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Reactions of Heterocyclic *beta*-enaminoester, Synthesis and Antimicrobial Activity of Novel PyrimidinoPyrimidine Derivatives

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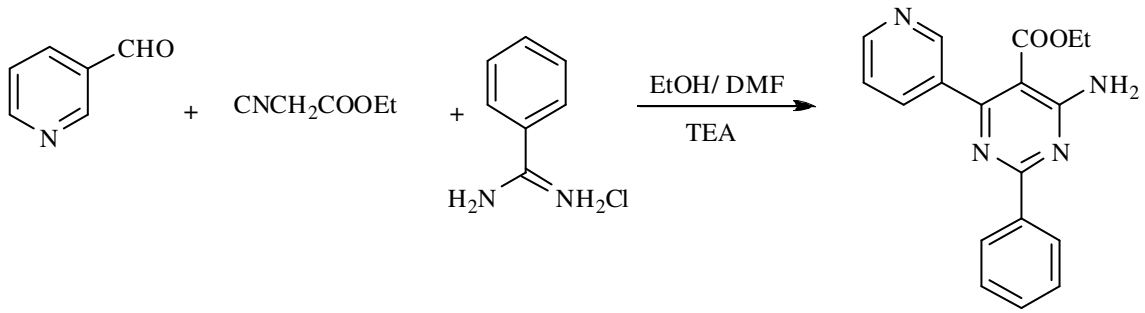
Abstract

In this work the corresponding 4-amino-6-pyridyl-2-phenyl-5-carbethoxypyrimidine **(3)** was synthesized through a one-pot, three-component reaction of pyridine-3-carbaldehyde, ethyl cyanoacetate and benzamidine hydrochloride in the presence of TEA in refluxing ethanol-DMF mixture. The synthesized pyrimidine **(3)** was directly transformed to medicinally important pyrimido[4,5-d]pyrimidine derivatives. All the synthesized products were tested and evaluated as antimicrobial agents.

Keywords: Pyrimidine-5-ethyl carboxylate derivative, Pyrimido[4,5-d]pyrimidine, Chloroacetyl chloride

1. Introduction

Pyrimido pyrimidines are annelated uracils that have attracted considerable interest in recent years. Derivatives of pyrimido pyrimidine are known to display a wide range of pharmacological activities, and their potent inhibitory properties regarding the tyrosine kinase domain of epidermal growth factor receptor (Rewcastle *et al.* 1997), 5-phosphoribosyl-1-pyrophosphate synthetase (Fry *et al.* 1995) and dihydrofolate reductase (Gready *et al.* 2003) have been fully demonstrated. Numerous reports delineate the antitumor (Sanghhvi *et al.* 1989), antiviral (Tenser *et al.* 2001), antioxidant (De la Cruz *et al.* 1992), antifungal and heptatoprotective activities (Ram *et al.* 2002). Combinatorial chemistry is playing an increasingly important role as one of the tool of modern medicinal chemistry for the rapid discovery of new leads (Gordan et al. 1994; Virgilio & Ellman 1994; Freier *et al.* 1995; Gordeev *et al.* 1997). Multi-component reactions (MCRs) (Zhu & Bienayme 2005; Domling 2006; Ram *et al.* 2002) are masterpieces of synthetic efficiency and reaction design. Inevitably, many classical heterocyclic syntheses are MCR that are based upon carbonyl group condensations. Hence, medicinal chemistry is largely found on these easily accessible heterocyclic frameworks. The use of multicomponent reactions (MCRs) to generate interesting and novel, druglike scaffolds is replete in the recent chemical literature (Ugi *et al.* 1997; Armstrong *et al.* 1996). This prompted us to synthesize some new pyrimido-pyrimidine derivatives starting from 4-amino-5-carbethoxy pyrimidine derivative **3** which prepared as shown in Scheme 1.



1 **2** **3**

Scheme 1. Synthesis of 4-amino-6-pyridyl-2-phenyl-5-carbethoxypyrimidine **(3)**

2. Results and discussion

2.1. Chemistry

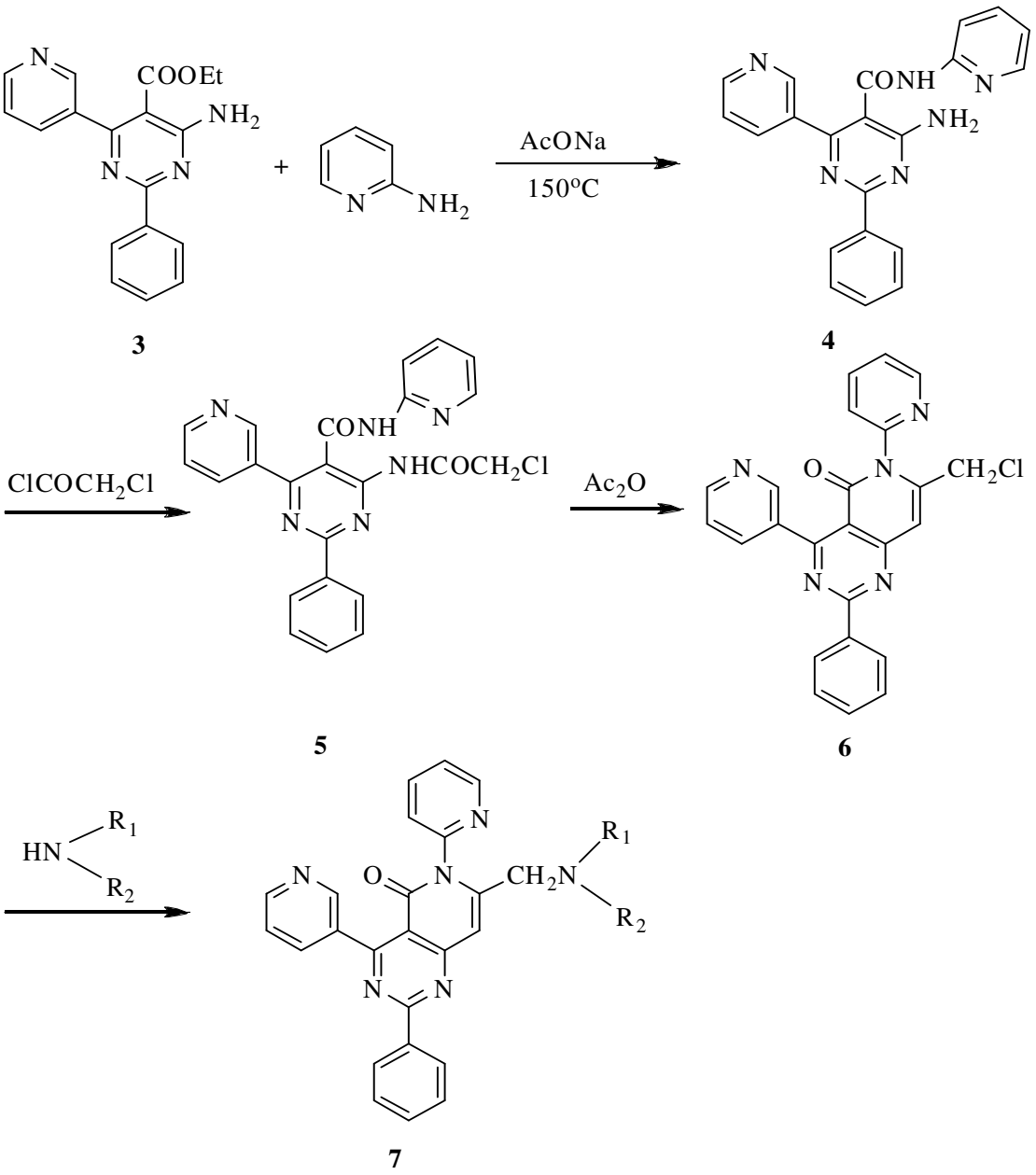
Initially, pyridine-3-carboxaldehyde **1**, ethyl cyanoacetate and benzamidine hydrochloride **2**, were mixed and heated in ethanol-DMF (1:1) in presence of a catalytic amount of TEA. The product **3** was prepared with excellent yield. The IR spectrum showed bands at 3500-3400 (NH_2), 3100 (NH), 1690 (CO), 1595 cm^{-1} (C=C), ^{1}HNMR spectrum showed peaks at δ 1.09 (CH_3), 4.30 (CH_2), 6.90 (NH_2) and 7.50-8.36 (m, Ar-H).

Fusion of **3** with 2-amino pyridine in the presence of a catalytic amount of freshly fused sodium acetate afforded the amide derivative **(4)** whose IR spectrum showed bands at 3500-3400 (NH_2), 3100 (NH), 1680 (C=O), 1595 cm^{-1} (C=C).

This amide reacted with chloroacetyl chloride in chloroform at room temperature to give the chloro-acetylamino derivative **(5)**, the structure of which was supported by IR spectrum showing bands at 3100 (NH), 1680 (C=O), 1687 (C=O), 1595 (C=C) and 760 cm^{-1} (C-Cl), and PMR spectrum exhibiting a proton singlet at δ 4.27 (CH_2) in addition to a multiplet due to aromatic and NH protons.

The chloroacetylamino derivative **(5)** was cyclized by refluxing with acetic anhydride to give the corresponding pyrimidine derivative **(6)**. Its structure was established on the basis of elemental analysis and spectral data. The IR spectrum showed bands at 2950 (C-H methylene), 1700 (C=O), 1595 (C=C) and 760 cm^{-1} (C-Cl), while PMR spectrum exhibited two-protons singlet at δ 4.37 (CH_2).

The Mannich base moiety is known to have diverse pharmacological properties including antibacterial, antiviral, and antiallergic. This prompted us to synthesis pyrimidine derivatives possessing a Mannich base moiety. Thus, the chloro compound **6** and an appropriate secondary amine when left at room temperature for several days gave the corresponding Mannich base **(7a,b)** (Scheme 2).



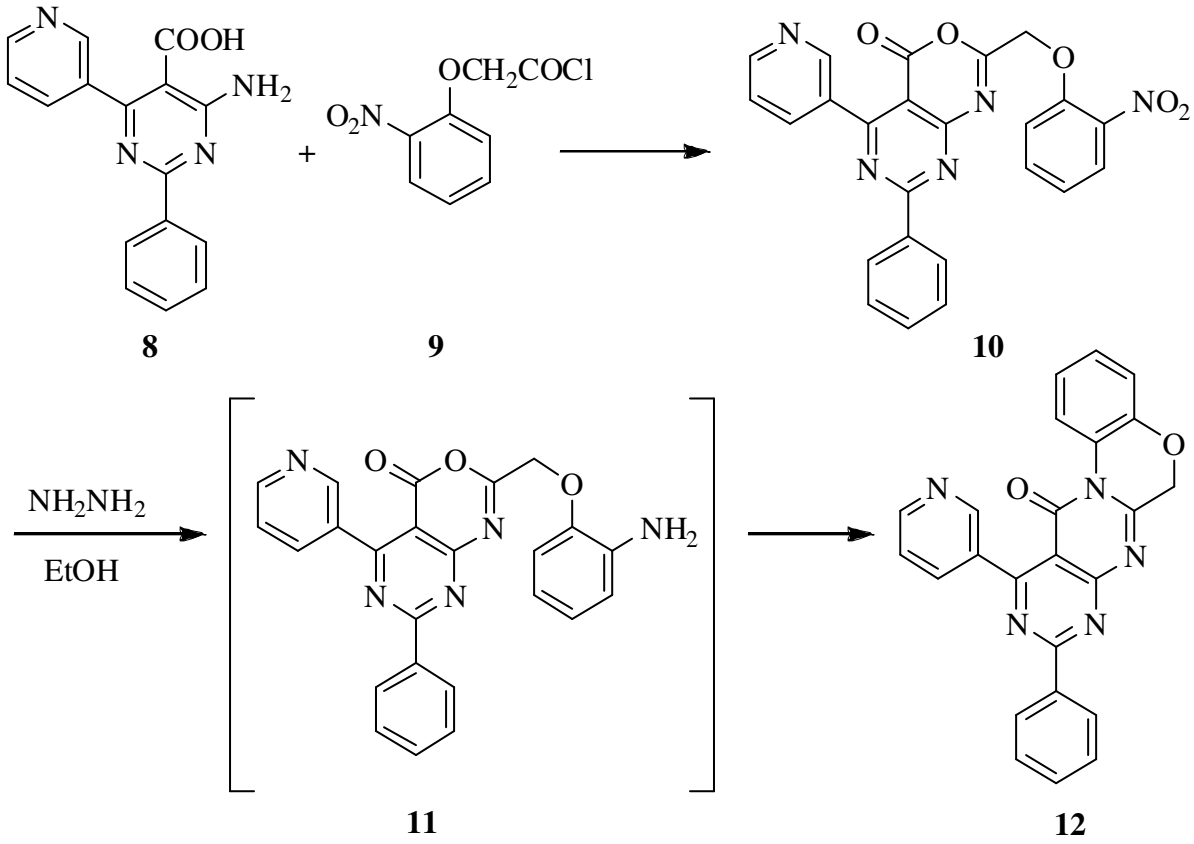
7 , a, $R_1 = R_2 = CH_3$

b, $R_1 = R_2 = C_2H_5$

Scheme 2. Synthesis of pyrido[4,3-d]pyrimidine derivatives **6** and **7**

The IR spectra of **7** displayed bands around 1565,1620 and 2900 cm^{-1} assignable to C-N, C=N and CH_2 stretching, respectively. The PMR spectrum of VIIa in $CDCl_3$ exhibited signals at δ 3.91 (s, 2H, -C-CH_2-N), 3.18 [s, 6H, $(NMe)_2$], and 7.50-8.30 (m, 12H, Ar-H). The PMR data of the other member **7b** of the series were also in agreement with its structure.

In continuation of our study on various reactions (Mohamed *et al.* 2013; Fadda *et al.* 2008; Fadda & Abdel-Razek 2001; Fadda 2012), we report herein the results of our investigation on the reaction of **3** with 2-nitrophenoxyacetic acid **(9).** It was found that the amino acid **8**, obtained by hydrolysis of the aminoester **3**, reacted with **9** to give the corresponding oxazine **10** (Scheme 3). Its IR spectrum showed bands at 1680 (C=O), 1520, 1360 cm^{-1} aromatic (NO_2).



Scheme 3. Synthesis of pyrimido-oxazinone derivatives **10** and **12**

The oxazine derivative **10** on reduction afforded a product which was assigned the structure **12** based on elemental analysis and spectral data. The formation of **12** may be assumed to proceed as shown in Scheme 3.

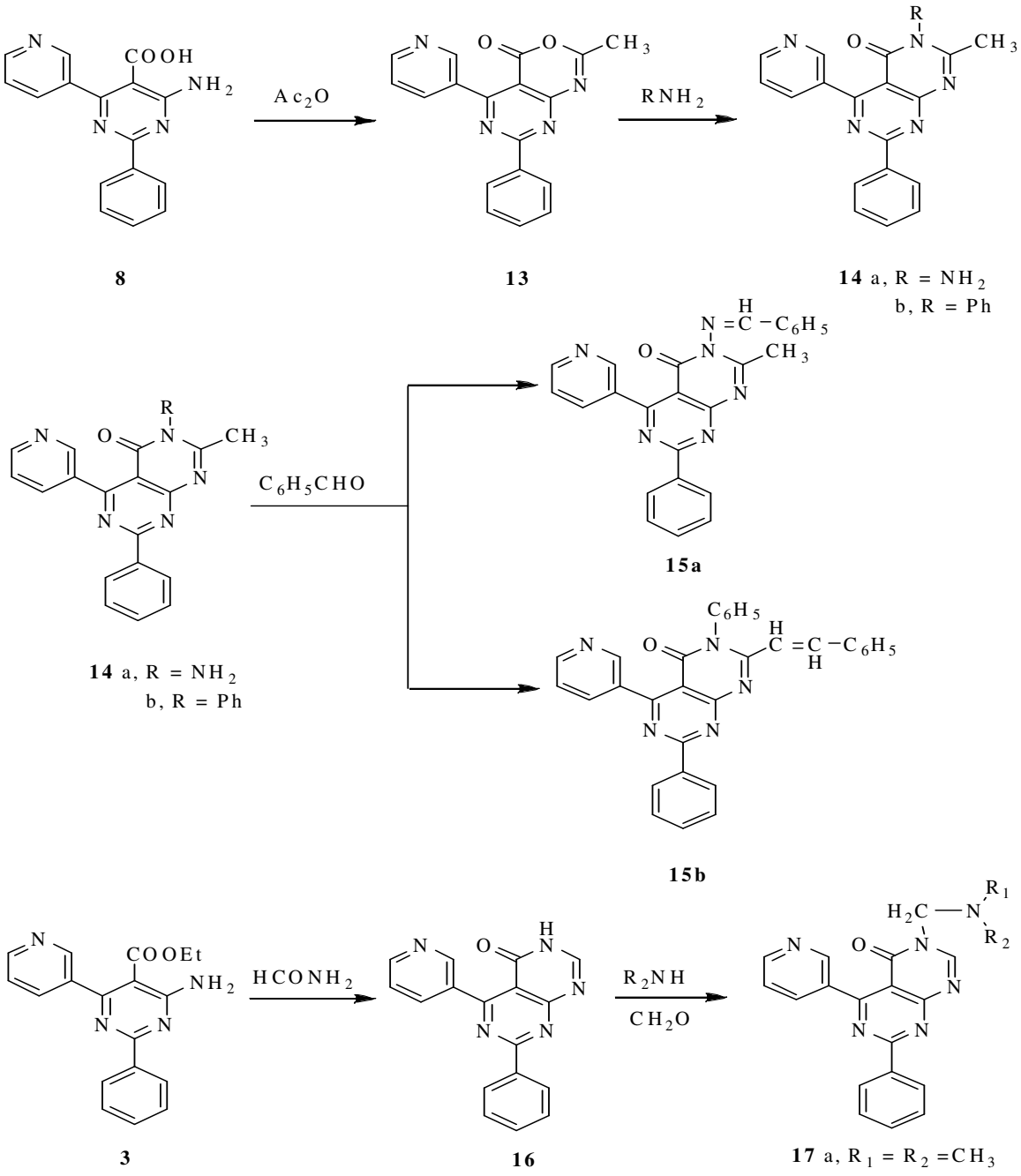
Attempts to isolate the intermediate **11** remained unsuccessful. Absence of IR bands at 1360 and 1525 cm^{-1} due to the aromatic nitro group, present in its precursor, indicated that the reduction of nitro group was complete and also the absence of 3300-3400 cm^{-1} band confirmed that the amino group formed during this reduction had reacted interamolecularly with oxazine oxygen to form **12**.

Reaction of the amino acid **8** with acetic anhydride seemed to be a logical method for the preparation of the corresponding oxazine derivative. Such a reaction furnished 2-methyl-7-phenyl-5-(pyridin-3-yl)-4*H*-pyrimido[4,5-d][1,3]oxazin-4-one **(13)** (Scheme 4).

We also report herein the synthesis of new pyrimido-pyrimidines **(14)** *via* the reaction of **13** with hydrazine hydrate and aromatic amines. The IR spectrum of **14a** showed bands at 3400-3350 (NH_2), 2950 (CH_3), 1680 cm^{-1} (C=O). The pyrimido-pyrimidines **14a,b** were reacted with benzaldehyde to give the corresponding Schiff base **(15a)** and the arylidene derivative **(15b)**, respectively (Scheme 4).

Compound **15a** showed IR peaks at 1690 ($-\overset{\overset{O}{\|}}{C}-N$) and 1610 cm^{-1} (C=N), whereas compound **15b** gave peaks at 1680 (C=O) and 1590 cm^{-1} (C=C). The PMR spectrum of **15a** exhibited signals at δ 2.3 (s, 3H, CH_3), 7.50-8.35 (m, 13H, Ar-H) and one azomethine proton at δ 9.01 ppm, whereas that of **15b** showed signals at 5.3 (d, 1H, CH=CH, J = 7.4 Hz), 6.2 (d, 1H, -CH=CH-, J = 7.4 Hz) and 7.50-8.33 (m, 13H, Ar-H). From the J values of CH-CH protons we can say that these protons are *cis*-oriented.

The pyrimido amino ester **3** also reacted with formamide to yield the corresponding pyrimido-pyrimidine **(16)** which formed the Mannish base **17** (Scheme 4). The IR spectrum of **17** displayed bands around 1565,



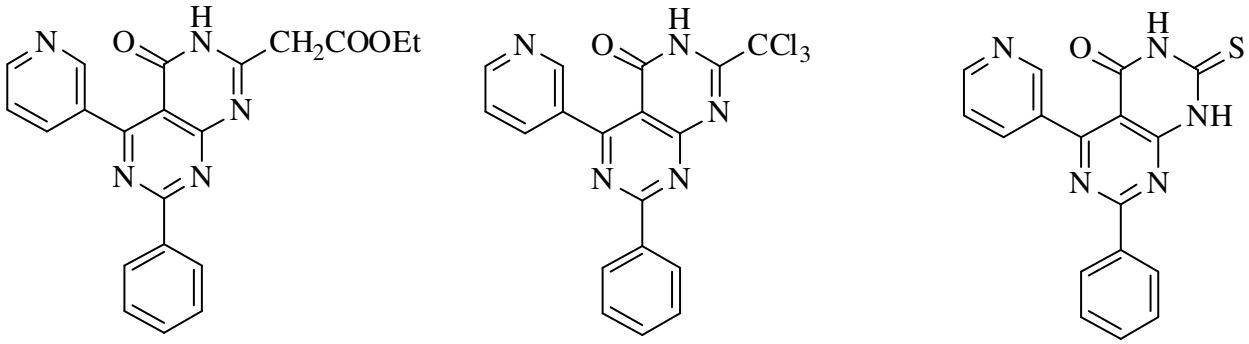
Scheme 4. Synthesis of pyrimido[4,5-d]pyrimidine derivatives **14-17**

1622, 1681 and 2900 cm^{-1} assignable to C-N, C=N, C=O and CH_2 stretching, respectively. The PMR spectrum of **17a** in DMSO exhibited signals at δ 3.18 [s, 6H, -N(Me)$_2$], 4.31 (s, 2H, -N-CH_2-N), , 7.48-8.57 (m, 8H, 10 Ar-H), 9.23 (s, 1H, C_2-H pyridine), 9.44 (s, 1H, N=CH).

Recently, activated nitriles have been used for the synthesis of a large variety of heterocyclic compounds (Fadda *et al.* 2013; Fadda *et al.* 2013; Fadda *et al.* 2012; Fadda *et al.* 2007; Fadda *et al.* 2006; Fadda *et al.* 2003). This prompted us to synthesize some new pyrimido-pyrimidine derivatives *via* the reaction of **3** with different activated nitriles.

Thus, fusion of **3** with ethyl cyanoacetate in an oil-bath afforded the pyrimido-pyrimidine derivative **18.** The

structure assignment of which was based on both elemental and spectral data. The IR spectrum showed absorption bands at 3500 (OH), 1720 cm^{-1} (CO). ^{1}HNMR spectrum showed signals at δ 1.2 for CH_3 protons, 4.1 CH_2 protons and 4.2 for $\underline{CH_2}CH_3$ protons. In addition, when **3** was treated with trichloroacetonitrile in refluxing ethanol afforded the corresponding pyrimido-pyrimidine **19**.



18 **19** **20**

Fig 1.: Structures of pyrimido[4,5-d]pyrimidine derivatives **18-20**

2.2. Antimicrobial evaluation

Twenty one of newly synthesized target compounds were evaluated for their in *vitro* antibacterial activity against *Bacillus subtilis* and *Bacillus thuringiensis* as example of Gram positive bacteria and *Escherichia coli* and *Pseudomonas aeruginosa* as examples of Gram-negative bacteria. They were also evaluated for their *in vitro* antifungal potential against *Fusarium oxysporum* and *Botrytis fabae* fungal strains.

Agar-diffusion method was used for the determination of the preliminary antibacterial and antifungal activity. Chloramphenicol, cephalothin and cycloheximide were used as reference drugs. The results were recorded for each tested compound as the average diameter of inhibition zones (IZ) of bacterial or fungal growth around the disks in mm. The minimum inhibitory concentration (MIN) measurement was determined for compounds showed significant growth inhibition zones (> 14 mm) using two fold serial dilution method (Fadda *et al.* 2013). The MIC (µg/mL) and inhibition zone diameters values are recorded in Table 1.

Table 1: Minimal inhibitory concentration (MIC, µg/mL) and inhibition zone (mm) of some new synthesized compounds

Compound No.	MIC[a] in µg/mL, and inhibition zone (mm)					
	Bacteria				Fungi	
	Gram-positive bacteria		Gram-negative bacteria			
	B. subtilis	*B. thuringiensis*	*E. coli*	*P. aeruginosa*	*F. oxysporum*	*B. fabae*
3	50 (18)	50 (14)	100 (15)	50 (19)	100 (16)	50 (18)
4	25 (27)	50 (15)	100 (15)	100 (16)	50 (19)	100 (16)
5	6.25 (45)	6.25 (38)	25 (25)	12.5 (33)	100 (16)	25 (27)
6	6.25 (33)	6.25 (37)	50 (20)	50 (19)	12.5 (33)	50 (20)
7a	3.125 (32)	6.25 (20)	100 (15)	100 (15)	12.5 (25)	6.25 (16)
7b	3.125 (44)	6.25 (37)	100 (14)	50 (20)	12.5 (38)	6.25 (19)
8	12.5 (32)	50 (20)	100 (15)	100 (15)	25 (25)	100 (16)
9	12.5 (32)	50 (15)	100 (15)	100 (16)	50 (19)	50 (20)
10	6.25 (38)	6.25 (30)	100 (14)	100 (15)	100 (16)	100 (16)
12	12.5 (32)	6.25 (38)	100 (15)	50 (19)	100 (15)	100 (15)
13	6.25 (37)	6.25 (37)	100 (15)	100 (15)	12.5 (33)	12.5 (32)
14a	6.25 (38)	6.25 (37)	100 (15)	50 (19)	100 (15)	100 (16)
14b	6.25 (38)	6.25 (37)	100 (15)	50 (19)	100 (15)	100 (16)
15a	3.125 (40)	6.25 (37)	100 (15)	50 (19)	100 (15)	100 (16)
15b	3.125 (41)	6.25 (38)	100 (15)	50 (19)	100 (15)	100 (16)
16	25 (26)	12.5 (44)	50 (20)	50 (19)	100 (15)	50 (20)
17a	3.125 (44)	6.25 (38)	100 (15)	100 (16)	12.5 (33)	100 (15)
17b	3.125 (40)	6.25 (37)	25 (25)	12.5 (33)	12.5 (33)	50 (20)
18	25 (27)	50 (20)	100 (15)	100 (16)	100 (15)	100 (15)
19	12.5 (32)	12.5 (44)	6.25 (37)	50 (19)	50 (19)	100 (16)
20	3.125 (40)	6.25 (37)	6.25 (37)	50 (19)	100 (16)	100 (15)
Chloramphenicol	3.125 (44)	3.125 (44)	6.25 (37)	6.25 (38)	NT	NT
Cephalothin	6.25 (36)	6.25 (37)	6.25 (38)	6.25 (37)	NT	NT
Cycloheximide	NT	NT	NT	NT	3.125 (43)	3.125 (42)

MIC: Minimal inhibitory concentration values with SEM = 0.02. NT: Not tested.

The results depicted in Table 1 revealed that the most of tested compounds displayed variable inhibitory effects on the growth of the tested Gram-positive and Gram negative bacterial strains, and also against antifungal strain.

In general most of the tested compounds revealed better activity against the Gram-positive rather than the Gram-negative bacteria.

Regarding the structure-activity relationship of the pyrimido pyrimidine derivatives against Gram positive bacteria, the results revealed that compounds **5**, **6**, **7a,b, 17a,b,** and **20** exhibited broad spectrum antibacterial profile against the tested organisms. Mannish base derivatives **7a,b** and **XVII** recorded higher activity than the other pyrimido pyrimidine dervatives. In this view, compounds **17a,b** and **20** were equipotent to

chloramphenicol in inhibiting the growth of *B. subtilis* (MIC 3.125 µg/mL), while its activity was 50% lower than of chloramphenicol against *B. thuringiensis*. Compounds **5, 6, 10, 13** and **14a,b** showed 50% of the activity of chloramphenicol (MIC 6.25 µg/mL) but they were equipotent to cephalothin in inhibiting the growth of *B. subtilis* and *B. thuringiensis* (MIC 6.25 µg/mL).

On the other hand, compounds **3, 4**, **8**, **10**, **12**, **13, 14a,b, 16, 18** and **19** exhibited moderate growth inhibitory activity against Gram-positive bacteria as revealed from their MIC values (6.25-50 µg/mL). Among these compounds **10**, **13**, **14a** and **14b** showed good growth inhibitory against *B. subtilis* (MIC 6.25 µg/mL), while the rest of other compounds **8**, **9**, **12** and **19** showed relatively good growth inhibitory profiles against *B. subtilis* (MIC 12.5 µg/mL) which were about 25% of the activity chloramphenicol and 50% cephalothin against the same organism. Concerning the antibacterial activity of the compound **3** revealed weak growth inhibitory against the tested Gram-positive bacteria (MIC 50 µg/mL).

Regarding the activity of Mannish base derivatives, against antifungal strains, the results revealed that compound **7a,b** was 50% lower than cycloheximide in inhibitory the growth of *B. fabae* (MIC 6.25 µg/mL), while the activity of compound **7a,b** and **17a,b** were 25% lower than cycloheximide against *F. oxysporum* (MIC 12.5 µg/mL).

The tested compounds were more active against Gram-positive than Gram-negative bacteria, it may be concluded that the antimicrobial activity of the compounds is related to cell wall structure of the bacteria. It is possible because the cell wall is essential to survival of bacteria and some antibiotics are able to kill bacteria by inhibiting a step in the synthesis of peptidoglycan. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids, but in contrast, Gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins. These differences in cell wall structure can produce differences in antibacterial susceptibility and some antibiotics can kill only Gram-positive bacteria and are inactive against Gram-negative pathogens (Fadda 2012).

In conclusion, the objective of the present study was to synthesize and investigate the antimicrobial activities of some new functionalized pyrimido pyrimidine and their Mannish base derivatives with the hope of discovering new structure leads serving as antimicrobial agents. Our aim has been verified by the synthesis of four different rings.

3. Experimental

3.1. Instruments

Melting points were measured with a Gallenkamp apparatus and are uncorrected. IR spectra were recorded for KBr disc on a Mattson 5000 FTIR spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker AC 300 (300 MHz) in $CDCl_3$ or DMSO-d_6 as solvent, using TMS as an internal standard, and chemical shifts are expressed as δ_{ppm}. Mass spectra were determined on Finnigan Incos 500 (70 ev). Elemental analyses were carried out at the Microanalytical Center of Cairo University. All reactions were followed by TLC (Silica gel, aluminum sheets 60 F_{254}, Merck.

3.1.1. Synthesis of ethyl 4-amino-2-phenyl-6-(pyridin-3-yl)pyrimidine-5-carboxylate ***(3)***

Ethyl cyanoacetate (0.1 mol), pyridine-3-carboxyladehyde (0.1 mol) and benzamidine hydrochloride (0.1 mol), a catalytic amount of triethylamine was refluxed in ethanol-DMF (25 ml) for 4 hours. (The reaction was monitored by TLC using a mixture of EtOAc and Pet.ether 1:1). The reaction mixture was cooled and the precipitated solid material was filtered off and recrystallized from a mixture of ethanol-DMF (1:1) to give ethyl 4-amino-2-phenyl-6-(pyridin-3-yl)pyrimidine-5-carboxylate **(3)**.

Yield (72%); m.p. 181 ^{o}C; IR (KBr): ν/cm^{-1}= 3500-3400 (NH_2), 3100 (NH), 1690 (CO), 1595 (C=C); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.09 (t, 3H, CH_3), 4.30 (q, 2H, CH_2), 6.90 (s, 2H, NH_2), 7.57-8.70 (m, 3H, pyridine), 7.50-8.36 (m, 5H, Ar-H), 9.24 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 320 (M^+, 62). Anal. Calcd. for $C_{18}H_{16}N_4O_2$ (320.35): C, 67.49; H, 5.03; N, 17.49%. Found: C, 67.31; H, 5.00; N, 17.25%.

3.1.2. Synthesis of 4-amino-2-phenyl-N-(pyridin-2-yl)-6-(pyridin-3-yl)pyrimidine-5-carboxamide ***(4)***

A mixture of 2-aminopyridine (0.02 mol) and **3** (0.02 mol) was heated on an oil-bath at 150 $^{\circ}$C in the presence of sodium acetate (1g) for 2 hr. The solid product, so formed, was washed with water, filtered, dried, and crystallized from ethanol.

Yield (60%); m.p. 221 ^{o}C; IR (KBr): ν/cm^{-1}= 3500-3400 (NH_2), 3100 (NH), 1680 (CO), 1595 (C=C); ^{1}H-NMR

(DMSO-d_6) δ (ppm): 6.85 (s, 2H, NH_2), 7.19-8.06 (m, 8H, Ar-H), 9.30 (s, 1H, C_2-H py), 11.01 (s, 1H, NH); ^{13}C-NMR (DMSO-d_6) δ (ppm): 165 C_4-pyrimidine, 164 C=O, 161.0 C_2-pyrimidine, 158.0 C_6 pyrimidine, 151.0 C_2-NH, 148.0 3C_2-pyridine, 138.0 C4-pyridine, 134.0 C4-pyridine, 134.0 C_1-ph, 133.0 C_3-pyridine, 131.0 C_4-pyridine, 129.0 C_3, C_5-Ph, 127.0 C_2, C_6-ph, 124.0 C_3-pyridine, 117.9 C_3-pyridine, 114.4, 106.8 C_3-pyridine; MS (EI, 70 eV): *m/z* (%) = 368 (M^+, 72). Anal. Calcd. for $C_{21}H_{16}N_6O$ (368.40): C, 68.47; H, 4.38; N, 22.81%. Found: C, 68.31; H, 4.11; N, 22.63%.

3.1.3. Synthesis of 2-chloro-N-(2-phenyl-5-(2-pyridin-2-yl)-acetyl)-6-(pyridin-3-yl) pyrimidin-4-yl)acetamide ***(5)***

To a solution of **4** (0.005 mol) in chloroform (50 ml), chloroacetyl chloride (0.01 mol) was added and the mixture left overnight at room temperature. The reaction mixture was concentrated under reduced pressure and cooled to give **5**.

Yield (80%); m.p. 230 ^{o}C; IR (KBr): ν/cm^{-1}= 3100 (NH), 1680 (CO), 1687 (CO), 1595 (C=C) and 760 cm^{-1} (C-Cl); ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.27 (s, 2H, CH_2Cl), 7.50-8.30 (m, 5H, Ar-H), 7.57-8.70 (m, 3H, pyridine-H), 9.21 (s, 1H, C_2-H pyridine), 10.60 (s, 1H, NH-CO), 11.30 (s, 1H, NHCO-pyridine); MS (EI, 70 eV): *m/z* (%) = 444 (M^+, 82). Anal. Calcd. for $C_{23}H_{17}ClN_6O_2$ (444.88): C, 62.10; H, 3.85; N, 18.89%. Found: C, 62.00; H, 3.63; N, 18.71%.

3.1.4 Synthesis of 7-(chloromethyl)-2-phenyl-6-(pyridin-2-yl)-4- (pyridin-3-yl)pyrido[4,3-d]pyrimidin-5(6H)-one ***(6)***

A solution of **5**. (0.002 mol) in acetic anhydride (10 ml) was refluxed for 6 hr (TLC). The reaction mixture was poured over crushed ice and the solid separated was filtered, washed with water and recrystallized from benzene.

Yield (62%); m.p. 195 ^{o}C; IR (KBr): ν/cm^{-1}= 2950 (C-H), 1700 (C=O), 1595 (C=C) and 760 cm^{-1} (C-Cl); ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.37 (s, 2H, CH_2Cl), 7.50-8.30 (m, 5H, Ar-H), 7.51-8.57 (m, 3H, pyridine-H), 9.24 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 425 (M^+, 29). Anal. Calcd. for $C_{24}H_{16}ClN_5O$ (425.87): C, 64.72; H, 3.54; N, 19.69%. Found: C, 64.61; H, 3.33; N, 19.50%.

3.1.5. Synthesis of 7-((N,N-substitutedamino)methyl)-2-phenyl-6- (pyridin-2-yl)-4-(pyridin-3-yl)pyrido[4,3-d]pyrimidin-5(6H)-one ***(7a,b)***

General procedure

A mixture of **6** (0.002 mol) and an appropriate secondary amine (0.0025 mol) was left at room temperature in 25 ml pyridine for several days. The excess pyridine was distilled off under reduced pressure and the residue poured over crushed ice. The crude product was filtered, dried, and recrystallized from ethanol.

For **7a**: Yield (71%); m.p. 231 ^{o}C; IR (KBr): ν/cm^{-1}= 2900 (CH_2), 1620 (C=N), 1565 (C=C); ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.18 (s, 6H, 2CH_3), 3.91 (s, 2H, CH_2), 7.50-8.30 (m, 12H, Ar-H), 9.30 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 434 (M^+, 82). Anal. Calcd. for $C_{26}H_{22}N_6O$ (434.49): C, 68.72; H, 4.86; N, 22.33%. Found: C, 68.60; H, 4.70; N, 22.13%.

For **7b**: Yield (63%); m.p. 195 ^{o}C, (KBr): ν/cm^{-1}= 1620 (C=N), 1560 (C=C); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.02 (t, 6H, 2CH_3), 2.8 (q, 4H, 2CH_2), 3.35 (s, 2H, CH_2), 7.50-8.57 (m, 12H, Ar-H), 9.26 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 462 (M^+, 15). Anal. Calcd. for $C_{28}H_{26}N_6O$ (462.55): C, 69.96; H, 5.44; N, 21.15%. Found: C, 69.76; H, 5.31; N, 21.00%.

3.1.6. Synthesis of 4-amino-2-phenyl-6-(pyridin-3-yl)pyrimidine-5-carboxylic acid ***(8)***

To a solution of **3** (0.005 mol) in ethanol (5 ml) was added sodium hydroxide (0.5%, 25 ml). The reaction mixture was refluxed for 2hr, cooled and neutralized with cold dil. hydrochloric acid. The precipitate was filtered, washed with water, dried and recrystallized from methanol.

Yield (80%); m.p. 187 ^{o}C; IR (KBr): ν/cm^{-1}= 3500-3400 (NH_2), 1730 (CO); ^{1}H-NMR (DMSO-d_6) δ (ppm): 6.89 (s, 2H, NH_2), 7.50-8.70 (m, 8H, Ar-H), 9.25 (s, 1H, C_2-H pyridine), 12.75 (s, 1H, COOH); MS (EI, 70 eV): *m/z* (%) = 292 (M^+, 78). Anal. Calcd. for $C_{16}H_{12}N_4O_2$ (292.29): C, 65.75; H, 4.14; N, 19.17%. Found: C, 65.61; H, 4.00; N, 19.00%.

3.1.7. Synthesis of 2-((2-nitrophenoxy)methyl)-7-phenyl-5-(pyridin-3-yl)- 4H-pyrimido[4,5-d][1,3]oxazin-4-one ***(10)***

This compound was prepared following the method reported earlier (Sammour *et al.* 1973).

Yield (86%); m.p. 242 ^{o}C; IR (KBr): ν/cm^{-1}= 1680 (CO), 1620 (C=N), 1520, 1360 cm^{-1} (NO_2); ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.79 (s, 2H, CH_2-O), 6.84-8.25 (m, 12H, Ar-H), 9.21 (s , 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 453 (M^+, 15). Anal. Calcd. for $C_{24}H_{15}N_5O_5$ (453.41): C, 63.58; H, 3.33; N, 15.45%. Found: C,

63.33; H, 3.21; N, 15.33%.

3.1.8. Synthesis of 9-phenyl-11-(pyridine-3-yl)-benzo[b]-pyrimido[4′,5′:4,5] pyrimido[1,2-d][1,4]oxazin-12(6H)one ***(12)***

Pyrimidoxazine **10** (0.01 mol) was dissolved in ethanol (20 ml) and Rany nickel (0.5 g) placed into it. Hydrazine hydrate (10 ml, 35%) was then carefully added to it at room temperature. After the evolution of hydrogen had ceased the reaction mixture was allowed to reflux on a water-bath for 3 hr. It was then acidified with hot 5 N hydrochloric acid and filtered hot. The filtrate on cooling yielded the crude product which was recrystallized from ethanol.

Yield (60%); m.p. 261 ^{o}C; IR (KBr): ν/cm^{-1}= 1685 (CO), 1620 (C=N); ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.80 (s, 2H, CH_2), 7.50-8.70 (m, 12H, Ar-H), 9.24 (s, 1H, C_2-H pyridine); ^{13}C-NMR (DMSO-d_6) δ (ppm): 2x127, 2x129, 131, 134 (ph-carbon), 163, 181, 164, 115 pyrimidine, 160, 155, pyrimidone, 76 C-O, 152 O-C, 112, 128, 124, 115, 119, pyridine 132, 2x147, 124, 134; MS (EI, 70 eV): *m/z* (%) = 405 (M^+, 25). Anal. Calcd. for $C_{24}H_{15}N_5O_2$ (405.42): C, 71.10; H, 3.73; N, 17.27%. Found: C, 70.87; H, 3.55; N, 17.10%.

3.1.9. Synthesis of 2-methyl-7-phenyl-5-(pyridin-3-yl)-4H-pyrimido[4,5-d][1,3]oxazin-4-one ***(13)***

It was prepared by following the procedure as described earlier (Zentmeyer & Wanger 1949).

Yield (68%); m.p. 195 ^{o}C; IR (KBr): ν/cm^{-1}= 1690 (CO); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.16 (s, 3H, CH_3), 7.50-8.70 (m, 8H, Ar-H), 9.21 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 316 (M^+, 80). Anal. Calcd. for $C_{18}H_{12}N_4O_2$ (316.31): C, 68.35; H, 3.82; N, 17.71%. Found: C, 68.10; H, 3.65; N, 17.60%.

3.1.10. Synthesis of 3-substituted-2-methyl-7-phenyl-5-(pyridin-3-yl) pyrimido[4,5-d]pyrimidin-4(3H)-one ***(14a,b)***

General procedure

These compounds were synthesized by the well known procedure (Burckhalter & Tendick 1948).

For **14a**: Yield (81%); m.p. 221 ^{o}C; IR (KBr): ν/cm^{-1}= 3400-3350 (NH_2); 1680 (CO); ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.26 (s, 3H, CH_3), 5.70 (s, 2H, NH_2), 7.50-8.57 (m, 8H, Ar-H), 9.21 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 330 (M^+, 90). Anal. Calcd. for $C_{18}H_{14}N_6O$ (330.35): C, 65.44; H, 4.27; N, 25.44%. Found: C, 65.31; H, 4.11; N, 25.30%.

For **14b**: Yield (65%); m.p. 241 ^{o}C; IR (KBr): ν/cm^{-1}= 1686 (CO); ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.21 (s, 3H, CH_3), 7.50-8.55 (m, 8H, Ar-H), 9.24 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 391 (M^+, 65). Anal. Calcd. for $C_{24}H_{17}N_5O$ (391.43): C, 73.64; H, 4.38; N, 17.89%. Found: C, 73.38; H, 4.25; N, 17.77%.

3.1.11. Reaction of benzaldehyde with XIVa,b: Formation of compounds ***15a,b***

General procedure ***(15a,b)***

Benzaldehyde (0.01 mol) and **14** (0.01 mol) in dry abs. ethanol (30 ml) in the presence of piperidine (5 drops) were refluxed 3hr. The reaction mixture was concentrated under reduced pressure. The solid product, thus obtained, was filtered, dried and recrystallized from ethanol to give **15a,b**.

(Z)3-(Benzylideneamino)-2-methyl-7-phenyl-5-(pyridin-3-yl)pyrimido[4,5-d]pyrimidin-4(3H)-one (15a)

Yield (82%); m.p. 234 ^{o}C; IR (KBr): ν/cm^{-1}= 1690 (CO), 1640 (C=N); ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.30 (s, 3H, CH_3), 7.50-8.35 (m, 13H, Ar-H), 9.01 (s, 1H, N=CH), 9.24 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 418 (M^+, 15). Anal. Calcd. for $C_{25}H_{18}N_6O$ (418.41): C, 71.76; H, 4.34; N, 20.08%. Found: C, 71.63; H, 4.25; N, 19.77%.

(E)-3,7-Diphenyl-5-(pyridin-3-yl)-2-styrylpyrimido[4,5-d]pyrimidin-4(3H)-one ***(15b)***

Yield (73%); m.p. 260 ^{o}C; IR (KBr): ν/cm^{-1}= 1680 (CO), 1590 (C=C); ^{1}H-NMR (DMSO-d_6) δ (ppm): 5.30 (d, 1H, CH=C, J = 7.40 Hz), 6.20 (d, 1H, =CH-ph, J = 7.40 Hz), 7.50-8.33 (m, 13H, Ar-H), 9.24 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 479 (M^+, 65). Anal. Calcd. for $C_{31}H_{21}N_5O$ (479.53): C, 77.64; H, 4.41; N, 14.60%. Found: C, 77.45; H, 4.33; N, 14.51%.

3.1.12. Synthesis of 7-phenyl-5-(pyridin-3-yl)pyrimido[4,5-d]pyrimidin-4(3H)-one ***(16)***

It was obtained according to the literature method (Sherrill *et al.* 1946).

Yield (73%); m.p. 213 ^{o}C; IR (KBr): ν/cm^{-1}= 3310 (NH), 2900 (C-H), 1680 (CO) 1620 (C=N); ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.50-8.36 (m, 8H, Ar-H), 9.24 (s, 1H, C_2-H pyridine), 9.43 (s, 1H, N=CH), 12.14 (s, 1H, NH); MS (EI, 70 eV): *m/z* (%) = 301 (M^+, 100). Anal. Calcd. for $C_{17}H_{11}N_5O$ (301.31): C, 67.77; H, 3.68; N,

23.24%. Found: C, 67.51; H, 3.41; N, 23.11%.

3.1.13. Synthesis of 3-((N,N-substitutedamino)methyl) -7-phenyl-5-(pyridin-3-yl)pyrimido [4,5-d]pyrimidin-4(3H)-one ***(17a,b)***

These Mannish bases were prepared according to the method as described before for compound **7**.

For **17a**: Yield (63%); m.p. 178 ^{o}C; IR (KBr): ν/cm^{-1}= 1681 (CO), 1622 (C=N); ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.18 (s, 6H, 2CH_3), 4.31 (s, 2H, N-CH_2-N), 7.48-8.57 (m, 8H, Ar-H), 9.23 (s, 1H, C_2-H pyridine), 9.44 (s, 1H, N=CH); MS (EI, 70 eV): *m/z* (%) = 358 (M^+, 100). Anal. Calcd. for $C_{20}H_{18}N_6O$ (358.40): C, 67.02; H, 5.06; N, 23.46%. Found: C, 66.85; H, 4.79; N, 23.22%.

For **17b**: Yield (66%); m.p. 222 ^{o}C; IR (KBr): ν/cm^{-1}= 1680 (CO); ^{1}H-NMR (DMSO-d_6) δ (ppm): .1.02 (s, 6H, 2CH_3), 2.65 (q, 4H, 2CH_2), 4.30 (s, 2H, N- CH_2-N), 7.50-8.36 (m, 8H, Ar-H), 9.24 (s, 1H, C_2-H pyridine); 9.40 (s, 1H, N=CH). MS (EI, 70 eV): *m/z* (%) = 386 (M^+, 100). Anal. Calcd. for $C_{22}H_{22}N_6O$ (386.46): C, 68.38; H, 5.74; N, 21.75%. Found: C, 68.12; H, 5.66; N, 21.53%.

3.1.14. Synthesis of ethyl-2-(4-hydroxy-7-phenyl-5- (pyridine-3-yl)pyrimido [4,5-d]pyrimidin-2-yl)acetate ***(18)***

Fusion of **3** (0.01 mol) with ethyl cyanoacetate (0.01 mol) for 2 hr in an oil-bath at 150 ^{o}C gave a pale yellow product which recrystallized from ethanol to give **18**.

Yield (72%); m.p. 275 ^{o}C; IR (KBr): ν/cm^{-1}= 3340 (OH), 1730 cm^{-1} (ester CO); ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.20 (t, 3H, CH_2CH_3), 4.10 (s, 2H, CH_2), 4.20 (q, 2H, CH_2CH_3), 7.10-7.80 (m, 8H, Ar-H)CH_3), 9.24 (s, 1H, C_2-H pyridine); MS (EI, 70 eV): *m/z* (%) = 387 (M^+, 100). Anal. Calcd. for $C_{21}H_{17}N_5O_3$ (387.40): C, 65.11; H, 4.42; N, 18.08%. Found: C, 65.00; H, 4.19; N, 17.85%.

3.1.15. Synthesis of 7-phenyl-5-(pyridin-3-yl)-2-(trichloromethyl)pyrimido[4,5-d]pyrimidin-4(3H)-one ***(19)***

A solution of **3** (0.005 mol) in ethanol (20 ml) was refluxed with trichloroacetonitrile (0.005 mol) for 3 hr and evaporated *in vacuo*. The residue was triturated with ethanol and the resulting solid product was collected by filtration and crystallized from ethanol to give **19**.

Yield (53%); m.p. 233 ^{o}C; IR (KBr): ν/cm^{-1}= 3310 (NH), 1680 (CO), 760 (C-Cl); ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.50-8.60 (m, 8H, Ar-H), 9.24 (s, 1H, C_2-H pyridine), 12.10 (s, 1H, NH); MS (EI, 70 eV): *m/z* (%) = 417 (M^+, 95). Anal. Calcd. for $C_{18}H_{10}Cl_3N_5O$ (418.66): C, 51.64; H, 2.41; N, 16.73%. Found: C, 51.41; H, 2.31; N, 16.66%.

3.1.16. Synthesis of 7-phenyl-5-(pyridin-3-yl) -2-thioxo-2,3-dihydropyrimido [4,5-d]pyrimidin-4(1H)-one ***(20)***

A solution of **2** (0.005 mol) in acetone (20 ml) was stirred at room temperature with phenyl isothiocyanate (0.005 mol) for 0.5 hr. The solid product was collected and crystallized from ethanol to give **20**.

Yield (62%); m.p. 219 ^{o}C; IR (KBr): ν/cm^{-1}= 1700 (NH), 1595 (C=C), 1300 (C=S); ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.35-8.57 (m, 8H, Ar-H), 9.24 (s, 1H, C_2-H pyridine), 10.20 (s, 1H, NH), 12.10 (s, 1H, NH); MS (EI, 70 eV): *m/z* (%) = 333 (M^+, 100). Anal. Calcd. for $C_{17}H_{11}N_5OS$ (333.37): C, 67.47; H, 3.69; N, 17.10%. Found: C, 67.35; H, 3.45; N, 16.85%.

3.2. Antimicrobial evaluation

The disks of Whatman filter paper were prepared with standard size (5.0 mm diameter) and kept into 1.0 Oz screw capped wide mouthed containers for sterilization. These bottles are kept into hot air oven at temperature of 150°C. Then, the standard sterilized filter paper disks impregnated with a solution of the test compound in DMF (1 mg/mL) were placed on nutrient agar plate seeded with the appropriate test organism in triplicates. Standard concentrations of 10^6 CFU/mL (Colony Forming U/mL) and 10^4 CFU/mL were used for antibacterial and antifungal assay, respectively. Pyrex glass Petri dishes (9 cm in diameter) were used and two disks of filter paper were inoculated in each plate. The utilized test organisms were: *B. subtilis* and *B. thuringiensis* as examples of Gram-positive bacteria and *E. coli* and *P. aeruginosa* as examples of Gram-negative bacteria. They were also evaluated for their in vitro antifungal potential against *F. oxysporum* and *B. fabae* fungal strains. Chloramphenicol, cephalothin and cycloheximide were used as standard antibacterial and antifungal agents, respectively. DMF alone was used as control at the same above-mentioned concentration and due this there was no visible change in bacterial growth. The plates were incubated at 37°C for 24 h for bacteria and for 48 h for fungi. Compounds that showed significant growth inhibition zones (> 14 mm) using the twofold serial dilution technique, were further evaluated for their minimal inhibitory concentrations (MICs).

3.3. Minimal inhibitory concentration (MIC) measurement

The microdilution susceptibility test in Müller-Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) were used for the determination of antibacterial and antifungal activity, respectively. Stock solutions of the tested compounds, chloramphenicol, cephalothin and cycloheximide were prepared in DMF at concentration of 1000 mg/mL. Each stock solution was diluted with standard method broth (Difco) to prepare serial twofold dilutions in the range of 500-3.125 mg/mL 10 mL of the broth containing about 10^6 CFU/mL of test bacteria was added to each well of 96-well microtiter plate. The sealed microplates were incubated at 37°C for 24 h for antibacterial activity and at 37°C for 48 h for antifungal activity in a humid chamber. At the end of the incubation period, the minimal inhibitory concentrations (MIC) values were recorded as the lowest concentrations of the substance that had no visible turbidity. Control experiments with DMF and uninoculated media were run parallel to the test compounds under the same conditions. The substance that had no visible turbidity. Control experiments with DMF and uninoculated media were run parallel to the test compounds under the same conditions.

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