a,b** and **8c,d**

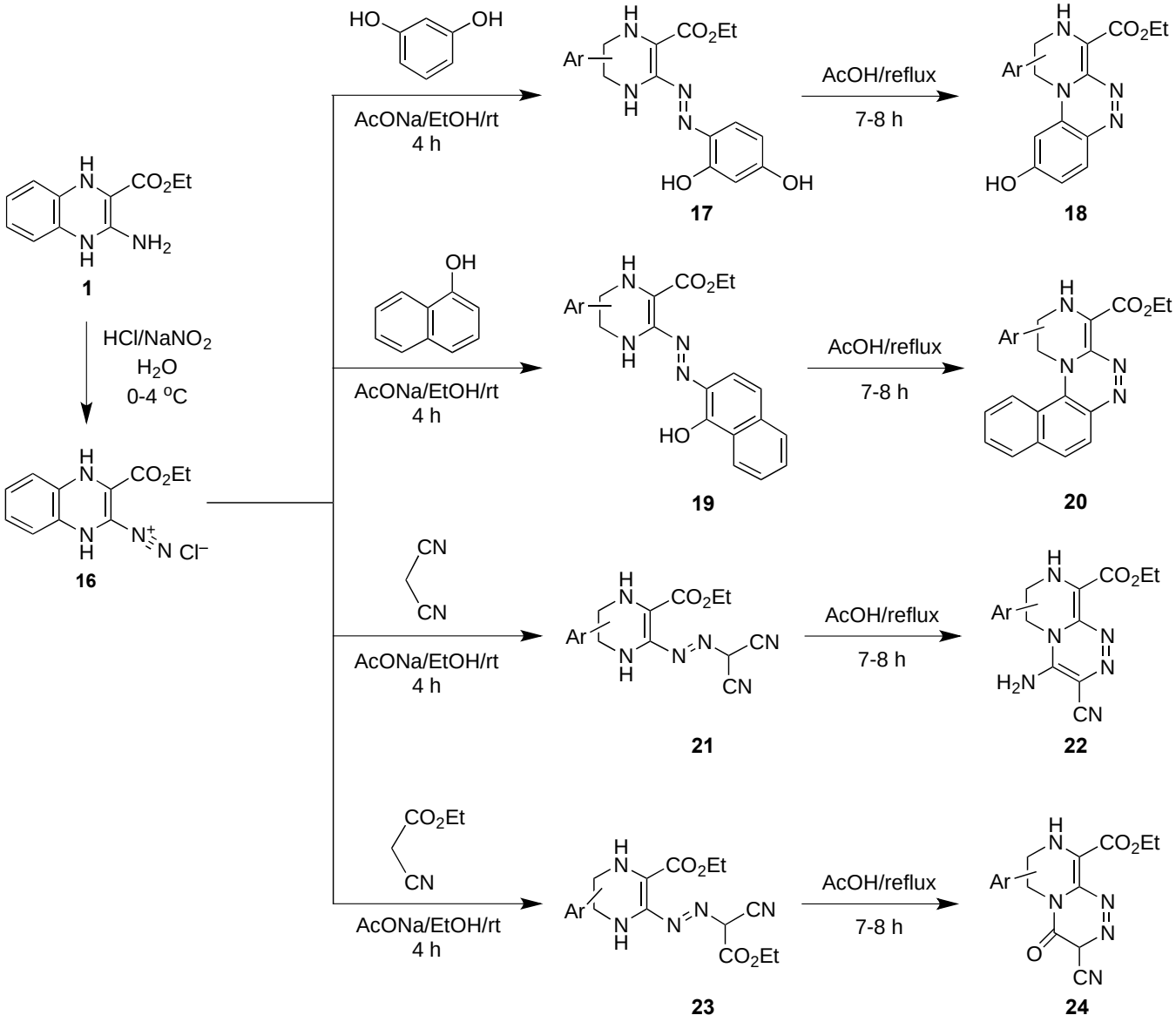
Furthermore, the treatment of the ethyl 3-amino-1,4-dihydroquinoxaline-2-carboxylate (**1**) with arylidene derivatives **11a** and **11b** yielded the fused dihydro-3*H*-pyrimido[1,2-*a*]quinoxalines **13a** and **13b** *via* intermediates **12a** and **12b**, rather than **14a** and **14b** (Scheme 2). This example clearly demonstrates the vital role of the substituent on controlling the regioselectivity of the reaction.[28] The addition of the amino group of the ethyl 3-amino-1,4-dihydroquinoxaline-2-carboxylate (**1**) to form intermediates **12a** and **12b** was followed by an intramolecular nucleophilic addition of the N*H* functionality on the cyano group. The spectroscopic data was found to be in good agreement with compounds **13a** and **13b**. The IR spectrum of compound **13a** showed the expected absorption bands of the NH_2, N*H*, CN and CO moieties at 3400–3100, 2199 and 1735 cm^{-1}. The ^{1}H NMR spectrum of **13a** showed a signal, corresponding to the triplet-quartet signal of the ethyl carboxylate functionality at δ_H 1.25 and 4.24 ppm. The NH and NH_2

signal appeared at 9.79 and 8.59 ppm, respectively. The EI-MS of **13a** showed the expected molecular ion peak at *m*/*z* 373.



Scheme 2. Synthesis of pyrimido[1,2-*a*]quinoxaline derivatives **13a,b**

On the other hand, the synthesis of the triazinoquinoxalinecarboxylates **18**, **20**, **22** and **24** was achieved through a multiple synthetic pathways, which included diazotization of the primary amino group of compound **1** to give diazonium salt **16** (Scheme 3). The reactions of salt **16** with resorcinol, α-naphthol and/or active methylene reagents, such as malononitrile and ethyl cyanoacetate in mixtures of ethanolic sodium acetate solutions gave arylazo compounds **17**, **19**, **21** and **23**, which were used without further analysis in the subsequent cyclization steps upon refluxing in acetic acid. The IR spectrum of triazinoquinoxaline carboxylate **18** showed the expected absorption band of the OH group at ν_{max} 3510 cm^{-1}. The ^{1}H NMR spectrum of **18** showed the phenolic OH signal appeared at 8.99 ppm as an abroad singlet. The EI-MS of **18** showed the expected molecular ion peak at *m*/*z* 322. Similarly, the reaction of salt **16** with α-naphthol proceeded through the azo-derivative **19**. The spectroscopic data of the obtained product was in good agreement with compound **20** as shown in Scheme 3. The ^{1}H NMR spectrum of **20** showed the ester triplet-quartet signals at δ_H 1.32 and 4.23, respectively. The molecular ion peak of **20** appeared at *m*/*z* 356. The reaction of malononitrile with **16** resulted in the formation of **21**, which upon treatment with acetic acid under refluxing conditions yielded **22**. The cyclization reaction of **21** took place by an attack of the ring NH on the cyano group. The IR spectrum of compound **22** showed the expected characteristic absorption bands of the functionalities existing in the molecule. The IR absorption bands of the CO, CN and NH, NH_2 moieties appeared at ν_{max} 1735, 2218 and 3100–3400 cm^{-1}, ester triplet-quartet and N*H*$_2$ appeared in the ^{1}H NMR spectrums of **22** at 1.32 and 4.23, 8.35 ppm.



Scheme 3. Synthesis of triazino[4,3-*a*]quinoxaline derivatives **18**, **20**, **22** and **24**

As similar to **22**; compound **24** was prepared from **16** and ethyl cyanoacetate through **23**. The spectroscopic data confirmed the structural framework of **24**. The IR spectrum of compound **24** showed the appearance of CO and CN groups at ν_{max} 1660 and 2218 cm^{-1}, respectively. Moreover, in ^1H NMR spectrum the presence of a singlet signal at 3.44 ppm due to the presence of CH proton confirmed the structure. The mass spectrum showed the molecular ion peak at *m*/*z* 297.

Antimicrobial activity

The candidates **1**, **4a**, **8c**, **13a**, **18**, **20**, **22** and **24** were tested for their antibacterial activity against a gram-negative bacterium (*Escherichia coli*) and a gram positive bacterium (*Micrococcus roseus*) using

levofloxacin as a standard. Results are presented in the next table (Table 1) which demostrated that the pyrimido[1,2-*a*]quinoxaline derivatives **1**, **4a** and **8c** exhibited antibacterial activity against both tested bacteria, *E. coli* and *M. roseus*. In addition, ethyl 3-amino-1,4-dihydroquinoxaline- 2-carboxylate **1** and ethyl 3-amino-2-cyano-2,6-dihydro-1*H*-pyrimido[1,2-*a*]quinoxaline-5-carboxylate (**4a**) recorded more antibacterial activity than the candidates **8c**. Moreover, the candidates **1** and **4a** revealed comparable antibacterial activity to that shown by the standard drug Levofloxacin. In case of antifungal activity; one isolate of the fungus of *Aspergillus niger*) and an isolate of *Aspergillus ochraceus* as well as two isolates of *Penicillium chrysogenum* and *Penicillium italicum* were used for the antifungal activity test using miconazole as a standard (Table 1, Figure 2). Ethyl 3-amino-2-cyano-2,6-dihydro-1*H*-pyrimido[1,2-*a*]-quinoxaline-5-carboxylate (**4a**) demonstrated comparable antifungal activity as recorded by miconazole against *A. niger* and *A. ochraceus*. Also, only the target compounds **1**, **4a** and **8c** displayed antifungal

Table 1. Antibacterial and antifungal activity of the tested compounds (**1**, **4a**, **8c**, **13a**, **18**, **20**, **22** and **24**)

Tested Compound	Bacteria		Fungi			
	Escherichia coli	***Micrococcus roseus***	***Aspergillus niger***	***Aspergillus ochraceus***	***Penicillium chrysogenum***	***Penicillium italicum***
1	+++	+++	++	++	++	++
4a	+++	+++	+++	+++	++	++
8c	++	++	+	+	+	+
13a	N	N	N	N	N	N
18	N	N	N	N	N	N
20	N	N	N	N	N	N
22	N	N	N	N	N	N
24	N	N	N	N	N	N
Control (the solvent) DEMFU	N	N	N	N	N	N
Levofloxacin	++++	++++	ND	ND	ND	ND
Miconazole	ND	ND	++++	++++	++++	++++

• N = No effect
• ND = Not determined
• Diameter of no growth zone: + = till 0.5 cm, ++ = till 1.0 cm, +++ = till 2.0 cm, ++++ = more than 2.0 cm
Sample concentration (included controls) = 3%, dissolved in *N*, *N*-dimethylformamide

activity. Regarding *A. niger* and *A. ochraceus*, the candidate **4a** recorded the highest antifungal than exhibited by compound **1** and **8c**. Concerning *P. chrysogenum* and *P. italicum*, both the target compounds **1** and **4a** showed higher antifungal activity than compound **8c**.

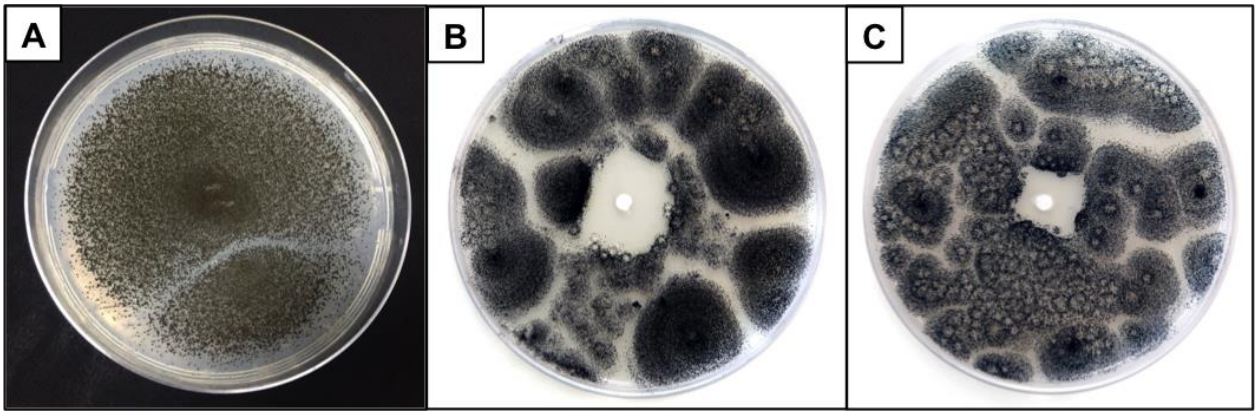
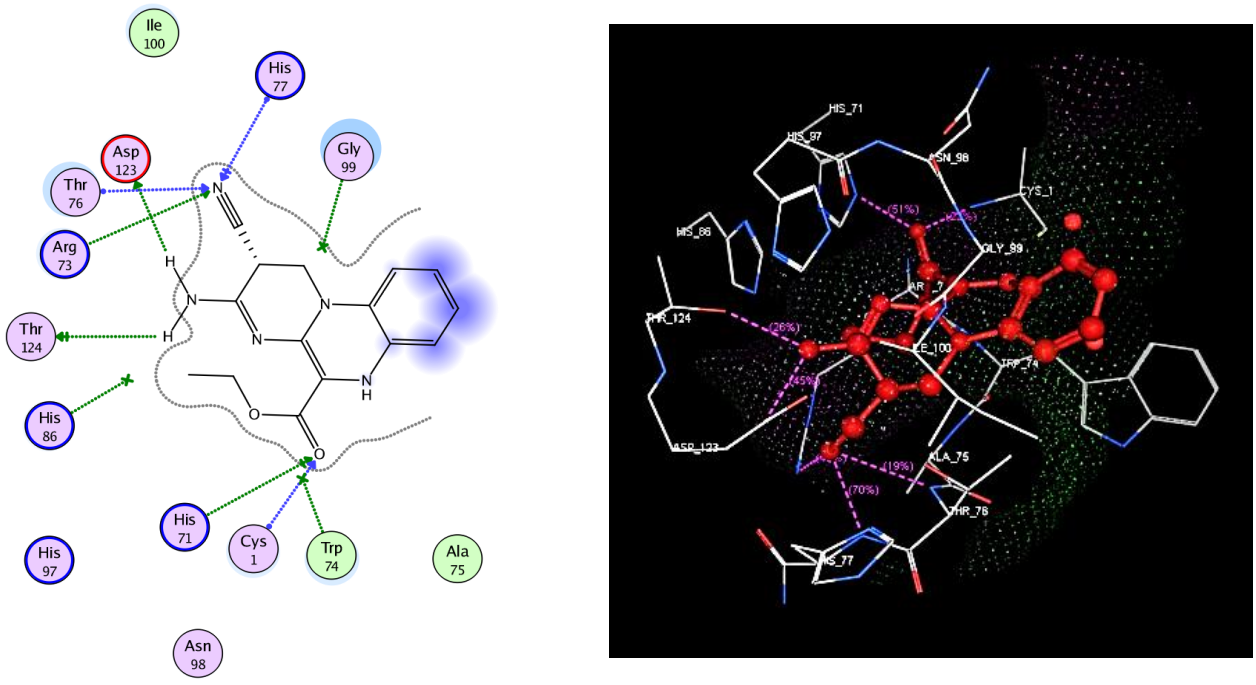


Figure 2. Effect of the tested compound (**4a**) on growth of *Asperigillus niger* cultivated in PDA medium after 6 days at 28 °C in the dark. Inhibition zones represent the influence.
(A) negative control, the filter paper disc embedded only on DEMFU, before culturing. (B) positive control, the filter paper disc embedded on Miconazole, before culturing. (C) the filter paper disc embedding in **4a**, before culturing.

Docking study

Molecular docking study had been carried out on the biologically active candidates to detect the mode of action of these target compounds. In this study, the target derivatives **1**, **4a** and **8c** were docked into binding site of glucosamine-6-phosphate synthase enzyme (GlcN-6-P). Glucosamine-6-phosphate synthase (*D*-fructose-6-phosphate amidotransferase) is one of most important enzyme for synthesis of cell wall[29] and is responsible for transferring ammonia from L-glutamine to Fru-6-P, then isomerization of the produced fructosamine-6-phosphate to glucosamine-6-phosphate.[30,31] This reaction is the first step for UDP-GlcNAc formation, which is required for living organisms, in fungi and bacteria and is necessary for building macromolecules for the cell wall assembly, as chitin and mannoproteins in fungi and peptidoglycan in bacteria. In human, UDP-GlcNAc is used for biosynthesis of glycoproteins and mucopolysaccharides.[32,33] Therefore, targeting GlcN-6-P synthase is a selective trend for preventing fungal growth with better selectivity and low toxicity. Many quinoline derivatives had been reported to inhibit GlcN-6-P synthase and so showed good antimicrobial activity. In this study we prepared many quinoxaline derivatives since they are considered as quinoline isosteres. This work was performed by the use of Molecular Operating Environment (MOE, 2010, Version 8, Chemical Computing Group Inc., Montreal, Quebec, Canada) as a software of choice used in the docking experiments. The X-ray crystal structure of GlcN-6-P was downloaded from the protein data bank with code (PDB: ID 1gdo). In this

study the target compounds **1**, **4a** and **8c** were docked into binding site of GlcN-6-P to confirm the ability of the novel candidates to act as antimicrobial agents. The obtained results of docking study including the energy associated with intermolecular interactions (affinity in kcal/mol) of the target compounds (**1**, **4a** and **8c**) within glucosamine-6-phosphate synthase active site and hydrogen bonding interactions between the amino acid residues and functional groups of compounds are showed in Table 2. From these data compound **4a** demonstrated the highest docking score (-18.22 kcal/mol), it showed seven hydrogen bonding as following: i) Cys1 with C=O, ii) His71 with C=O, iii) Thr124 with NH, iv) Asp123 with NH, v) Arg73 with CN, vi) Thr76 with CN, and vii) His77 with CN (Figure 3).

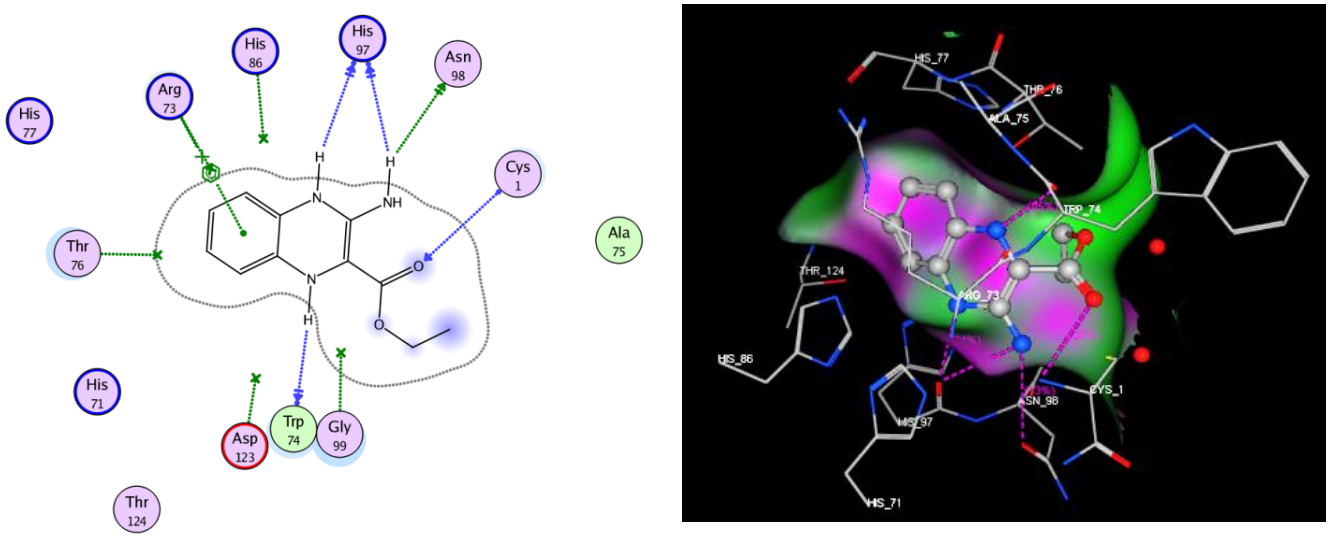


(**A**) (**B**)

Figure 3. Binding of compound **4a** inside glucosamine-6-phosphate synthase. (A) 2D interactions of **4a** within glucosamine-6-phosphate synthase (using MOE site finder program), the dotted lines represents seven H-bonding interactions. (B) 3D interactions of **4a** with glucosamine-6-phosphate synthase.

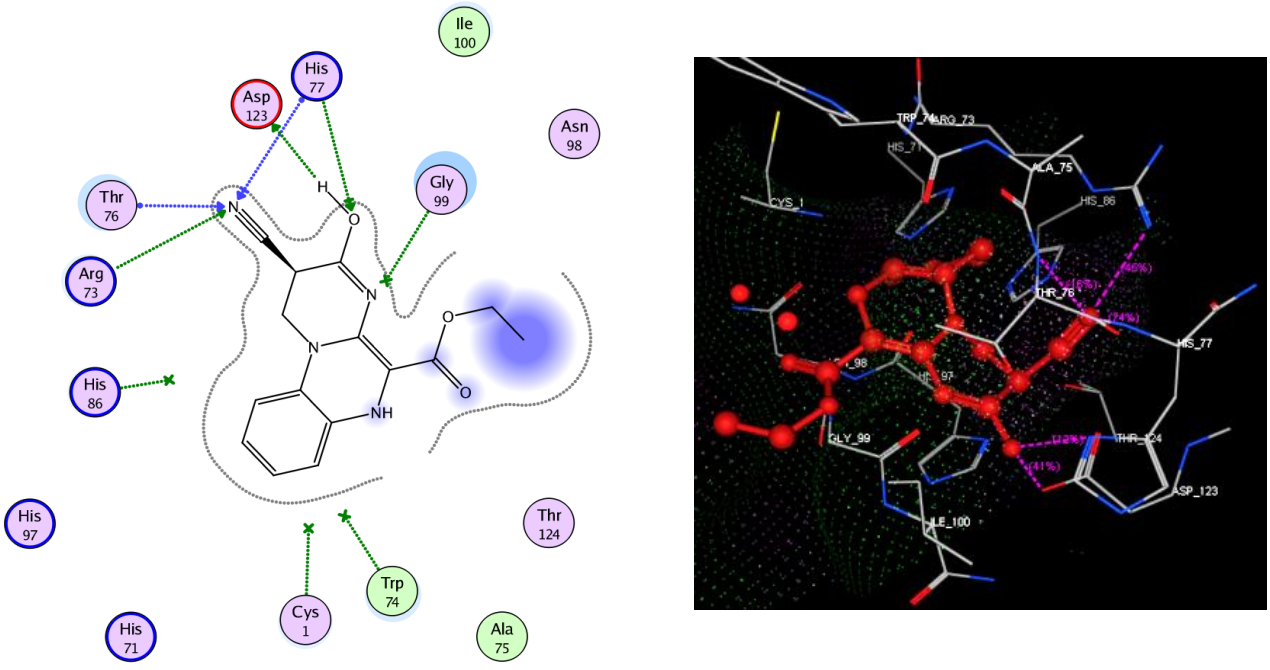
Furthermore, compound **1** exhibited docking score = -17.99 kcal/mole and showed five hydrogen bonding interactions: i) quinoxaline NH with His97, ii) NH with His97, iii) NH with Asn98, iv) C=O with Cys1 and v) NH and Trp 74. In addition, this compound performed arene-cation interactions between Arg73 and phenyl moiety (Figure 4).

Moreover, the candidate **8c** recorded energyscore = -15.32 kcal/mole and was detected to show five hydrogen bonding interactions: i) His77 with OH, ii) His77 with CN, iii) Asp123 with OH, iv) Thr76 with CN, and V) Arg73 with CN (Figure 5).



(**A**) (**B**)

Figure 4. Binding of compound **1** inside glucosamine-6-phosphate synthase. (A) 2D interactions of **1** within GlcN-6-P synthase (using MOE site finder program), the dotted lines represents seven H-bonding interactions. (B) 3D interactions of **1** with GlcN-6-P synthase.



(**A**) (**B**)

Figure 5. Binding of compound **8c** inside glucosamine-6-phosphate synthase. (A) 2D interactions of **8c** within GlcN-6-P synthase (using MOE site finder program), the dotted lines represents seven H-bonding interactions. (B) 3D interactions of **8c** with GlcN-6-P synthase.

Table 2. Molecular modeling data for compounds **1**, **4a** and **8c** in the active site of glucosamine-6-phosphate synthase enzyme (PDB: ID 1gdo)

Compound no.	Affinity kcal/mol	No. of Hydrogen bonds	Distance (Å) from main residue		Functional group
1	-17.99	5	His97	2.55	QuinoxalineNH
			His97	2.16	NH
			Asn98	2.67	NH
			Cys1	3.11	C=O
			Trp74	3.05	NH
4a	-18.22	7	Cys1	2.10	C=O
			His71	2.38	C=O
			Thr124	3.11	NH
			Asp123	2.89	NH
			Arg73	3.25	CN
			Thr76	2.89	CN
			His77	2.12	CN
8a	-15.32	5	His77	2.10	OH
			His77	2.38	CN
			Asp123	3.01	OH
			Thr76	2.89	CN
			Arg73	3.23	CN

EXPERIMENTAL

Chemistry: Reagents were purchased from Sigma Aldrich (Bayouni Trading Co. Ltd., Al-Khobar, Saudi Arabia) and used without further purification. Reaction progress was monitored by thin-layer chromatography on silica gel pre-coated F254 Merck plates (Darmstadt, Germany). Spots were visualized by ultraviolet irradiation. Melting points were determined on a Gallenkampelectrothermal melting point apparatus (Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. IR spectra were recorded as potassium bromide disks using Bruker-Vector 22 Fouriertransform infrared spectrophotometer (Billerica, MA). The NMR spectra were recorded with a Varian Mercury VXR-300 NMR spectrometer (Palo Alto, CA) at 300 and 75 MHz for ^{1}H and ^{13}C NMR spectra, respectively, is using DMSO-d_6 as solvents. Mass spectra were recorded on a Hewlett Packard MS-5988 spectrometer (Palo Alto, CA) at 70 eV. Elemental analyses were carried out at the Micro-analytical Center of Cairo University, Giza, Egypt. Ethyl 3-amino-1,4-dihydroquinoxaline-2-carboxylate (**1**),[34] alkylidene derivatives (**2a-d**)[27] and arylidene derivatives (**11a**, **11b**)[35] were synthesized according to literature methods.

General procedure for the synthesis of compounds (4a, 4b) and (8c, 8d). A mixture of compound (**1**) (0.01 mol) and the corresponding alkylidene derivatives (**2a-d**) (0.01 mol) in EtOH (30 mL) and few drops of piperidine was refluxed for 12-13 h and the solvent was evaporated *in vacuo*. The remaining

solid was triturated with water-ice and acidified with concentrated HCl. The formed product was collected by filtration and recrystallized from acetone to afford compounds **4a**, **4b**, **8c**, and **8d**.

Ethyl 3-amino-2-cyano-2,6-dihydro-1*H*-pyrimido[1,2-*a*]quinoxaline-5-carboxylate (4a). Yield 57%; mp 185-187 °C; IR (KBr): ν (cm^{-1}), 1735 (CO ester), 2215 (CN), 3100–3400 (NHstr., NH_2str.); ^{1}H NMR (δ, DMSO-d_6): 1.23 (t, *J* = 7.54 Hz, 3H, CH_2CH_3), 2.73 (t, 1H, *J* = 4.10 Hz, C*H*CN), 3.41 (d, 2H, *J* = 4.10 Hz, CH_2N), 4.17 (q, *J* = 7.55 Hz, 2H, CH_2CH_3), 7.10–8.01 (m, 4H, Ar-H), 9.15 (brs, 2H, NH_2), 11.35 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): 14.23 (CH_3), 61.53 (CH_2), 117.95, 118.62, 119.8, 120.3, 128.52, 132.36, 116.86 (CN), and 165.4 (CO); MS (*m/z*, %): 297.0 (M^+, 40). Anal. Calcd for $C_{15}H_{15}N_5O_2$ (297.31): C, 60.60; H, 5.09; N, 23.56. Found: C, 60.62; H, 5.30; N, 23.59.

Ethyl 3-amino-2-cyano-1-methyl-2,6-dihydro-1*H*-pyrimido[1,2-*a*]quinoxaline-5-carboxylate (4b). Yield 63%; mp 265-267 °C; IR (KBr): ν_{max} (cm^{-1}), 3100–3400 (NHstr., NH_2str.), 2215 (CN), 1735 (CO ester); ^{1}H NMR (δ, DMSO-d_6): 1.09 (t, 3H, CH_3), 1.23 (t, *J* = 7.54 Hz, 3H, CH_2CH_3), 4.17 (q, *J* = 7.56 Hz, 2H, CH_2CH_3), 2.73 (d, 1H, *J* = 3.41 Hz, C*H*CN), 3.71 (d, 1H, *J* = 4.10 Hz, C*H*N), 7.10–8.01 (m, 4H, Ar-H), 9.17 (brs, 2H, NH_2), 11.38 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): 14.23 (CH_3), 61.53 (CH_2), 117.95, 118.62, 119.8, 120.3, 128.52, 132.36, 116.86 (CN), and 165.4 (CO); MS (*m/z*, %): 311.0 (M^+, 40). Anal. Calcd for $C_{16}H_{17}N_5O_2$ (311.34): C, 61.72; H, 5.50; N, 22.49. Found: C, 61.74; H, 5.54; N, 22.52.

Ethyl 2-cyano-3-hydroxy-2,6-dihydro-1*H*-pyrimido[1,2-*a*]quinoxaline-5-carboxylate (8c). Yield 77%; mp 250-253 °C; IR (KBr): ν_{max} (cm^{-1}) = 3430 (OH), 2216 (CN), 1735 (CO ester), 3100–3400 (NHstr); ^{1}H NMR (δ, DMSO-d_6): 1.24 (t, *J* = 7.54 Hz, 3H, CH_2CH_3), 4.30 (q, *J* = 7.56 Hz, 2H, CH_2CH_3), 2.71 (t, 1H, C*H*CN), 3.74 (d, 2H, *J* = 4.10 Hz, CH_2N), 7.10–8.01 (m, 4H, Ar-H), 10.25 (s, 1H, OH), 11.38 (s, 1H, NH, D_2O exchangeable).^{13}C NMR (DMSO-d_6, ppm): 14.25 (CH_3), 64.48 (CH_2), 26.77 (C-CN), 105.76, 131.25 (C=C), 117.95, 118.62, 119.8, 120.3, 128.52, 132.36, 116.86 (CN), 161.73 (C-OH) and 165.4 (CO); MS, *m*/*z* (%): 298 (M^+, 66). Anal. Calcd for $C_{15}H_{14}N_4O_3$ (298.30); C, 60.40; H, 4.73; N, 18.78. Found: C, 60.43; H, 4.77; N, 18.81.

Ethyl 2-cyano-3-hydroxy-1-methyl-2,6-dihydro-1*H*-pyrimido[1,2-*a*]quinoxaline-5-carboxylate (8d). Yield 76%; mp 270-272 °C; IR (KBr): ν_{max} (cm^{-1}) = 3400–3100 (NHstr), 3430 (OH), 2216 (CN), 1735 (CO ester); ^{1}H NMR (δ, DMSO-d_6): 1.23 (t, *J* = 7.54 Hz, 3H, CH_2CH_3), 1.18 (s, 3H, CH_3), 4.17 (q, *J* = 7.56 Hz, 2H, CH_2CH_3), 2.71 (t, 1H,C*H*CN), 7.10–8.01 (m, 4H, Ar-H), 10.25 (s, 1H, OH), 11.38 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, ppm): 14.26 (CH_3), 15.84 (CH_3), 51.49 (CH), 31.17 (C-CN), 105.76, 131.25, 117.95, 118.62, 119.8, 120.3, 128.52, 132.36, 116.86 (CN), 161.73 (C-OH) and

165.4 (CO); MS, *m*/*z* (%): 312 (M^+, 66). Anal. Calcd For $C_{16}H_{16}N_4O_3$ (312.32); C, 61.53; H, 5.16; N, 17.94. Found: C, 61.55; H, 5.20; N, 17.97.

General procedure for the synthesis of compounds 13a and 13b. A mixture of (**1**) (0.01 mol) and cinnamonitriles (**11a**) or (**11b**) (0.01 mol) in absolute EtOH (20 mL), DMF (5 mL) and piperidine (0.5 mL) was refluxed for 12-13 h and the solvent was evaporated. The remaining solid product was treated with ice-water and acidified with concentrated HCl. The product was collected by filtration and recrystallized from MeOH to afford compounds (**13a**) or (**13b**).

Ethyl 1-amino-2-cyano-3-phenyl-4,6-dihydro-3*H*-pyrimido[1,2-*a*]quinoxaline-5-carboxylate (13a). Yield 55%; mp 295-297 °C; IR (KBr): ν_{max} (cm^{-1}): 3100–3400 (2NH, NH_2), 2199 (CN), 1735 (CO ester); ^{1}H NMR (DMSO-d_6, ppm): 1.25 (t, J = 7.54 Hz, 3H, CH_2CH_3), 4.64 (s, 1H, C*H*Ph), 4.24 (q, J = 7.56 Hz 2H, CH_2CH_3), 7.01–8.02 (m, 9H, CH_{Ar}), 8.59 (brs, 2H, NH_2), 9.79 (s, 1H, NH), 11.38 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, ppm): 14.26 (CH_3), 61.48(CH_2), C-Ph (50.32), 59.31 (C-CN), 105.76, 131.25 (C=C), 117.95, 117.38 (CN), 118.62, 119.8, 120.30, 128.52, 132.36, 164.93 (CO); MS, *m*/*z* (%): 373 (M^+, 50). Anal. Calcd for $C_{21}H_{19}N_5O_2$ (373.41); C, 67.55; H, 5.13; N, 18.76. Found: C, 67.58; H, 5.17; N, 18.78.

Diethyl 1-amino-3-phenyl-4,6-dihydro-3*H*-pyrimido[1,2-*a*]quinoxaline-2,5-dicarboxylate (13b). Yield 57%; mp 240-242 °C; IR (KBr): ν_{max} (cm^{-1}): 1735 (CO ester), 3100–3400 (2NH, NH_2), 2199 (CN); ^{1}H NMR (DMSO-d_6, ppm): 1.25 (t, J = 7.54 Hz, 6H, $2CH_2CH_3$), 4.21 (q, J = 7.56 Hz, 4H, $2CH_2CH_3$), 4.45 (s, 1H, C*H*Ph), 7.01–8.02 (m, 9H, CH_{Ar}), 8.58 (brs, 2H, NH_2), 9.79 (s, 1H, NH), 11.37 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, ppm): 14.26 (CH_3), 61.48 (CH_2), C-Ph (50.32), 59.42 (C-CN), 87.04, 137.76 (C=C), 117.95, 118.62, 119.8, 120.24, 126.73, 128.52, 132.36, 117.38 (CN), 164.93, 167.24 (CO); MS, *m*/*z* (%): 420 (M^+, 45). Anal. Calcd for $C_{23}H_{24}N_4O_4$ (420.46); C, 65.70; H, 5.75; N, 13.33. Found: C, 65.72; H, 5.78; N, 13.37.

General procedure for the reaction of diazotized compound (16) with active methylene compounds. A solution of diazotized compound (**16**) (0.01 mol) was added dropwise with stirring at 0-5 °C over a course of 30 min to a cold solution of resorcinol, α-naphthol, malononitrile or ethyl cyanoacetate in EtOH (50 mL) containing 5 g of sodium acetate. The reaction mixture was stirred for further 4 h, then kept in an ice chest for additional 12 h and finally diluted with water. The precipitated solid was collected by filtration, washed with water, dried and recrystallized from MeOH to afford the corresponding arylazo compounds (**17**), (**19**), (**21**) and (**23**) and the products were used in following steps without further analysis.

General procedure for the cyclization of compounds 17, **19**, **21 and 23.** A solution of compounds (**17**), (**19**), (**21**) or (**23**) (0.00327 mol) in acetic acid (30 mL) was refluxed for 4 h. The solvent was poured onto ice cold water and the solid precipitate was filtered off, washed with water and dried. The crude product was recrystallized from EtOH to afford the corresponding fused ring systems (**18**), (**20**), (**22**) or (**24**).

Ethyl 2-hydroxy-8*H*-benzo[5,6][1,2,4]triazino[4,3-*a*]quinoxaline-7-carboxylate (18). Yield 70%; mp 230-232 °C; IR (KBr): ν_{max} (cm^{-1}): 3510 (OH), 3100–3400 (NH), 1735 (CO ester), ^{1}H NMR (DMSO-d_6, ppm): δ_H = 1.25 (t, 3H, J = 7.02 Hz, CH_3), 4.20 (q, 2H, J = 7.21 Hz, O$\underline{CH_2}CH_3$), 7.02–8.02 (m, 7H, CH_{Ar}), 8.99 (s, 1H, OH), 11.37 (s, 1H, NH, D_2O exchangeable.), ^{13}C NMR (DMSO-d_6, ppm): 14.26 (CH_3), 61.48 (CH_2), 112.84, 122.26 (C=C), 103.94, 118.62, 119.8, 120.24, 126.73, 130.53, 132.36, 144.41, 106.58 (C-N=N), 164.94 (CO); MS, *m*/*z* (%): 322 (M^+, 30). Anal. Calcd for $C_{17}H_{14}N_4O_3$ (322.32); C, 63.35; H, 4.38; N, 17.38. Found: C, 63.37; H, 4.41; N, 17.42.

Ethyl 1*H*-naphtho[1',2':5,6][1,2,4]triazino[4,3-*a*]quinoxaline-2-carboxylate (20). Yield 55%; mp 275-277 °C; IR (KBr): ν_{max} (cm^{-1}): 3400–3100 (NH), 1735 (CO ester); ^{1}H NMR (DMSO-d_6, ppm): δ_H = 1.32 (t, 3H, J = 7.02 Hz, CH_3), 4.23 (q, 2H, J = 7.21 Hz, O$\underline{CH_2}CH_3$), 7.02–8.02 (m, 10H, CH_{Ar}), 11.37 (s, 1H, NH, D_2O exchangeable.),^{13}C NMR (DMSO-d_6, ppm): 14.26 (CH_3), 61.48 (CH_2), 112.84, 122.26 (C=C), 103.94, 118.62, 119.8, 120.24, 126.73, 130.53, 132.36, 144.41, 108.58 (C-N=N), 164.94 (CO); MS, *m*/*z* (%): 356 (M^+, 25). Anal. Calcd for $C_{21}H_{16}N_4O_2$ (356.38); C, 70.77; H, 4.53; N, 15.72. Found: C, 70.79; H, 4.56; N, 15.76.

Ethyl 1-amino-2-cyano-6*H*-[1,2,4]triazino[4,3-*a*]quinoxaline-5-carboxylate (22). Yield 45%; mp 255-257 °C; IR (KBr): ν_{max} (cm^{-1}): 3400–3100 (NH, NH_2), 2218 (CN), 1735 (CO ester): ^{1}H NMR (DMSO-d_6, ppm): δ_H = 1.32 (t, 3H, J = 7.02 Hz, CH_3), 4.23 (q, 2H, J = 7.21 Hz, O$\underline{CH_2}CH_3$), 7.02–8.02 (m, 4H, CH_{Ar}), 8.35 (s, 2H, NH_2), 11.37 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR (DMSO-d_6, ppm): 14.26 (CH_3), 61.48 (CH_2), 42.85, 122.81 (C=C), 53.47, 173.41, 103.94, 118.62, 119.8, 120.24, 126.73, 130.53, 132.36, 144.41 (C=C), 114.68 (CN), 164.94 (CO); MS, *m*/*z* (%): 296 (M^+, 30). Anal. Calcd for $C_{14}H_{12}N_6O_2$ (296.28); C, 56.75; H, 4.08; N, 28.36. Found: C, 56.78; H, 4.12; N, 28.38.

Ethyl 2-cyano-1-oxo-2,6-dihydro-1*H*-[1,2,4]triazino[4,3-*a*]quinoxaline-5-carboxylate (24). Yield 63%; mp 236-238 °C; IR (KBr): ν_{max} (cm^{-1}): 1660 (CO), 1735 (CO ester), 2218 (CN), 3100–3400 (NH); ^{1}H NMR (DMSO-d_6, ppm): δ_H = 1.32 (t, 3H, J = 7.02 Hz, CH_3), 4.23 (q, 2H, J = 7.21 Hz, O$\underline{CH_2}CH_3$), 3.44 (s, 1H, CH), 7.02–8.02 (m, 4H, CH_{Ar}), 11.37 (s, 1H, NH, D_2O exchangeable), ^{13}C NMR (DMSO-d_6, ppm): 14.26 (CH_3), 61.48 (CH_2), 55.46 (CH), 112.86, 122.81 (C=C), 46.18, 192.73, 103.94, 118.62, 119.8, 120.24, 126.73, 130.53, 132.36, 135.41 (C=C), 114.69 (CN), 162.87 (CO), 164.94 (CO); MS, *m*/*z* (%): 297 (M^+, 16). Anal. Calcd for $C_{14}H_{11}N_5O_3$ (297.27); C, 56.56; H, 3.73; N, 23.56. Found: C, 59.56; H,

3.77; N, 23.60.

Antimicrobial activity

This technique was performed using the filter paper disc method.[36] For antibacterial activity; an isolate of Gram-negative bacterium (*E. coli*) which was isolated freshly from human feces in toilet for students, College of Science, Jouf University, Saudi Arabia, and an isolate of gram positive bacterium (*M. roseus*) which was obtained from soil around Dawmat Aljandal lake, Dawmat Aljandal city, Aljouf, Saudi Arabia were used. Bacteria were isolated and purificated on Nutrient Agar (NA) medium for subsequent operations. For preparation of bacterial inocula, each isolate was cultured on a Nutrient Broth (NB) and incubated at 35 °C for 24 h. In isolation cabinet and under the aseptic conditions, 1 mL of NB medium containing the bacterial inocula were placed in a sterile Petri dish and then 15 mL of the warm melted NA medium poured over the inocula. Dishes were left until solidification and then each dish was supplemented with filter paper disc (5 mL diameter) which was saturated with 3% concentration of each chemical sample, dissolved, in DMF. Plates were sealed with parafilm, placed in sterile bags and closed with rubber bands, and incubated at 35 °C for 48 h. Diameter of inhibition zones was measured and results were recorded. All effects were done against a standard control sample of the antibiotic of levofloxacin.

Antifungal activity; Four isolates of *A. niger*, *A. ochraceus*, *P. chrysogenum* and *P. italicum* which were isolated recently from air of car repair workshops in the industrial area, Sakaka city, Aljouf, Saudi Arabia, were used in this study. Fungi were cultured and maintained on Potato Dextrose Agar (PDA) medium. In isolation cabinet and under the aseptic conditions, 1 mL of conidial suspension of each fungus were placed in a sterile Petri dish and then 15 mL of the warm melted PDA medium poured over the conidial suspension. Dishes were left until solidification and then each dish was supplemented with filter paper disc (5 mm diameter) which was saturated with 3% concentration of each chemical sample, dissolved, in DMF. Tested plates were wrapped with parafilm, placed in sterile bags and closed with rubber bands, and incubated at 28 °C for 4 days. The diameter of inhibition zones was measured and results were recorded. All effects were done against a standard control sample of the antifungal of miconazole.

Docking study

In this work, we carried out molecular simulation modelling by the aid of Molecular Operating Environment software (MOE, Version 2010.08, Canada). We obtained the active site by downloading glucosamine-6-phosphate synthase enzyme (GlcN-6-P) (PDB: ID 1gdo) from RCSB Protein Data Bank. The novel candidates **1**, **4a** and **8c** for docking was done *via* their 3D structure. Before docking, certain steps were performed including running conformational analysis using systemic search, 3D protonation of the structures, selecting the least energetic conformer and applying the docking protocol. Hydrogen bond

lengths, amino acid interactions, and the summarized in (Table 2).

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