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Synthesis, characterization and antimicrobial activity of new 1,2,4-triazole and pyridine derivatives bearing methyl 4-aminosalicylate moiety as a substructure

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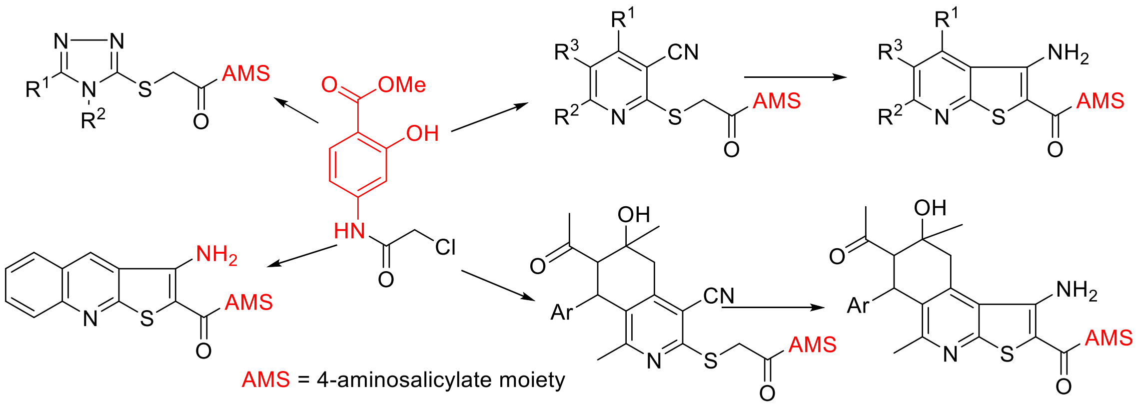
Abstract

A general procedure was developed to synthesize and characterize some new 1,2,4-triazole, 3-cyanopyridine, 3-aminothienoquinoline and 5,6,7,8-tetrahydroisoquinoline derivatives bearing a 4-aminosalicylic acid ester moiety as substructure. Some of them were further modified to give new derivatives. This work allowed the preparation of 18 new compounds obtained in good yields and under simple experimental conditions. These compounds were evaluated for their antimicrobial activity against four strains of bacteria and two fungal species.



Keywords: Methyl 4-aminosalicylate, 1,2,4-Triazoles, pyridine-3-carbonitriles, Tetrahydroisoquinolines

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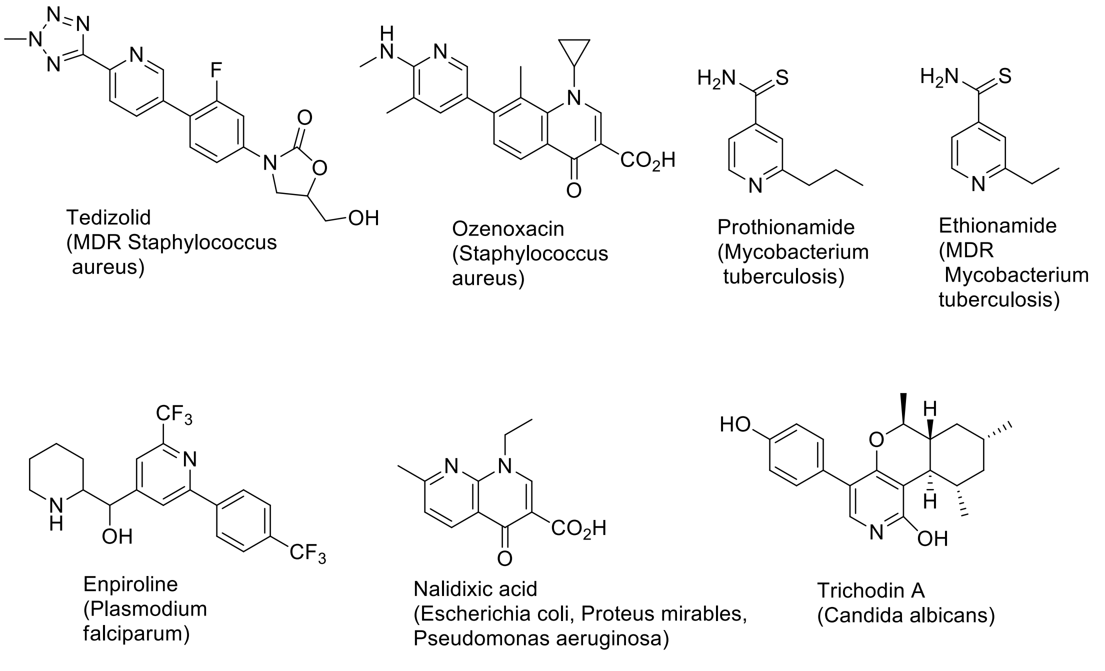
Introduction

1,2,4-Triazoles are a very important class of compounds which attracted the attention of many chemists and biologists in organic synthesis and pharmaceutical fields due to their various biological activities such as antimigrain,[1,2] antioxidant,[3] anti-urease,[4] antimicrobial,[5,6] anti-inflammatory,[6,7] anticonvulsant,[8] anticancer,[9,10] antiviral,[11] and antiparasitic.[12] Numerous medicines containing triazole moiety available in market are antifungals,[1,12-14] anticancers,[1,9] antimigraine [1,2] and antivirals.[1,15]

Various pharmacological activities of 1,2,4-triazoles as antifungal have been observed which included fluconazole, isavuconazole, itraconazole, voriconazole, ravuconazole, and posaconazole.[16] Several 1,2,4-triazole-based drugs are under clinical use for the treatment of different diseases. Some of the most effective drugs available in the market.[16]

The pyridine nucleus is found in many natural products, such as vitamins, alkaloids and coenzymes, as well as in many drugs and pesticides.[17,18] The recent literature mentions various series of compounds containing the pyridine nucleus, as a unique heterocycle, and attached to or fused with other heterocycles, which have remarkable antibacterial, antifungal, and antiviral properties.[17-19]

Pyridine is particularly an important nitrogen-containing heteroarene with great utility in medicinal chemistry.[20-23] More than 50 drugs in the database of US FDA stem from pyridine, including pyridostigmine for myasthenia gravis, dexamethasone isonicotinate for inflammation and allergy, piroxicam for arthritis, crizotinib for cancer, tacrine for Alzheimer's, abiraterone acetate for prostate cancer, delavirdine for HIV/AIDS, and many more.[22] For the treatment of bacterial and fungal infections, many FDA-approved drugs also contain pyridine scaffolds (Figure 1), such as tedizolid and ozenoxacin mainly for Staphylococcus aureus infections, prothionamide for Mycobacterium tuberculosis, ethionamide for MDR Mycobacterium tuberculosis, enpiroline for Plasmodium falciparum, nalidixic acid for Escherichia coli, Proteus mirables and Pseudomonas aeruginosa, and trichodin A for Candida albicans.



Tedizolid
(MDR Staphylococcus aureus)

Ozenoxacin
(Staphylococcus aureus)

Prothionamide
(Mycobacterium tuberculosis)

Ethionamide
(MDR Mycobacterium tuberculosis)

Enpiroline
(Plasmodium falciparum)

Nalidixic acid
(Escherichia coli, Proteus mirables, Pseudomonas aeruginosa)

Trichodin A
(Candida albicans)

Figure 1. FDA approved drugs containing pyridine scaffold to treat bacterial and fungal infections

4-Aminosalicylic acid (4-ASA) is a drug that is largely used for the multi-therapy of tuberculosis. Particularly, as a second-line anti-tuberculosis drug in the treatment of drug-resistant tuberculosis caused by *Mycobacterium tuberculosis*.[24] 4-ASA is one among the World Health Organization's (WHO) List of Essential Medicines.[25] There are strong evidences for mechanisms of bacteriostatic action of 4-ASA. Firstly, it inhibits folic acid synthesis, cell growth and multiplication slows.[26-28] Secondly, 4-ASA has been proven to inhibit the synthesis of mycobactins, which are crucial siderophores for tuberculous bacilli, and hence the drug disrupts the iron intake of susceptible acid-fast bacteria.[29]

Apart from the significant anti-tubercular activity, 4-ASA has also shown promising anti-inflammatory effects in inflammatory bowel diseases (IBD). It has been used in the treatment of mild to moderate ulcerative colitis in patients who are intolerant of sulfasalazine and in the treatment of Crohn's disease;[30] the drug is designated as an orphan drug by the FDA for use in mild to moderate ulcerative colitis.[31,32] Several prodrugs of 4-ASA were investigated for their colonic anti-inflammatory activity.[33]

In view of the above observations, the current work was planned to synthesize and characterize of some new triazoles and pyridines bearing the moiety of methyl 4-aminosalicylic acid ester as substructure with the hope that these new compounds will find good applications in both biological and medicinal fields owing to their incorporation of various pharmacophores. Actually, most synthesized compounds were evaluated for their antimicrobial activity against four strains of bacteria and two fungal species and promising results obtained for some compounds as antifungal agents.

Results and Discussion

Synthesis and characterization

The current work started from methyl 4-(2-chloroacetamido-2-hydroxybenzoate (**3**)[34] which underwent some rational straightforward or successive reactions[35,36] to give the target compounds (Scheme 1-).

Compound **3** underwent some nucleophilic substitution reactions upon treatment with different *S*-nucleophiles. Thus, reaction of **3** with substituted 1,2,4-triazolinethiones **4a-f** [37-40] by refluxing in ethanol containing slightly excess molar amount of sodium acetate trihydrate for one hour furnished the corresponding thioethers **5a-f** in high yields (Scheme 1). IR spectra of **5a-f** showed the disappearance of absorption bands of triazole NH-SH tautomeric mixture and appearance of three absorption bands in the region from 3310 to 3240 cm^{-1} for (NH), 3200 to 3193 cm^{-1} for (OH), and 1670 to 1664 cm^{-1} for (C=O, anilide). ^1H- NMR spectra of **5a-f** exhibited a singlet signal at δ value ranged from 4.13 to 4.23 due to SCH_2 group beside other signals which are in agreement with their proposed structures.

In a similar manner, reaction of compound **3** with functionally substituted pyridinthiones **6**[41] or **8a-e**[42-45] under the same (above) conditions produced the corresponding sulfanylpyridine derivatives **7** or **9a-e**, respectively. Upon heating compound **9b** in ethanol containing slightly excess amount of sodium ethoxide, it underwent interamolecular Thorpe-Zeigler cyclization accompanied by ester group exchange affording thieno[2,3-*b*]pyridine **10** (Scheme 2). IR spectra of **7** or **9a-e** showed the absence of absorption bands of pyridine NH-SH tautomeric mixture and presence of three absorption bands in the region from 3312 to 3258 cm^{-1} for (NH), 3202 to 3193 cm^{-1} for (OH), 2230 to 2221 cm^{-1} for (C≡N), and 1679 to 1667 cm^{-1} for (C=O, anilide) beside other absorption bands which are in accordance with their proposed structures . ^1H NMR spectra of **7** or **9a-e** exhibited a singlet signal at δ value ranged from 3.97 to 4.26 attributed to SCH_2 group. IR spectrum of **10** revealed the disappearance of the absorption band characteristic for C≡N group at 2226 cm^{-1} and presence of absorption bands characteristic for amino group. Also, ^1H NMR spectrum of **10** revealed the disappearance of SCH_2 singlet signal at δ 4.26 and appearance of new one at δ 6.09 equivalent to NH_2 group.

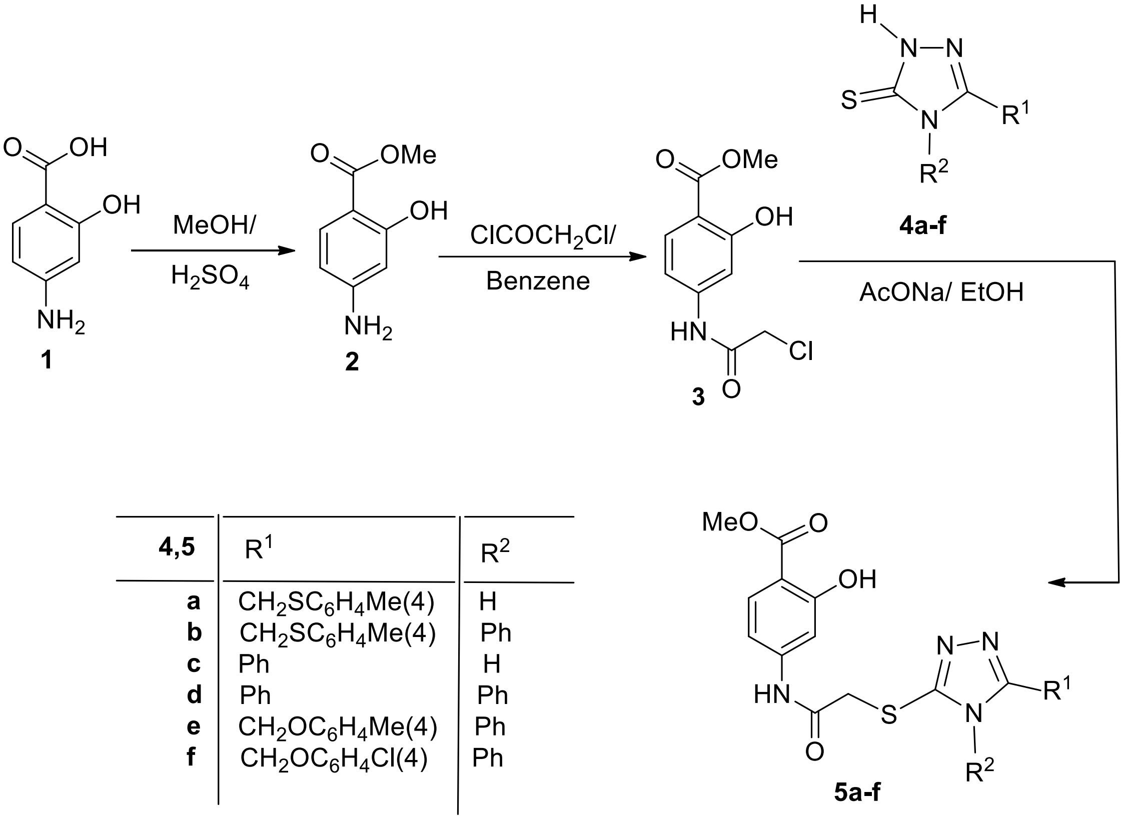
Also, The ^{1}H NMR spectrum of compound **10** confirms the presence of two ethyl ester groups in the molecule since each ester group shows a quartet signal and a triplet one.

Moreover, 5,6,7,8-tetrahydroisoquinoline derivative **12** was synthesized in excellent yield *via* reaction of **3** with isoquinolinethiones **11**[46] by refluxing in ethanol containing slightly excess molar quantity of sodium acetate trihydrate for two hours. Upon treatment of compound **12** in boiling methanol with sodium methoxide solution, it underwent intramolecular Thorpe-Zeigler cyclization to afford the corresponding isomer, 6,7,8,9-tetrahydrothieno{2,3-*c*]isoquinoline **13** (Scheme 3). IR spectrum of compound **12** showed absorption bands at 3487 cm^{-1} for (2 O-H), at 3359 cm^{-1} for (N-H), at 2220 cm^{-1} for (C≡N) 1722 cm^{-1} for (C=O, ester), 1698 cm^{-1} for (C=O, acetyl), and at 1671 cm^{-1} for (C=O, anilide). ^{1}H NMR spectrum of **12** displayed a double doublet at δ 4.13-4.14 due to SCH$_2$ group. IR spectrum of compound **13** showed absorption bands at 3458, 3386, 3338 cm^{-1} for (O-H, NH$_2$, N-H); 1724 cm^{-1} for (C=O, ester); 1699 cm^{-1} for (C=O, acetyl); and 1671 cm^{-1} for (C=O, anilide). ^{1}H NMR spectrum of both **12** and **13** revealed the presence of specific signals corresponding to the proton of cyclohexene ring as reported for similar compounds.

In contrast, refluxing of **3** with 3-cyanoquinoline-2(1*H*)-thione (**14**)[47] in ethanol containing slightly excess amount of sodium acetate trihydrate for two hours furnished the corresponding thieno[2,3-*b*]quinoline **16** directly without isolation of the intermediate **15**. This may be due to the absence of any substituent at position 4 of intermediate **15** which facilities the intramolecular Thorpe-Zeigler cyclization (Scheme 3). IR spectrum of compound **16** showed the absence of $\nu_{C≡N}$ absorption bands. ^{1}H NMR spectrum of **16** revealed no singlet signal corresponds to SCH$_2$ group.

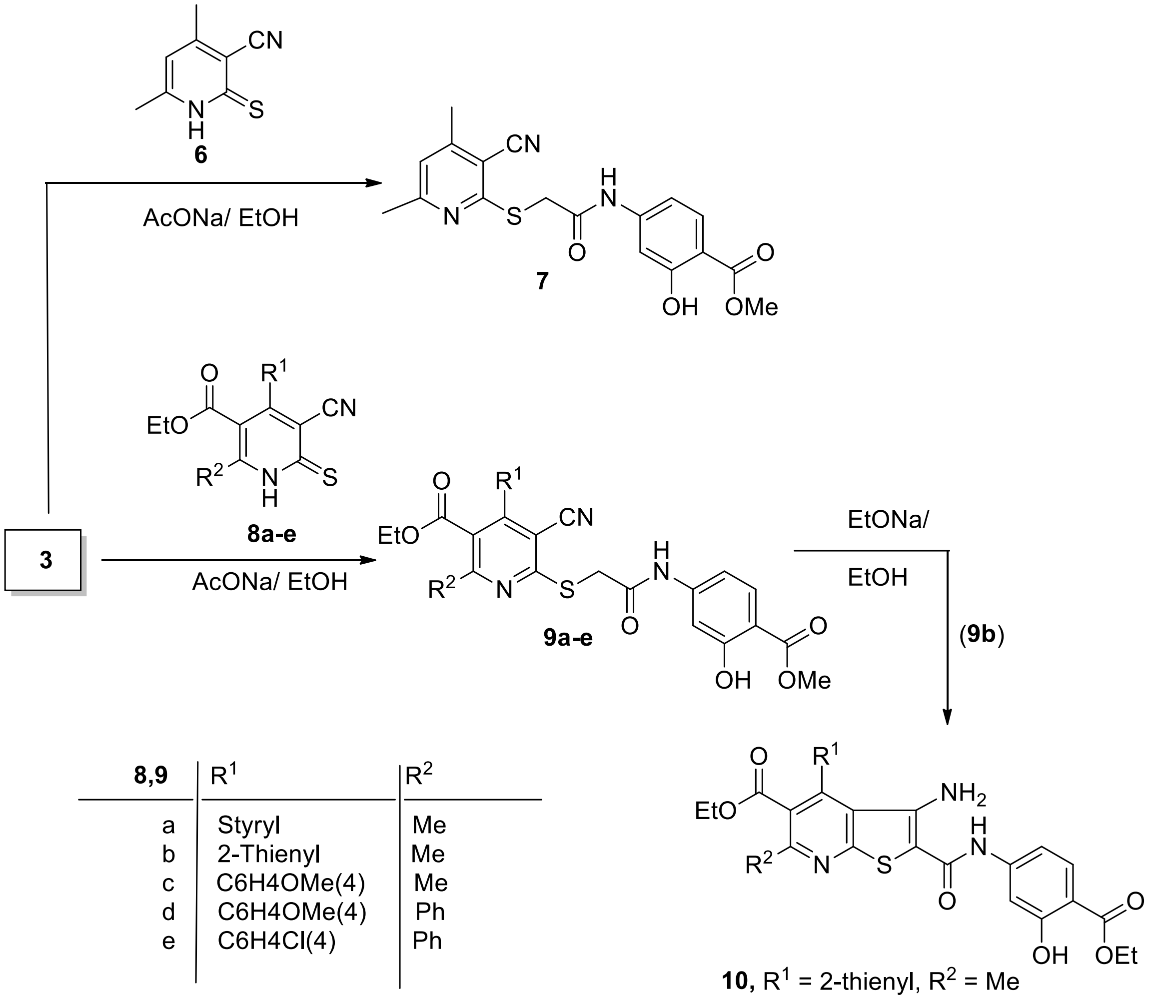
Compound **3** underwent substitution reaction with two *N*-nucleophiles. Thus, reaction of compound **3** with nicotinamide **17** by refluxing in ethanol afforded pyridinium chloride **19** in very pure state. Heating compound **3** with triazineone **18** [48] in DMF containing anhydrous potassium carbonate at 100 °C produced *N*-substituted 1,2,3-triazineone derivative **20** in a high yield (Scheme 3). IR and ^{1}H NMR spectral data of compounds **18** and **20** are in agreement with their proposed structures.

It is noteworthy that the results of elemental analyses for all compounds and ^{13}C NMR spectroscopy of most compounds are in agreement with their proposed structures (*cf.* Materials and Methods).



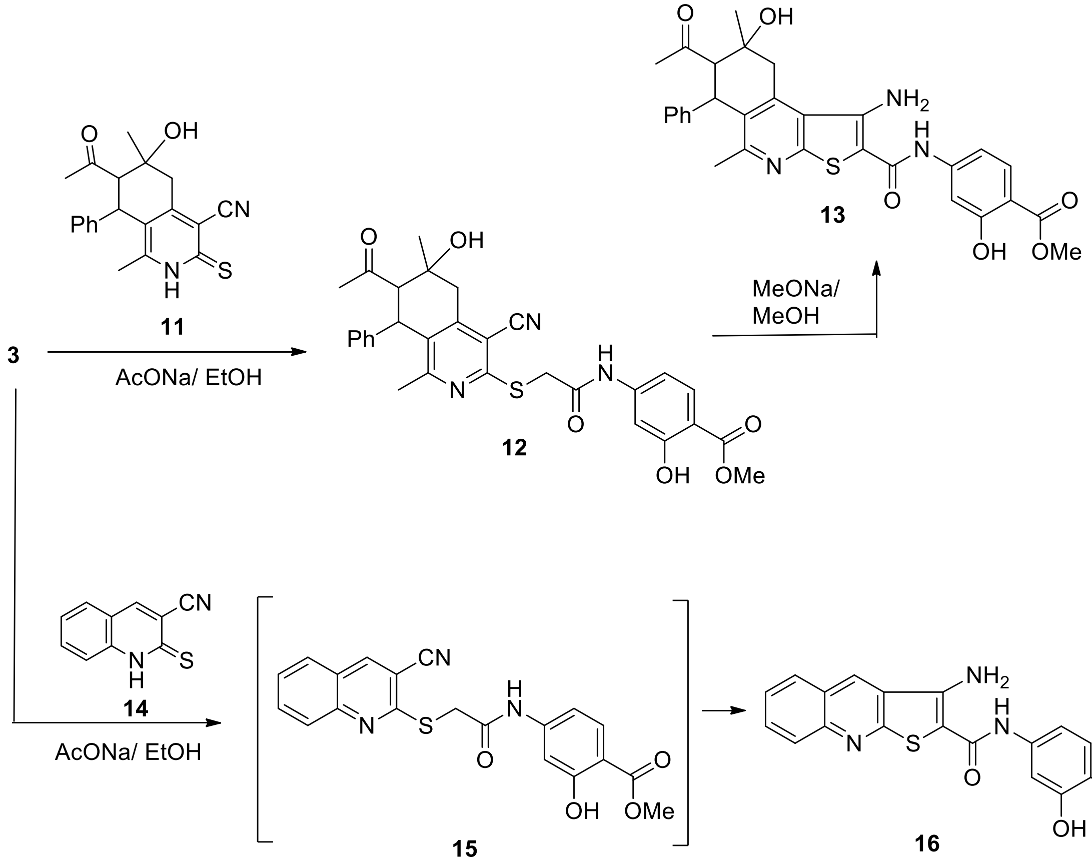
4,5	R^1	R^2
a	$CH_2SC_6H_4Me(4)$	H
b	$CH_2SC_6H_4Me(4)$	Ph
c	Ph	H
d	Ph	Ph
e	$CH_2OC_6H_4Me(4)$	Ph
f	$CH_2OC_6H_4Cl(4)$	Ph

Scheme 1. Synthesis of compounds **2,3** and **5a-f**.

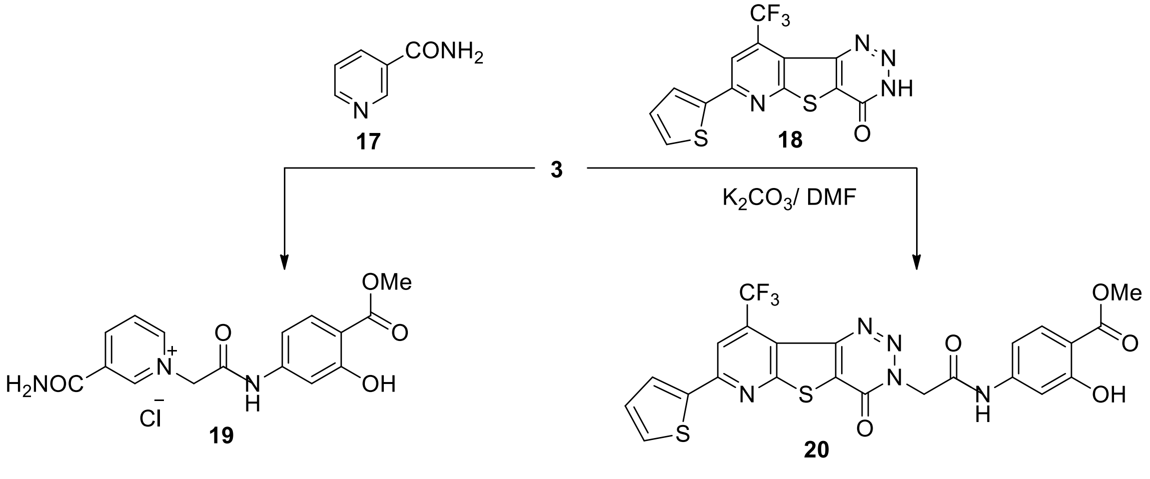


8,9	R^1	R^2
a	Styryl	Me
b	2-Thienyl	Me
c	C6H4OMe(4)	Me
d	C6H4OMe(4)	Ph
e	C6H4Cl(4)	Ph

Scheme 2. Synthesis of compounds **7**, **9a-e** and **10**.



Scheme 3. Synthesis of compounds **12 13** and **16**.



Scheme 4. Synthesis of compounds **19** and **20**

Biological activity

Most synthesized compounds were evaluated as bactericidal agents towards four strains of bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*) and as antifungals towards two fungal species (*Aspergillus flavus* and *Candida Albicans*) according to the dilution method.[49] The results obtained are expressed as Minimal Inhibition Concentration (MIC) (µg/ml) and are depicted in Table 1. These results revealed that (i) all compounds showed either very weak or no antimicrobial activity against all bacterial strains under investigated; (ii) most compounds exhibited antifungal activity that ranged from weak to strong towards the two fungal species and (iii) compounds **9b, 9d, 9e** and **19** showed strong antifungal activity near to that of the reference drug.

Table 1. Minimal Inhibition Concentration (MIC) of the synthesized compounds (µg/ml)

Compd. No.	Gram negative bacteria		Gram positive bacteria		*A. flavus* 1276	*C. Albicans* 2091
	S. aureus 19213	*S. pneumoniae* 19258	*P.* aeruginosa 9027	*E. Coli* 8739		
5a	-	-	128	128	64	-
5b	256	256	128	128	32	256
5c	-	-	-	256	128	128
5d	256	-	-	256	-	256
5e	256	256	256	256	-	-
5f	-	-	-	-	-	-
7	-	-	256	128	64	128
9a	-	-	-	-	16	8
9b	-	-	256	-	8	4
9c	-	256	256	-	16	256
9d	-	-	256	-	8	4
9e	128	256	-	128	4	4
10	256	-	-	-	8	16
12	-	256	256	256	8	128
13	256	-	256	128	32	256
16	-	-	-	128	-	128
19	128	32	128	-	4	4
20	256	256	-	-	8	128
Colistin	1	1				
Ciprofloxacin			4	4		
Fluconazole					≤ 4	≤ 4

Conclusions

Some new triazole, pyridine and thieno-fused pyridine derivatives, containing methyl 4-aminosalicylate moiety as a substructure, were synthesized and characterized. The synthesized compounds were evaluated for their antibacterial and antifungal activity and some of them showed promising activity as fungicidal agents.

Experimental Section

General. Melting points were determined on a Gallankamp apparatus and are uncorrected. The IR spectra were recorded on a Shimadzu 470 IR-spectrophotometer (KBr; ν_{max} in cm^{-1}). The ^{1}H and ^{13}C NMR spectra were recorded on a Varian A5 500 MHz spectrometer using DMSO-d_6 as a solvent and tetramethylsilane (TMS) as internal reference. Coupling constants (*J* values) are given in Hertz (Hz).

Synthesis of methyl 4-(2-chloroacetamido)-2-hydroxybenzoate (3). This compound was synthesized according to our reported method.[34]

Synthesis of compounds 4a-d, 6, 8a-e, and 11

Compounds **4a,**[37]**4b,**[37]**4c,**[38] **4d,**[39] **4e,**[40] **4f,**[40] **6,**[41] **8a,**[42] **8b,**[43] **8c,** [44] **8d,** [45] **8e**[45] and **11**[46] were prepared according to the reported methods**.**

Reaction of compound 3 with 4a-d, 6, 8a-e, and 11. Synthesis of compounds 5a-d, 7, 9a-e, and 12, respectively; General procedure. To a mixture of compound **3** (1.21 g, 0.005 mol) and the respective thione **4a-f, 6, 8a-e** or **11** (0.005 mol) in ethanol (35 ml), sodium acetate trihydrate (0.005 mol) was added. The reaction mixture was heated under reflux for 2 h and then allowed to cool. The precipitate that formed was collected by filtration, dried in air and recrystallized from ethanol to give colorless crystals of compounds **5a-f, 7, 9a-e** or **12**, respectively.

Methyl 2-hydroxy-4-(2-((5-((*p*-tolylthio)methyl)-4*H*-1,2,4-triazol-3-yl)thio) acetamido)benzoate (5a). It was synthesized by reaction of compound **3** with triazolinethione **4a**; yield: 72 %; m.p.:156-158 ºC. IR: 3310 (N-H), 3192 (O-H), 3110 (C-H, sp^2), 2955, 2916 (C-H, sp^3), 1691 (C=O, ester), 1664 (C=O, anilide). ^{1}H NMR: δ 9.54 (s, 1H, NH); 9.27 (s, 1H, OH); 7.79-7.81 (d, 1H, ArH); 7.30 (s, 1H, triazole-NH), 7.24 (s, 1H, ArH), 7.18-7.20 (d, 2H, ArH), 7.04-7.06 (d, 1H, ArH), 6.92-6.94 (d, 2H, ArH), 4.21 (s, 2H, SCH$_2$); 4.01 (s, 2H, SCH$_2$); 3.94 (s, 3H, OCH$_3$); 2.25 (s, 3H, CH$_3$ of 4-tolyl group). ^{13}C NMR: δ 170.1, 167.9, 161.8, 159.8, 155.9, 142.5, 139.6, 132.4, 130.5, 127.8, 118.01, 115.7, 113.5, 109.9, 52.6, 36.3, 29.4, 20.9. Anal. calcd. for $C_{20}H_{20}N_4O_4S_2$ (444.09), %: C, 54.04; H, 4.53; N, 12.60; S, 14.43. Found, %: C, 54.11; H, 4.32; N, 12.46; S, 14.15.

Methyl 2-hydroxy-4-(2-((4-phenyl-5-((*p*-tolylthio)methyl)-4*H*-1,2,4-triazol-3-yl)thio)acetamido)benzoate (5b). It was synthesized by reaction of compound **3** with triazolinethione **4b**; yield: 79 %; m.p.: 168-170 ºC. IR: 3300 (N-H), 3187 (O-H), 3105, 3026 (C-H, sp^2), 2967 (C-H, sp^3), 1691 (C=O, ester), 1671 (C=O, anilide). ^{1}H NMR: δ 10.61 (s, 1H, NH); 10.58 (s, 1H, OH); 7.72-7.74 (d, 1H, ArH); 7.57-7.58 (m, 3H, ArH); 7.44-7.45 (d, 2H, ArH); 7.33-7.34 (d, 1H, ArH); 7.03-7.13 (m, 5H, ArH); 4.13 (s, 2H, SCH$_2$); 4.12 (s, 2H, SCH$_2$); 3.85 (s, 3H, OCH$_3$); 2.22 (s, 3H, CH$_3$ of 4-tolyl group). ^{13}C NMR: δ 168.8, 166.2, 161.1, 152.5, 150.4, 145.0, 136.6, 132.5, 130.8, 130.3, 130.0, 129.8, 129.6, 127.1, 110.3, 107.7, 106.1, 52.2, 36.9, 27.9, 20.5. Anal. calcd. for $C_{26}H_{24}N_4O_4S_2$ (520.12), %: 59.98; H, 4.65; N, 10.76; S, 12.32. Found, %: C, 59.72; H, 4.42; N, 10.59; S, 12.18.

Methyl 2-hydroxy-4-(2-((5-phenyl-4*H*-1,2,4-triazol-3-yl)thio)acetamido)benzoate (5c). It was synthesized by reaction of compound **3** with triazolinethione **4c**; yield: 78 %; m.p.:190-192º C. IR: 3253 (N-H), 3187 (O-H), 3108, 3056 (C-H, sp^2), 2930 (C-H, sp^3), 1694 (C=O, ester), 1670 (C=O, anilide). ^{1}H NMR: δ 10.71 (s, 1H, NH); 10.67 (s, 1H, OH); 7.78-7.80 (d, 2H: NH and ArH); 7.54-7.58 (t, 3H, ArH); 7.31-7.42 (m, 3H, ArH); 7.06-7.11 (dd, 1H, ArH); 4.26 (s, 2H, SCH$_2$); 3.88 (s, 3H, OCH$_3$). Anal. calcd. for $C_{18}H_{16}N_4O_4S$ (384.09), %: C, 56.24; H, 4.20; N, 14.57; S, 8.34 Found, %: C, 56.00; H, 4.13; N, 14.42; S, 8.17

Methyl 4-(2-((4,5-diphenyl-4*H*-1,2,4-triazol-3-yl)thio)acetamido)-2-hydroxybenzoate (5d). It was synthesized by reaction of compound **3** with triazolinethione **4d**; yield: 85 %; m.p.: 218-220º C. IR: 3236 (N-H), 3165 (O-H), 3094 (C-H, sp^2), 2969, 2925 (C-H, sp^3), 1693 (C=O, ester), 1669 (C=O, anilide). ^{1}H NMR: δ 10.66 (s, 1H, NH); 10.63 (s, 1H, OH); 7.75-7.77 (d, 1H, ArH); 7.55-7.57 (t, 3H, ArH); 7.34-7.45 (m, 8H, ArH); 7.07-7.10 (dd, 1H, ArH); 4.23 (s, 2H, SCH$_2$); 3.87 (s, 3H, OCH$_3$). Anal. calcd. for $C_{24}H_{20}N_4O_4S$ (460.12), %: C, 62.60; H, 4.38; N, 12.17; S, 6.96. Found, %: C, 62.48; H, 4.08; N, 12.22; S, 7.21.

Methyl 2-hydroxy-4-(2-((4-phenyl-5-((4-tolyloxy)methyl)-4*H*-1,2,4-triazol-3-yl)thio)acetamido)benzoate (5e). It was synthesized by reaction of compound **3** with triazolinethione **4e**; yield: 86 %; m.p.: 222-224 ºC. IR: 3240 (N-H), 3182 (O-H), 3103, 3018 (C-H, sp^2), 2952 (C-H, sp^3), 1693 (C=O, ester), 1667 (C=O, anilide). ^{1}H NMR: δ 10.59 (s, 1H, NH); 10.57 (s, 1H, OH); 7.71-7.72 (d, 1H, ArH); 7.46-7.56 (m, 4H, ArH); 7.32 (s, 1H, ArH); 6.98-7.05 (m, 3H, ArH); 6.70-6.71 (d, 2H, ArH); 5.01 (s, 2H, OCH_2); 4.18 (s, 2H, SCH_2); 3.83 (s, 3H, OCH_3), 2.15 (s, 3H, CH_3 of 4-tolyl group). ^{13}C NMR: δ 169.5, 166.8, 161.7, 155.8, 152.4, 151.9, 145.6, 133.2, 131.4, 130.8, 130.6, 130.3, 127.5, 115.3, 111.0, 108.4, 106.8, 60.5, 52.7, 37.5, 20.6. Anal. calcd. for $C_{26}H_{24}N_4O_5S$ (504.15), C, 61.89; H, 4.79; N, 11.10; S, 6.36. Found, %: 62.12; H, 4.74; N, 11.00; S, 6.51.

Methyl 4-(2-((5-((4-chlorophenoxy)methyl)-4-phenyl-4*H*-1,2,4-triazol-3-yl)thio)acetamido)-2-hydroxybenzoate (5f). It was synthesized by reaction of compound **3** with triazolinethione **4f**; yield: 85 %; m.p.: 292-294 º C. IR: 3243 (N-H), 3182 (O-H), 3103, 3018 (C-H, sp^2), 2952 (C-H, sp^3), 1682 (C=O, ester), 1666 (C=O, anilide). ^{1}H NMR: δ 10.61 (s, 1H, NH); 10.60 (s, 1H, OH); 7.70-7.72 (d, 1H, ArH); 7.51-7.58 (m, 3H, ArH); 7.28-7.32 (d, 2H, ArH); 6.98-7.05 (m, 4H, ArH); 6.84-6.91 (d, 2H, ArH); 5.06 (s, 2H, OCH_2); 4.18 (s, 2H, SCH_2); 3.83 (s, 3H, OCH_3). ^{13}C NMR: δ 169.5, 166.8, 161.7, 156.7, 152.1, 145.6, 133.1, 131.5, 130.7, 130.4, 129.7, 127.5, 125.8, 117.4, 117.2, 111.0, 108.4, 106.8, 60.7, 52.8, 37.5. Anal. calcd. for $C_{25}H_{21}ClN_4O_5S$ (524.09), %: C, 57.20; H, 4.03; N, 10.67; S, 6.11. Found, %: C, 57.50; H, 4.17; N, 10.42; S, 6.00.

Methyl 4-(2-((3-cyano-4,6-dimethylpyridin-2-yl)thio)acetamido)-2-hydroxybenzoate. (7). It was synthesized by reaction of compound **3** with pyridinethione **6**; yield: 88 %; m.p.: 238-240º C. IR: 3307 (O-H), 3284 (N-H), 2230 (C≡N), 1695 (C=O, ester), 1673 (C=O, anilide). ^{1}H NMR: δ 10.58 (s, 1H, NH); 7.71-7.73 (d, 1H, ArH); 7.33-7.34 (d, 1H, ArH); 7.07-7.09 (t, 2H, ArH); 4.16 (s, 2H, SCH_2); 3.85 (s, 3H, OCH_3); 2.40 (s, 3H, CH_3); 2.36 (s, 3H, CH_3). ^{13}C NMR: δ 178.1, 169.5, 167.5, 161.7, 157.4, 153.1, 145.9, 131.5, 121.0, 116.8, 115.4, 113.9, 110.9, 108.1, 35.6, 24.7, 21.3, 19.4. Anal. calcd. for $C_{18}H_{17}N_3O_4S$ (371.09), %: C, 58.21; H, 4.61; N, 11.31; S, 8.63. Found, %: C, 58.00; H, 4.32; N, 11.23; S, 8.79.

Ethyl 5-cyano-6-((2-((3-hydroxy-4-(methoxycarbonyl)phenyl)amino)-2-oxoethyl)thio)-2-methyl-4-styrylnicotinate (9a). It was synthesiszed by reaction of compound **3** with **8a**; yield: 77 %; m.p.: 210-212 º C. IR: 3260 (N-H), 3190 (O-H), 3108 (sp^2, C-H); 3953 (sp^2, C-H); 2221 (C≡N), 1723 (2 C=O, ester), 1674 (C=O, anilide). ^{1}H NMR: δ 10.65 (s, 1H, NH); 10.63 (s, 1H, OH); 7.73-7.74 (d, 1H, CH=C); 7.61-7.62 (d, 2H, ArH); 7.37-7.45 (m, 4H, ArH); 7.21 (s, 2H, ArH); 7.10-7.12 (d, 1H, C=CH); 4.29-4.34 (q, *J* = 8, 2H, OCH_2), 4.25 (s, 2H, SCH_2); 3.86 (s, 3H, OCH_3); 2.44 (s, 3H, CH_3); 1.19-1.22 (t, *J* = 8, 3H, CH_3). Anal. calcd. for $C_{28}H_{25}N_3O_6S$ (531.15), %: C, 63.26; H, 4.74; N, 7.90; S, 6.03. Found, %: C, 63.04; H, 4.51; N, 7.97; S, 6.22

Ethyl 5-cyano-6-((2-((3-hydroxy-4-(methoxycarbonyl)phenyl)amino)-2-oxoethyl)thio)-2-methyl-4-(thiophen-2-yl)nicotinate (9b). It was synthesized by reaction of compound **3** with **8b**; yield: 74 %; m.p.:172-174 º C. IR: 3378 (N-H), 3301 (O-H), 3091 (C-H, sp^2); 1988, 2951 (C-H, sp^3); 2225 (C≡N), 1728 (2 C=O, ester), 1678 (C=O, anilide). ^{1}H NMR: δ 10.67 (s, 1H, NH); 10.62 (s, 1H, OH); 7.89-7.90 (d, 1H, ArH); 7.73-7.75 (d, 1H, ArH); 7.36-7.40 (m, 2H, ArH); 7.24-7.25 (d, 1H, ArH); 7.10-7.12 (d, 1H, ArH); 4.26 (s, 2H, SCH_2); 4.07-4.11 (q, *J* = 10, 2H, OCH_2), 3.86 (s, 3H, OCH_3); 2.44 (s, 3H, CH_3); 0.98-1.01 (t, *J* = 10, 3H, CH_3). ^{13}C NMR: δ 169.5, 167.1, 166.3, 163.1, 161.7, 158.7, 145.8, 144.9, 133.6, 131.5, 131.1, 128.5, 125.8, 115.1, 110.9, 108.3, 106.6, 104.4, 100.0, 62.4, 52.8, 35.9, 23.5, 13.9. Anal. calcd. for $C_{24}H_{21}N_3O_6S_2$ (511.09), %: C, 56.35; H, 4.14; N, 8.21; S, 12.54. Found, %: C, 56.12; H, 4.01; N, 8.54; S, 12.42

Ethyl 5-cyano-6-((2-((3-hydroxy-4-(methoxycarbonyl)phenyl)amino)-2-oxoethyl) thio)-4-(4-methoxyphenyl)-2-methylnicotinate (9c). It was synthesized by reaction of compound **3** with **8c**; yield: 79 %; m.p.: 194-196 º C. IR: 3258 (N-H), 3194 (O-H), 3110 (C-H, sp^2), 3954, 3837 (C-H, sp^3), 2225 (C≡N), 1731 (2 C=O, ester), 1672 (C=O, anilide). ^{1}H NMR: δ 10.84 (s, 1H, NH); 9.16 (s, 1H, OH); 7.76-7.78 (d, 1H, ArH); 7.30-7.32 (d, 2H, ArH); 7.07.10 (t, 2H, ArH); 6.98-7.00 (d, 2H, ArH); 4.06-4.10 (q, *J* = 5, 2H, OCH_2), 4.02 (s, 2H, SCH_2); 3.92 (s, 3H, OCH_3); 3.85 (s, 3H, CO_2CH_3); 2.71 (s, 3H, CH_3); 0.98-1.01 (t, *J* = 5, 3H, CH_3). ^{13}C NMR: δ 170.7, 167.4, 167.1, 163.2, 162.5, 161.8, 159.5, 153.6, 144.8, 131.8, 130.4, 127.4, 126.8, 115.0, 114.9, 111.2, 109.1, 107.7, 106.2, 62.7, 56.1, 52.9, 35.8, 24.3, 14.3. Anal. calcd. for $C_{27}H_{25}N_3O_7S$ (535.14), %: 60.55; H, 4.71; N, 7.85; S, 5.99, Found, %: C, 60.49; H, 4.51; N, 7.72; S, 6.23.

Ethyl 5-cyano-6-((2-((3-hydroxy-4-(methoxycarbonyl)phenyl)amino)-2-oxoethyl) thio)-4-(4-methoxyphenyl)-2-phenylnicotinate (9d). It was synthesized by reaction of compound **3** with **8d**; yield: 79 %; m. p.: 234-236 º C. IR: 3312 (N-H), 2210 (O-H), 3098, 3000 (C-H, sp^2), 2960, 2909, 2837 (C-H, sp^3), 2223 (C≡N), 1732 (C=O, ester), 1685 (C=O, anilide). ^{1}H NMR: δ 10.66 (s, 2H: NH and OH); 7.77-7.79 (d, 1H, ArH); 7.39-7.48 (m, 6H, ArH); 7.20-7.21 (d, 2H, ArH); 7.09-7.18 (m, 3H, ArH); 4.29 (s, 2H, SCH_2), 3.83-3.88 (m, 8H: OCH_2 and two OCH_3) 0.74-0.77 (t, 3H, CH_3). ^{13}C NMR: δ 169.5, 166.9, 166.6, 162.9, 161.8, 161.0, 157.6, 153.2, 145.9, 138.1, 131.4, 130.6, 130.4, 129.1, 128.9, 128.8, 125.3, 115.2, 114.6, 110.9, 108.2, 106.6, 105.5, 62.1, 55.9, 52.8, 36.0, 13.7. Anal. calcd. for $C_{32}H_{27}N_3O_7S$ (597.16), %: C, 64.31; H, 4.55; N, 7.03; S, 5.37. Found, %: C, 64.00; H, 4.51; N, 7.27; S, 5.47.

Ethyl 4-(4-chlorophenyl)-5-cyano-6-((2-((3-hydroxy-4-(methoxycarbonyl)phenyl) amino)-2-oxoethyl)thio)-2-phenylnicotinate (9e). It was synthesized by reaction of compound **3** with **8e**; yield: 81 %; m.p.: 170-172 ºC. IR: 3297 (N-H), 2200 (O-H), 3095 (C-H, sp^2), 2960, 2909, 2837 (C-H, sp^3), 2223 (C≡N), 1732 (C=O, ester), 1677 (C=O, anilide). ^{1}H NMR: δ 10.70 (s, 1H, NH); 9.06 (s, 1H, OH); 7.66 (s, 2H, ArH); 7.53-7.55 (d, 2H, ArH); 7.47-7.49 (m, 4H, ArH); 6.33-6.35 (d, 2H, ArH); 6.63 (s, 1H, ArH); 6.38-6.40 (d, 1H, ArH); 3.97 (s, 2H, SCH_2), 3.90-3.95 (q, *J* = 10, 2H, OCH_2) 3.88 (s, 3H, OCH_3), 0.84-0.87 (t, *J* = 10, 3H, CH_3). ^{13}C NMR: δ 170.5, 167.1, 166.5, 163.1, 163.0, 159.2, 153.7, 144.7, 138.1, 137.2, 132.7, 131.4, 131.6, 130.2, 129.8, 129.0, 126.5, 114.3, 110.7, 108.7, 107.3, 106.5, 62.8, 52.7, 35.9, 14.0. Anal. calcd. for $C_{31}H_{24}ClN_3O_6S$ (601.11), %: C, 61.84; H, 4.02; Cl, 5.89; N, 6.98; S, 5.33. Found, %: C, 61.66; H, 4.12; Cl, 5.52; N, 6.84; S, 5.21.

Cyclization of compound 9b; formation of compound 10. To a suspension of **9b** (0.01 mol), in ethanol (40 ml), sodium ethoxide solution (0.51 g sodium dissolved in 20 ml ethanol) was added. The reaction mixture was warmed at 50 °C for 10 mins. The precipitate that obtained upon recrystallization from dioxane and subjection to elemental and spectral analyses was identified as **ethyl 3-amino-2-((4-(ethoxycarbonyl)-3-hydroxyphenyl)carbamoyl)-6-methyl-4-(thiophen-2-yl)thieno[2,3-b] pyridine-5-carboxylate (10);** yield: 50 %; m.p.: 230-232 º C. IR: 3471, 3353 (O-H, NH_2, N-H), 3107 (C-H, sp^2); 2976, 2928 (C-H, sp^3); 1710 (C=O, ester), 1671 (C=O, anilide). ^{1}H NMR: δ 10.67 (s, 1H, NH); 9.76 (s, 1H, OH); 7.89-7.91 (m, 1H, ArH); 7.69-7.71 (d, 1H, ArH); 7.46-7.47 (d, 1H, ArH); 7.25-7.29 (m, 3H, ArH); 6.09 (s, 2H, NH_2); 4.30-4.34 (q, *J* = 5, 2H, OCH_2), 4.03-4.08 (q, *J* = 10, 2H, OCH_2), 2.50 (s, 3H, CH_3); 1.30-1.33 (t, 3H, CH_3); 0.97-1.00 (t, 3H, CH_3). ^{13}C NMR: δ 168.8, 166.6, 163.8, 161.1, 159.4, 155.0, 147.4, 145.6, 136.9, 132.1, 130.1, 129.6, 128.0, 127.6, 120.2, 111.8, 107.5, 107.4, 97.7, 61.4, 61.0, 22.7, 14.0, 13.5. Anal. calcd. for $C_{25}H_{23}N_3O_6S_2$ (525.10), %: C, C, 57.13; H, 4.41; N, 7.99; S, 12.20. Found, %: C, 56.86; H, 4.22; N, 7.71; S, 12.09

Methyl 4-(2-((7-acetyl-4-cyano-6-hydroxy-1,6-dimethyl-8-phenyl-5,6,7,8-tetrahydroisoquinolin-3-yl) thio)acetamido)-2-hydroxybenzoate (12). It was synthesized by reaction of compound **3** with **11**; yield: 83 %; m. p.:147-149 º C. IR: 3487, 3359 (2 O-H, N-H), 2220 (C≡N), 1722 (C=O, ester), 1698 (C=O, acetyl), 1671 (C=O, anilide). ^{1}H NMR: δ 10.76 (s, 1H, NH); 9.43 (s, 1H, phenolic OH); 7.66-7.68 (d, 1H, ArH); 7.29-7.34 (m, 3H, ArH); 6.96-6.97 (m, 3H, ArH); 6.87-6.89 (m, 1H, ArH); 4.36-4.38 (d, *J* =10, 1H, C^8H); 3.89-3.91 (m, 5H: OCH_3 and SCH_2); 3.44 (s, 1H, alcoholic OH); 3.14-3.17 (d, *J* = 15, 1H, C^5H); 3.08-3.11 (d, *J* = 15, 1H, C^7H); 2.94-2.98 (d, *J* = 15, 1H, C^5H); 2.06 (s, 3H, $COCH_3$); 1.81 (s, 3H, CH_3); 1.37 (s, 3H, CH_3). ^{13}C NMR: δ 215.9, 170.6, 168.0, 163.1, 162.1, 158.5, 150.0, 144.9, 143.1, 131.5, 130.8, 130.3, 128.3, 128.2, 114.8, 111.0, 108.8, 107.5, 106.4, 69.6, 64.0, 52,8, 46.3, 42.6, 35.5, 28.3, 26.0. Anal. calcd. for $C_{30}H_{29}N_3O_6S$ (559.18), %: **C**, 64.39; H, 5.22; N, 7.51; S, 5.73. Found, %: C, 64.22; H, 5.04; N, 7.17; S, 5.45.

7-Acetyl-1-amino-5,8-dimethyl-8-hydroxy-2-[*N*-(3-hydroxy-4-methoxycarbonyl) phenyl]carbamoyl-6-phenyl-6,7,8,9-tetrahydrothieno[2,3-*c*]isoquinoline (13). To a hot solution of **12** (0.28 g, 0.005 mol) in methanol (30 ml), sodium methoxide solution (0.51 g sodium dissolved in 20 ml methanol) was added. The reaction mixture was warmed for 10 mins. The precipitate that formed on cooling was collected and recrystallized from ethanol to give canary yellow crystals of compound **13**; yield: 79 %; m.p.:254-256 °C. IR: 3458, 3386, 3338 (O-H, NH_2, N-H); 3027 (C-H, sp^2); 2963, 2911 (C-H, sp^3); 1724 (C=O, ester); 1699 (C=O, acetyl); 1671 (C=O, anilide). ^{1}H NMR: δ 10.56 (broad s, 2H, NH and OH), 9.59 (s, 1H, OH); 7.31-7.72 (m, 4H: NH_2 and ArH); 7.15-7.22 (m, 3H, ArH); 6.96-6.97 (d, 3H, ArH); 4.60-4.62 (d, *J* =10, 1H, C^6H); 3.87 (s, 3H, OCH_3), 3.57-3.61(d, *J* =20, 1H, C^9H), 3.38-3.42 (d, *J* = 20, 1H, C^9H); 2.90-2.92 (d, *J* =10, 1H, C^7H); 2.08 (s, 3H, $COCH_3$); 2.03 (s, 3H, CH_3); 1.31 (s, 3H, CH_3). ^{13}C NMR: δ 210.4, 169.7, 165.4, 161.7, 159.4, 157.0, 145.3, 143.0, 130.8, 129.9, 129.1, 128.6, 127,7, 126.8, 123.3, 112.6, 108.0, 99.9, 67.8, 66.7, 44.0, 42.6, 31.8, 28.5, 25.2. Anal. calcd. for $C_{30}H_{29}N_3O_6S$ (559.18), %: **C**, 64.39; H, 5.22; N, 7.51; S, 5.73. Found, %: C, 64.17; H, 5.00; N, 7.61; S, 5.89.

Synthesis of 3-cyanoquinoline-2(1*H*)-thione (14). It was synthesized according to our reported method.[47]

Methyl 4-(3-aminothieno[2,3-*b*]quinoline-2-carboxamido)-2-hydroxybenzoate (16). A mixture of compound **3** (1.21 g, 0.005 mol), 3-cyanoquinoline-2(1*H*)-thione (**14**) (0.93 g, 0.005 mol), and sodium acetate trihydrate (0.005 mol) in ethanol (35 ml),was heated under reflux for 2 hours and then allowed to cool. The precipitate that formed was collected by filtration, dried in air and recrystallized from ethanol to give canary needles of compound **16**; yield: 77%; m.p.: 271-273 º C. IR: 3420, 3307 (O-H, NH_2, N-H), 1712 (C=O, ester), 1662 (C=O, anilide). ^{1}H NMR: δ 10.62 (s, 1H, NH); 9.62 (s, 1H, OH); 9.13 (s, 1H, quinoline-H); 8.03-8.05 (d, 2H, ArH); 7.79-7.82 (t, 1H, ArH); 7.67-7.71 (d, 3H: ArH and NH_2); 7.54-7.59 (m, 2H, ArH); 7.35-7.36 (d, 1H, ArH); 3.84 (s, 3H, OCH_3). ^{13}C NMR: δ 169.2, 164.1, 161.0, 159.3, 148.4, 147.5, 145.9, 131.4, 130.9, 130.2, 129.2, 127.7, 125.8, 125.6, 124.6, 111.8, 107.4, 107.1, 95.4, 52.1. Anal. calcd. for $C_{20}H_{15}N_3O_4S$ (393.42), %: C, 61.06; H, 3.84; N, 10.68; S, 8.15. Found, %: C, 60.78; H, 3.61; N, 10.85; S, 8.37.

3-Carbamoyl-1-[*N*-(3-hydroxy-4-methoxycarbonyl)pheny]carbamoylmethylpyridinium chloride (19). A mixture of compound **3** (1.21 g, 0.005 mol), nicotinamide **17** (0.93 g, 0.005 mol) in ethanol (30 ml) was heated under reflux for 2 hours and then allowed to cool. The precipitate that formed was collected by filtration, dried in air, and recrystallized from ethanol to give white crystals of **19**; yield: 87 %; m.p.: 279-280 ºC. IR: 3284 (O-H, N-H), 1678 (2C=O). ^{1}H NMR: δ 11.45 (s, 1H, NH); 10.62 (s, 1H, OH); 9.55 (s, 1H, CONH), 9.15-9.17 (d, *J* = 10, 1H, ArH), 9.05-9.07 (d, *J* = 10, 1H, ArH), 8.70 (s, 1H, CONH), 8.29-8.32 (dd, *J* = 5, 1H, ArH); 8.17 (s, 1H, ArH); 7.72-7.74 (d, *J* = 10, 1H, ArH), 7.35 (s, 1H, ArH); 7.12-7.14 (dd, *J* = 10, 1H, ArH); 5.76 (s, 2H, CH_2). 3.82 (s, 3H, OCH_3). ^{13}C NMR: δ 169.3, 164.5, 163.2, 161.5, 148.5, 147.2, 145.1, 144.7, 133.8, 131.5, 127.7, 111.0, 108.8, 107.0, 63.1, 52.8. Anal. calcd. for $C_{16}H_{16}ClN_3O_5$ (365.77), %: C, 52.54; H, 4.41; N, 11.49. Found, %: C, 52.61; H, 4.38; N, 11.62.

Synthesis of 7-(thiophen-2-yl)-9-(trifluoromethyl)pyrido[3',2':4,5]thieno[3,2-d][1,2,3]triazin-4(3H)-one (18). It was synthesizd according to our reported method.[48]

3-[*N*-(3-hydroxy-4-methoxycarbonylphenyl)carbamoylmethyl]-7-(thiophen-2-yl)-9-(trifluoromethyl) pyrido[3',2':4,5]thieno[3,2-*d*][1,2,3]triazin-4(3*H*)-one (20). To a mixture of compound **3** (1.21 g, 0.005 mol) and triazineone **18** (0.18 g, 0.005 mol) in DMF (25 ml), anhydrous K_2CO_3 (0.74 g, 0.005 mol) was added. The reaction mixture was heated at 100 C for 4 hours and then allowed to cool. The precipitate that formed was collected by filtration, washed several times with water, dried in air, and recrystallized from ethanol to give pale yellow crystals of compound **20**; yield: 75%; m. p.: 298-300 ºC. IR: 3280 (O-H, N-H), 1685 (C=O, ester), 1667 (2 C=O, pyrimidineone and anilide). ^{1}H NMR: δ 10.71 (s, 1H, NH); 8.65 (s, 1H, pyridine-H); 8.39 (s, 1H, ArH); 7.95-7.96 (d, *J* = 5, 1H, ArH); 7.77-7.79 (d, *J* =10, 1H, ArH); 7.33-7.35 (d, *J* =10, 2H, thiophene-H); 7.10-7.13 (d, *J* =15, 1H, thiophene-H); 5.44 (s, 2H, NCH_2); 3.88 (s, 3H, OCH_3). ^{13}C NMR: δ 169.5, 165.6, 163.7, 161.7, 155.1, 152.9, 146.0, 145.3, 142.6, 142.1, 133.1, 131.5, 131.0, 129.8, 128.0, 119.8, 115.6, 114.1, 111.0, 108.6, 106.9, 53.7, 52.8. Anal. calcd. for $C_{24}H_{16}F_3N_5O_5S_2$ (575.05), %: C, 50.08; H, 2.80; N, 12.17; S, 11.14. Found, %: C, 50.13; H, 2.96; N, 12.00; S, 11.28.

Biological Activity. Determination of MIC of newly synthesized compounds

All synthesized compounds were evaluated as bactericidal agents and as antifungals following the dilution method.[49]

Bacterial strains and culture conditions

MIC was determined by antimicrobial susceptibility testing according to CLSI guidelines.[50] In brief, overnight bacteria cultured in Lysogeny Broth (LB) medium were diluted at 1:100 in fresh LB and cultured at 37°C with shaking at 200 rpm to an optical density at 600 nm of 0.5. Then, 10 µL of 5 × 104 CFU was added into each well of a 96-well microtiter polystyrene tray with 100 µL LB of a series of 2-fold dilutions of an antibiotic/ substance. The mixtures were incubated at 37°C for 16 h. MIC was defined as the lowest antibiotic/substance concentration that inhibited visible bacteria growth. By using spectrophotometer (STAT FAX-2100, AWARENESS- Tech.com, USA) at 600 nm (turbidity method).

Preparation of cation adjusted MHB (20 mg Ca^{2+} and 10 mg Mg^{2+} per liter). A 10 mg/ml (1000 mg/100 ml) Mg^{2+} stock solution was prepared by dissolving 8.36 g of $MgCl_2.6H_2O$ in 100 ml deionized water. Also, a 10 mg/ml (1000 mg/100 ml) Ca^{2+} stock solution was prepared by dissolving 3.68 g of $CaCl_2.2H_2O$ in 100 ml deionized water. Filter-sterilize both stock solutions using 0.2-mm pore size cellulose-acetate filters. MHB (Mueller-Hinton Broth) was prepared according to the manufacturer's instructions, autoclaved and cooled the medium to 2 - 8 °C before the addition of the cation solutions. 100 ml of stock solution per 1 mg/L needed for 1 L of medium was added. For example, 2 ml of Ca^{2+} stock solution was added if 20 mg needs to be added to 1 L MHB (Mueller-Hinton Broth).

Preparation of inoculum. Using pure colonies from fresh agar plates to prepare bacterial suspension with a turbidity of 0.5 mcfarland ($1X10^8$ cfu/ml). Mix the bacterial suspension adjusted to $1X10^8$ cfu/ml and dilute it by a factor of 1:100 by adding 200 µl bacterial suspension to 19.8 ml sterile MHB in a sterile 25 ml Erlenmeyer flask to prepare a 20 ml inoculum of a concentration of $1X10^6$ cfu/ml. Each well containing the antibiotic solution (50 µl) and the growth control (50 µl) well is intended to be supplied with with 50 µl of the bacterial suspension. This results in the final desired inoculum of $5X10^5$ cfu/ml.

Microtiter plate pipetting and testing. Each tested compound is first dissolved in DMSO to obtain a stock solution of 1024 µg/ml. Each row of 12 wells is desired to test only one compound (10 testing wells represent serial dilution of the tested compound and the last two wells represent negative control (sterility) and positive control (fertility). The whole plate is desired to test a total of 8 compounds. Each testing well and positive control well is first supplied with 50 µl MHB. Each negative control well is supplied with 100 µl MHB. For each testing row, Take 50 µl from each tested compound stock solution in the first testing well to obtain a concentration of 512 µg/ml. From the first well of each row, transfer 50 µl to the second well to obtain a concentration of 256 µg/ml. Complete serial dilution of each compound by repeating the previous step till well 10 to obtain concentrations of each compound of 128, 64, 32, 16, 8, 4,2, and 1 µg/ml. Discard 50 µl from well 10 in each row. Finally, Add 50 µl of working bacterial suspension (1X10^6 cfu/ml) to each testing and growth control well to obtain final inoculum of 5X10^5 cfu/ml in these well. Now, the final concentrations for each tested compound will be 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 µg/ml.

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Supplementary Material

Copies of IR, ^{1}H NMR and ^{13}C NMR spectra of synthesized compounds are available in the supplementary material file associated with this manuscript.

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