Synthesis, Antimicrobial Activity, DNA-Binding Affinity and Molecular Docking of Certain 1,2,4-Triazolo[1,5-*a*]Pyrimidines as Nalidixic Acid Isosteres

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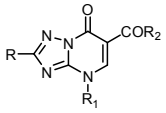
Abstract: A series of substituted 1,2,4-triazolo[1,5-*a*]pyrimidines, of the general formula A, was synthesized and tested for *in vitro* activities against a panel of Gram positive and Gram negative bacteria and the yeast-like pathogenic fungus *Candida albicans*. The results revealed that the G+ve bacteria *Bacillus subtilis* and to a lesser extent *Staphylococcus aureus*, and the G-ve *E. coli*, are sensitive to the majority of the synthesized compounds. In addition, the DNA binding affinity of the synthesized compounds was tested, and results of the qualitative preliminary assay showed that compounds **8c**, **7c** and **7a** were the most active analogues. Consequently, docking studies were done for these compounds to identify the target receptors, and the results showed promising energy score of docking with enzymes related to bacterial infections.



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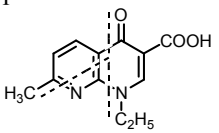
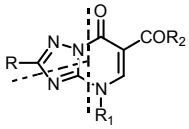
1. Introduction

4-Quinolones comprise a series of synthetic antibacterial agents patterned after nalidixic acid [1]. They have a proven track record over the past several decades for the treatment of bacterial infections. Their unique mechanism of action and bactericidal properties make them attractive therapeutic agents. Despite nearly forty years of research, quinolones still provide new analogues of both scientific and clinical interest. Compounds that are active against resistant strains, with improved pharmacokinetic and safety profiles are goals for current and future research in this area [2].

On the basis of the preceding information, coupled with the well-known antimicrobial activity of the acid hydrazides, thiosemicarbazides and their cyclic 1,2,4-triazole analogues [3-10], we synthesized new hybrid molecules through the combination of these moieties and the 1,2,4-triazolo[1,5-*a*]pyrimidine nucleus, as different pharmacophores, in one frame of the general formula **A**. Consequently, a series of 2-substituted-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carboxylates and their derivatives were synthesized as isosteres of nalidixic acid with the hope of finding interesting pharmacological profiles.



Nalidixic acid



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2. Results and Discussion

Chemical Synthesis. 5-Amino-3-substituted-1*H*-1,2,4-triazoles **2a-c**, the key starting materials necessary for this study, were synthesized from dimethyl *N*-cyanodithioiminocarbonate **1** *via* reaction with the appropriate amine in acetonitrile [11], to yield the intermediate of isothiourea, which was subsequently reacted with excesss of hydrazine hydrate to yield **2a-c**. Interaction of **2a-c** with diethyl ethoxymethylene malonate (DEEM) in refluxing acetic acid afforded the cyclized compounds, **3a** and **3b,c** [12]. Interaction of compounds **3a-c** with methyl iodide or ethyl bromide in dimethylformamide, in the presence of anhydrous potassium carbonate yielded the corresponding *N*-alkylated derivates **4a** and **4b,c**

[12], **5a** and **5b,c** [12]. Hydrolysis of the ethyl ester of compound **5a** with an aqueous solution of sodium hydroxide yielded the corresponding carboxylic acid **6** (**Scheme 1**, **Table 1**).

Reacting compounds **3a-c** with hydrazine hydrate in ethanol yielded the corresponding carboxylic acid hydrazides **7a-c**, which were subsequently treated with phenyl or butyl isothiocyanate in refluxing ethanol to yield the corresponding thiosemicarbazide derivatives **8a-c** and **9a-c**. Refluxing the ethanolic solution of **7b** and phenyl isothiocyanate in 2N sodium hydroxide solution yielded compound **10**.

The carboxylic acid hydrazides **7a,b** were treated with carbon disulphide and sodium hydroxide in dimethyl sulphoxide at room temperature. The sodium salts obtained were converted to the methyl derivatives **11a,b** by treating with dimethyl sulphate.

The reaction of the acid hydrazides **7a-c** with aryl sulphonyl chlorides in the presence of pyridine afforded the corresponding arylsulphonylhydrazines **12a-c** and **13a-c** (**Scheme 2**, **Table 2**).

***In vitro* antimicrobial activity.**

The synthesized compounds were tested for their *in vitro* antimicrobial activity against a panel of standard strains of the *Gram-positive* bacteria (*Staphylococcus aureus* IFO 3060, and *Bacillus subtilis* IFO 3007), the *Gram-negative* bacteria (*Escherichia coli* IFO 3301 and *Pseudomonas aeuroginosa* IFO 3448), and the yeast-like pathogenic fungus *Candida albicans* IFO 0583. The primary screen was carried out using the agar disc-diffusion method [13], using Müller-Hinton agar medium. The results of the preliminary antimicrobial testing of the synthesized compounds (200 µg/disc), the antibacterial antibiotic Ampicillin, Gentamycin (100 µg/disc), and the antifungal drug Clotrimazole (100 µg/disc), are shown in **Table 3**. The results revealed that the majority of the synthesized compounds showed varying degrees of inhibition against the tested microorganisms. Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and Gram negative *Escherichia coli* are considered the most sensitive among the tested microorganisms. The inhibitory activity against the tested Gram-negative bacteria *Pseudomonas aeuroginosa* was rather lower than the other tested microorganisms, The minimal inhibitory concentration (MIC) for the most active compounds, **3a, 4a, 5a, 7a, 7b, 7c, 8a, 8c, and 9a** against the same microorganisms used in the primary screening was carried out using the microdilution susceptibility method in Müller-Hinton Broth [14], as shown in **Table 4**. However, we can conclude that the Gram-positive bacteria *Bacillus subtilis* and to a lesser extent *Staphylococcus aureus* and the Gram-negative bacteria *Escherichia coli* are sensitive to the majority of the tested compounds. The inhibitory activity of the compounds against *Candida albicans* was rather weakly active or inactive.

According to the results of the antimicrobial activity, it seems difficult to abstract definite structure-activity relationship. However, we can conclude that introduction of 3,4-dimethoxybenzylamino moiety in the 2-position of the tested compounds led to increased activity against both G+ve and G-ve bacteria, while introduction of pyrrolidine moiety mainly increased the activity against G-ve bacteria, as in compound **7c**.

DNA-binding affinity

The mechanism of many antibacterial agents involves interaction with DNA. In the case of quinolones, the drugs are thought to act by stabilizing a cleavage complex between the gyrase enzyme and DNA that arrests polymerases *in vivo* [15]. DNA gyrase is involved in maintaining appropriate DNA supercoiling during DNA synthesis and transcription. Quinolones form a stable enzyme-DNA-quinolone cleavable complex. The formation of enzyme-quinolone-DNA complexes reversibly inhibits DNA synthesis and initially causes bacteriostatic growth inhibition. Irreversible lethal single- and double-stranded breaks are released when the complex dissociates [16].

Based on the interaction of small molecular weight ligands with DNA, some short-term procedures have been devised that are applicable for the discovery and evaluation of naturally occurring and synthetic compounds that function by this mechanism [17-20]. In addition, a variety of methods have been utilized for the interaction of small molecular weight compounds with DNA, such as equilibrium dialysis [21], DNA-binding assay [22], and methyl green-DNA displacement assay [23].

In the DNA-binding assay [22], a fixed amount of ligand is spotted on the RP-18 TLC plates followed by addition of known amount of DNA on the same spot. The plate was then developed and the position of the DNA was determined by spraying the plates with anisaldehyde reagent. The free DNA was detected as a blue spot (R_f; MeOH-H_2O, 8:2) on RP-18 TLC after spraying with anisaldehyde reagent. It is important to establish if the response of the test system is dependent on the dose of the test substance. In the presence of increasing quantities of DNA intercalators, a greater portion of DNA is bound to form a complex. On the other hand, compounds with

high binding affinity to DNA were retained on the base line. It was demonstrated that when DNA was applied to an RP-18 TLC plates, migration was observed when MeOH-H_2O (8:2) was used as the elution solvent. However, when the DNA was mixed with compounds with which it is known to interact (ethidium bromide), the complex was retained at the origin. Inactive compounds did not cause the DNA to be retained at the origin.

Methyl green [23] reversibly binds polymerised DNA, and the complex is stable at neutral pH, whereas free methyl green fades. Incubation for 24 h, in the buffer used for displacement reactions in this study, results in virtually a complete loss of methyl green absorbance. A colourimetric assay was used to measure the displacement of methyl green from DNA by compounds with ability to bind to DNA. The displacement was determined spectrophotometrically by a decrease in absorbance at 630 nm.

Nearly all compounds in this study showed high affinity to DNA which was demonstrated by retaining the DNA-compound complex at the origin or by migrating for very short distances. The most active compounds, **7a, 7c,** and **8c**, were subjected to methyl green/DNA displacement assay and the results are shown in **Table 5**.

These results are in accordance with the antimicrobial screening data and explain them, suggesting that binding with DNA may contribute to the activity of these compounds against bacterial infections.

Molecular docking

As an initial step in the way to identify the target receptors to which our compounds may exhibit antibacterial activity, the selected most active compounds, **7a, 7c,** and **8c**, were sketched and energy minimized by Schrodinger software, and then submitted to the website http://www.dddc.ac.cn/tarfisdock [24]. The compounds were docked into several enzymes whose inhibition may contribute to antibacterial activity (**Table 6**).

As revealed from the obtained data, the tested compounds showed promising scores of docking with enzymes related to bacterial infections, with energy score values ranging from -44.88 to -36.35 kcal/mol, so, these compounds are expected to possess promising antibacterial activity through interfering with protein synthesis as shown by the energy scores of compounds **7a** and **8c**, or damaging DNA as seen from the binding scores of compound **7c** with DNA gyrase B subunit and topoisomerase II.

In conclusion, the newly synthesized 1,2,4-triazolo[1,5-*a*]pyrimidines could be considered potential candidates for further derivatization to determine the scope and limitation of their activity as antimicrobial agents.

Experimental section

Melting points (ºC) were recorded using a *Fisher-Johns* melting point apparatus and are uncorrected. Microanalyses were performed in the Microanalytical unit, Cairo University. IR spectra (KBr) were recorded on Mattson 5000 FT-IR spectrometer (ν in cm^{-1}). ^{1}H-NMR spectra were obtained on FT-NMR spectrometer (200 MHz) Gemini Varian using TMS as internal standard (chemical shifts in ppm, δ units). MS analyses were performed on JEOL JMS-600H spectrometer.

Chemical Synthesis.

5-Amino-3-substituted-1*H*-1,2,4-triazoles 2a-c. A solution of the appropriate amine (0.011 mole) in acetonitrile (5ml) was added dropwise to a stirred solution of dimethyl *N*-cyano-dithioiminocarbonate **1** (1.46 g, 0.01 mole) in acetonitrile (15ml). The mixture was heated under reflux for 4 hours. On cooling, hydrazine hydrate (0.6 g, 0.012 mole) was added and heating under reflux was continued for further 1 hour. The reaction mixture was concentrated under vacuum to afford a precipitate which was collected by filtration washed with acetonitrile and air dried. The crude product was recrystallized from acetonitrile (**Table 1**).

2a: ^{1}H-NMR (DMSO-d_6): δ 3.36 (s, 1H, NH), 3.75 (s, 6H, $2OCH_3$), 4.15 (d, 2H, CH_2), 5.62 (br s, 2H, NH_2), 6.85-6.96 (m, 3H, Ar-H), 10.72 (br s, 1H, NH-triazole).

Ethyl 2-substituted-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carboxylates 3a-c. Diethyl ethoxymethylenemalonate (DEEM) (0.864 g, 0.004 mole) was added dropwise to a stirred solution of 5-amino-3-substituted-1*H*-1,2,4-triazoles **2a-c** (0.0025 mole) in glacial acetic acid (5ml). The reaction mixture was refluxed for 2 hours and refrigerated overnight. The precipitated solid was filtered, washed with ethyl acetate and air dried . The crude products were rescystallized from acetic acid (**Table 1**).

3a: IR: 1596 (C=O), 1700 (COOEt), 3158 and 3378 (NH) cm^{-1}. ^{1}H-NMR (DMSO-d_6): δ 1.28 (t, 3H, $CH_2\underline{CH_3}$), 3.37 (br s, IH, $CH_2\underline{NH}$) 3.75 (s, 6H, $2OCH_3$), 4.24 (q , 2H, $\underline{CH_2}CH_3$) 4.34 (d, 2H, $\underline{CH_2}NH$), 7.00-7.22 (m, 3H, Ar-H), 7.30 (s, 1H, NH-pyrimidine), 8.42 (s, 1H, CH-pyrimidine) .

Ethyl 2-substituted-4-alkyl-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carboxylates 4a-c and 5a-c. Methyl iodide or ethyl bromide (0.003 mole) was added dropwise over a period of 15 minutes during stirring to a suspension of ethyl 2-substituted- 4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carboxylates **3a-c** (0.001 mole) and anhydrous potassium carbonate (0.15 g, 0.0011 mole) in dry dimethylformamide (2.5 ml). The mixture was heated under reflux for 1 hour and cooled. The mixture was poured onto ice, extracted three times with chloroform. The combined chloroformic extract was dried over anhydrous sodium sulphate, evaporated under vacuum, and recrystallized from ethanol to give yellowish white crystals of compounds **4a-c** and **5a-c** respectively (**Table 1**).

4a: ^{1}H-NMR (DMSO-d_6): δ 1.37 (t, 3H, $OCH_2\underline{CH_3}$), 3.82 (s, 3H, NCH_3), 3.86 (s, 6H, $2OCH_3$), 4.39 (q , 2H, $O\underline{CH_2}CH_3$), 4.52 (d, 2H, $\underline{CH_2}NH$), 4.93 (t, 1H, $CH_2\underline{NH}$), 6.82 – 6.89 (m, 3H, Ar-H), 8.27 (s,1H, CH-pyrimidine).

5a: ^{1}H-NMR (DMSO-d_6): δ 1.38 (t, 3H, $OCH_2\underline{CH_3}$), 1.53 (t, 3H, $NCH_2\underline{CH_3}$), 3.87 (s, 6H, $2OCH_3$), 4.20 (q, 2H, $N\underline{CH_2}CH_3$), 4.39 (q, 2H, $O\underline{CH_2}CH_3$), 4.53 (d, 2H, $\underline{CH_2}NH$), 4.82 (t, 1H, -$CH_2\underline{NH}$), 6.90-6.93 (m, 3H, Ar-H), 8.29 (s, 1H, CH-pyrimidine).

2-(3,4-Dimethoxybenzylamino)-4-ethyl-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carboxylic acid 6. The ethyl ester **5a** (0.401 g, 0.001 mole) was dissolved in a solution of sodium hydroxide (0.08 g, 0.002 mole) in water (10ml) and heated under reflux for 3 hours. After cooling, the reaction mixture was filtered and acidified by dropwise addition of 4N hydrochloric acid . The carboxylic acid, precipitated as orange crystals, was collected by filtration, washed with water, air dried, and recrystallized from aqueous ethanol to yield compound **6** (**Table 1**).

^{1}H-NMR (DMSO-d_6):δ 1.38 (t, 3H, $NCH_2\underline{CH_3}$), 3.49 (s, 1H, $CH_2\underline{NH}$), 3.70 (s, 6H, $2OCH_3$), 4.24 (q, 2H, $N\underline{CH_2}CH_3$), 4.35 (d, 2H, $Ar\underline{CH_2}NH$), 6.86-7.02 (m, 3H, Ar-H), 8.80 (s, 1H, CH-pyrimidine), 12.86 (br s, 1H, D_2O-exchangeable, COOH).

2-Substituted-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carboxylic acid hydrazides 7a-c. Hydrazine hydrate (5.5 ml) was added dropwise to (0.004 mole) of the ethyl ester **3a-c**. The hot reaction mixture was stirred at room temperature overnight. The separated hydrazide was filtered , washed with ethanol, air dried and recrystallized from aqueous ethanol (**Table 2**).

7a: IR: 1644 (CONH) 1708 (C=O), 2998, and 3100 (NH), 3348, and 3385 (NH_2) cm^{-1}. ^{1}H-NMR (DMSO-d_6): 3.37 (br s, IH, $CH_2N\underline{H}$), 3.42 (d, 2H, $NH\underline{NH_2}$), 3.72 (s, 6H, $2OCH_3$), 4.31 (d, 2H, $\underline{CH_2}NH$) , 6.48 (t, 1H, $\underline{NH}$ NH_2), 6.86-7.00 (m, 3H, Ar-H), 7.15 (br s, IH, NH-pyrimidine), 8.39 (s, 1H, CH-pyrimidine).

7b: IR: 1625 (CONH), 1672 (C=O), 3056, and 3098 (NH), 3402 and 3446 (NH_2) cm^{-1}. ^{1}H-NMR (DMSO-d_6): 3.35 (t, 4H, $\underline{CH_2}N\underline{CH_2}$), 3.45 (d, 2H, $NH\underline{NH_2}$), 3.68 (t, 4H, $\underline{CH_2}O\underline{CH_2}$), 6.9-7.4 (br s, 2H, $\underline{NH}NH_2$ and NH-pyrimidine), 8.43 (s, 1H, CH-pyrimidine).

7c: MS: m/z (%) 264 (M^++1, 7.00), 263 (M^+, 40.78).

2-Substituted-6-(4-substituded thiosemicarbazidocarbonyl)-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidines 8a-c and 9a-c. A mixture of the acid hydrazide **7a-c** (0.005 mole), the proper substituted isothiocyanate (0.005 mole), and absolute ethanol (25ml) was heated under reflux for 5 hours. The product was collected by filtration, dried, and recrystallized from aqueous ethanol (**Table 2**).

8b: ^{1}H-NMR (DMSO-d_6): δ 3.40 (t, 4H, morpholine-$CH_2N\underline{CH_2}$), 3.69 (t, 4H, morpholine-$CH_2O\underline{CH_2}$), 7.14-7.55 (m, 7H, Ar-H, NH-pyrimidine, NH), 8.64 (s, 1H, CH-pyrimidine), 9.72 (br s, 1H, NH), 9.90 (br s, 1H, NH). MS: m/z (%)410 (M-4, 5.30).

9a: ^{1}H-NMR (DMSO-d_6): δ 0.88 (t, 3H, $CH_2CH_2CH_2\underline{CH_3}$), 1.28 (m, 2H, CH_2 $CH_2\underline{CH_2}CH_3$), 1.49 (m, 2H, $CH_2\underline{CH_2}CH_2CH_3$), 3.11 (m, 2H, $NH\underline{CH_2}CH_2CH_2CH_3$), 3.34 (s, 6H, $2OCH_3$), 3.82 (t, 2H, $Ar\underline{CH_2}NH$), 4.35 (t, 1H, $ArCH_2\underline{NH}$), 6.48-6.99 (m, 4H, ArH & NH), 7.41 (s, 1H, NH-pyrimidine), 8.12 (s, 1H, NH), 8.48 (s, 1H, CH-pyrimidine), 9.58 (s, 1H, NH).

9b: ^{1}H-NMR (DMSO-d_6): δ 0.88 (t, 3H, $CH_2\underline{CH_3}$), 1.28 (m, 2H, CH_2 $\underline{CH_2}CH_3$), 1.49 (m, 2H, $CH_2\underline{CH_2}CH_2CH_3$), 3.30 (t, 4H, morpholine-$CH_2N\underline{CH_2}$), 3.33 (t, 4H, morpholine-$CH_2O\underline{CH_2}$), 3.45 (dt, 2H, $NH\underline{CH_2}CH_2CH_2CH_3$), 6.85 (s, 1H, NH), 7.00 (s, 1H, NH-pyrimidine), 7.85 (s, 1H, NH), 8.38 (s, 1H, CH-pyrimidine), 9.15 (s, 1H, NH).

9c: ^{1}H-NMR (DMSO-d_6): δ 0.85 (t, 3H, $CH_2\underline{CH_3}$), 1.29 (m, 2H, CH_2 $\underline{CH_2}CH_3$), 1.49 (m, 2H, $CH_2\underline{CH_2}CH_2CH_3$), 1.94 (m, 4H, pyrrolidine-$2CH_2$), 3.25 (m, 2H, $NH\underline{CH_2}CH_2CH_2CH_3$), 3.35 (m, 4H, pyrrolidine-$CH_2N\underline{CH_2}$), 7.91 (d, 1H, NH), 8.48 (s, 1H, CH-pyrimidine), 9.26 (br d, 1H, NH), 10.15 (br s, 1H, NH), 10.60 (br s, 1H, NH). MS: m/z (%) 378 (M^+, 12.78).

6-(4,5-Dihydro-4-phenyl-5-thioxo-1*H*-1,2,4-triazol-3-yl)-2-(4-morpholinyl)-4*H*-1,2,4-triazolo[1,5-*a*]pyrimidin-7-one 10. A mixture of the

acid hyrazide **7b** (0.558 g, 0.002 mole) in ethanol (7ml), phenyl isothiocyanate (0.27 g, 0.002 mole), and 2N aqueous solution of sodium hydroxide (10 ml) was heated under reflux for 5 hours. The reaction mixture was allowed to cool to room temperature and acidified with 10% hydrochloric acid while cooling. The product was collected by filtration, washed with water, dried, and recrystallized from ethanol (**Table 2**).

^{1}H-NMR (DMSO-d_6): δ 3.32 (t, 4H, morpholine-CH_2NCH_2), 3.73 (t, 4H, morpholine-CH_2OCH_2), 6.92-7.57 (m, 6H, ArH, NH-pyrimidine), 8.35 (s, 1H, CH-pyrimidine), 13.35 (s, 1H, NH).

***N*'-(2-Substituted-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carbonyl)hydrazinecarbo-dithioic acid methyl esters 11a,b.** To a vigorously stirred solution of the 2-substituted-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carboxylic acid hydrazide **7a,b** (0.002 mole) in dimethyl sulfoxide (2 ml) at room temperature, carbon disulphide (0.198 g, 0.0026 mole) and 5 M sodium hydroxide (0.48 ml) were added dropwise during 15 minutes. The mixture was allowed to stirr for 30 minutes more. Dimethyl sulphate (0.252 g, 0.002 mole) was added at 5-10 °C , stirring was continued for 2 hours and the reaction mixture was poured onto ice water. The solid so obtained was filtered, washed with water, dried and recrystallized from ethanol (**Table 2**).

11a: ^{1}H-NMR (DMSO-d_6): 2.70 (s, 3H, CH_3), 3.38 (br s, 1H, ArCH_2NH), 3.68 (s, 6H, 2OCH_3), 4.31 (d, 2H, ArCH_2NH), 6.83-6.96 (m, 3H, Ar-H), 7.14 (s, 1H, NH-pyrimidine), 8.40 (s, 1H, CH-pyrimidine), 10.72 (s, 1H, NH), 11.71 (s, 1H, NH).

11b: IR: 3440, 3204, 3131 (NH), 1698 (cyclic C=O) , 1643 (CONH) and 1111 (C=S) cm^{-1}. ^{1}H-NMR (DMSO-d_6): 3.00 (s, 3H, CH_3), 3.35 (t, 4H, morpholine- CH_2NCH_2), 3.67 (t, 4H, morpholine-CH_2OCH_2), 7.10 (s, 1H, NH-pyrimidine), 8.47 (s, 1H, CH-pyrimidine), 10.77 (s, 1H, NH), 11.75 (s, 1H, NH).

***N*-(2-Substituded-4,7-dihydro-7-oxo-1,2,4-triazolo[1,5-*a*]pyrimidine-6-carbonyl)-*N'*-(arylsulphonyl)-hydrazines 12a-c and 13a-c.** A mixture of the acid hydrazide **7a-c** (0.001 mole), pyridine (3 ml) and the appropriate aryl sulphonyl chloride (0.001 mole) was stirred at room temperature overnight. The mixture was poured into ice cold water, filtered, washed with water, air dried, and recrystallized from aqueous ethanol (**Table 2**).

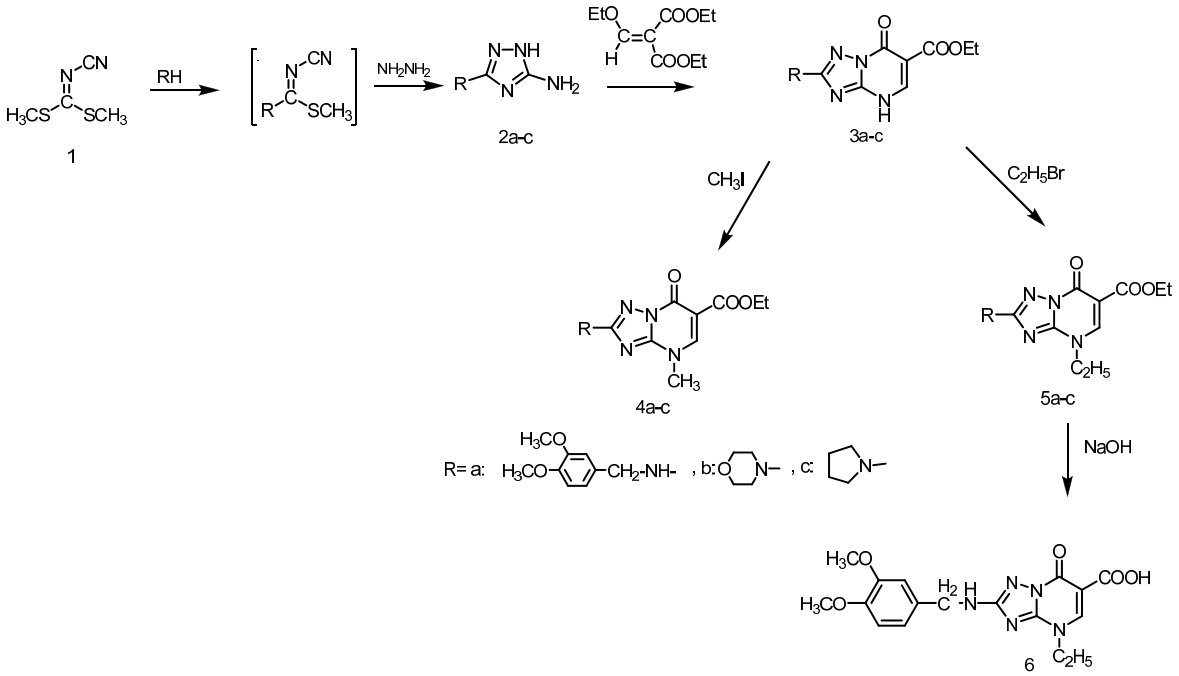
12a: IR: 1160 and 1340 (SO_2), 1636 (CONH), 1700 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.40 (s, 3H, CH_3), 3.35 (br s, 1H, ArCH_2NH), 3.72 (s, 6H, 2OCH_3), 4.33 (d, 2H, ArCH_2NH), 6.87 (m, 3H, Ar-H), 7.00 (s, 1H, NH-pyrimidine), 7.28 (br s, 1H, NH), 7.39-7.66 (m, 4H, tolyl-H), 8.41 (s, 1H, CH- pyrimidine), 9.59 (s, 1H, NH).

12c: ^{1}H-NMR (DMSO-d_6): 1.92 (m, 4H, pyrrolidine-2CH_2), 2.40 (s, 3H, CH_3) , 3.50 (m, 4H, pyrrolidine-CH_2NCH_2), 7.35-7.76 (m, 5H, Ar-H, NH-pyrimidine), 8.48 (s, 1H, CH- pyrimidine), 9.57 (s, 1H, NH) , 10.17 (br s, 1H, NH).

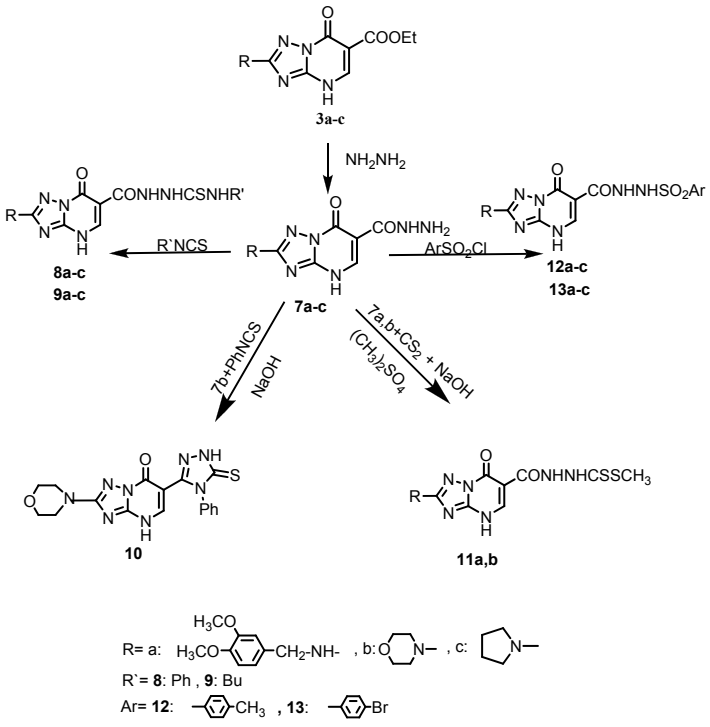
13a: ^{1}H-NMR (DMSO-d_6): 3.63 (br s, 1H, ArCH_2NH) , 3.72 (s, 6H, 2OCH_3), 4.33 (d, 2H, ArCH_2NH), 6.87 (m, 3H, Ar-H), 7.00 (s, 1H, NH-pyrimidine), 7.26 (br s, 1H, NH), 7.67-7.85 (m, 4H, Ar-H), 8.41 (s, 1H, CH-pyrimidine), 9.85 (s, 1H, NH).

Determination of *in vitro* antimicrobial activity

The primary screen was carried out using the agar disc-diffusion method according to the guidelines of the Clinical laboratory Standard Institute (CLSI 2008) [13], using Müller-Hinton agar medium. Sterile filter paper discs (8 mm diameter) were moistened with the compound solution in dimethylsulphoxide of specific concentration 200 µg/disc, the antibacterial antibiotic gentamicin, ampicillin and the antifungal drug Clotrimazole (100 µg /disc) were carefully placed on the agar cultures plates that had been previously inoculated separately with the microorganisms. The plates were incubated at 37 ^{o}C, and the diameter of the growth inhibition zones were measured after 24 hours in case of bacteria and at 25 ^{o}C for 48 hours in case of *Candida albicans*. The minimal inhibitory concentrations (MIC) for the compounds **7a, 7b** and **9a** against the same microorganisms used in the primary screening were carried out using the microdilution susceptibility method in Müller-Hinton Broth [14]. The selected compounds, gentamicin and ampicillin were dissolved in dimethylsulphoxide at concentration of 64 µg /ml. The two fold dilutions of the solution were prepared (64, 32, …, 0.5 µg /ml). The microorganism suspensions at 10^6 CFU/ml (colony forming unit/ml) concentrations were inoculated to the corresponding wells. The plates were incubated at 37 ^{o}C for 24 hours. The MIC values were determined as the lowest concentration that completely inhibited visible growth of the microorganism as detected by unaided eye.



Scheme 1



Scheme 2

Table 1. Characterization data of compounds **2a-c**, **3a-c**, **4a-c**, **5a-c**, and **6**.

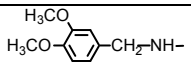
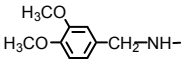
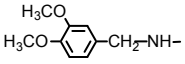
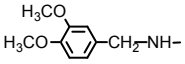
Compd. No.	R	Yield (%)	m. p. (ºC)	Mol. Form. (M. Wt.)		Analysis (%) Calcd.	Fd.
2a		88	243-245	$C_{11}H_{15}N_5O_2$ (249.27)	C H N	53.00 6.07 28.10	53.51 5.84 28.35
2b		68	163-165 as reported [12]	$C_6H_{11}N_5O$ (169.19)		-	
2c		94	215-217 as reported [12]	$C_6H_{11}N_5$ (153.19)		--	
3a		38	222-224	$C_{17}H_{19}N_5O_5$ (373.36)	C H N	54.69 5.13 18.76	54.73 5.60 18.77
3b		79	283-284 as reported [12]	$C_{12}H_{15}N_5O_4$ (293.28)		-	
3c		82	292-294 as reported [12]	$C_{12}H_{15}N_5O_3$ (277.28)		-	
4a		39	175-177	$C_{18}H_{21}N_5O_5$ (387.39)	C H N	55.81 5.46 18.08	55.75 5.51 18.90
4b		54	231-233 as reported [12]	$C_{13}H_{17}N_5O_4$ (307.31)		-	
4c		73	205-206 as reported [12]	$C_{13}H_{17}N_5O_3$ (291.31)		-	
5a		40	183-185	$C_{19}H_{23}N_5O_5$ (401.42)	C H N	56.85 5.87 17.45	56.90 5.90 17.62
5b		75	238-239 as reported [12]	$C_{14}H_{19}N_5O_4$ (321.33)		-	
5c		84	172-174 as reported [12]	$C_{14}H_{19}N_5O_3$ (305.33)		-	
6	-	31	210	$C_{17}H_{19}N_5O_5$ (373.36)	C H N	54.69 5.13 18.76	54.81 5.15 18.38

Table 2. Characterization data of compounds **7a-c**, **8a-c**, **9a-c**, **10**, **11a,b**, **12a-c**, and **13a-c**.

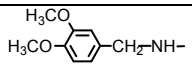
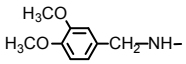
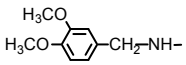
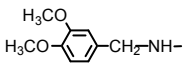
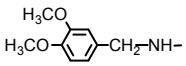
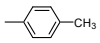
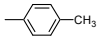
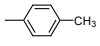
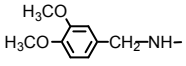
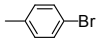
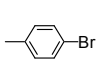
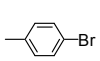
Compd. No.	R	R'	Ar	Yield (%)	m. p. (ºC)	Mol. Form. (M. Wt.)	Analysis (%)		
								Calcd.	Fd.
7a		-	-	92	231-233	$C_{15}H_{19}N_7O_4$ (359.34)	C H N	50.14 4.77 27.29	50.24 4.88 27.70
7b		-	-	75	290-291	$C_{10}H_{13}N_7O_3$ (279.26)	C H N	43.01 4.69 35.11	43.40 5.04 35.42
7c		-	-	81	> 300	$C_{10}H_{13}N_7O_2$ (263.26)	C H N	45.62 4.98 37.24	45.97 4.63 37.05
8a		Ph	-	99	193-195	$C_{22}H_{22}N_8O_4S$ (494.53)	C H N	53.43 4.48 22.66	53.93 4.40 22.73
8b		Ph	-	99	205-207	$C_{17}H_{18}N_8O_3S$ (414.44)	C H N	49.27 4.38 27.04	49.65 4.11 26.86
8c		Ph	-	88	>300	$C_{17}H_{18}N_8O_2S$ (398.44)	C H N	51.24 4.55 28.12	51.72 4.69 27.97
9a		Bu	-	90	decomp.	$C_{20}H_{26}N_8O_4S$ (474.54)	C H N	50.62 5.52 23.61	51.11 5.60 23.29
9b		Bu	-	41	199-201	$C_{15}H_{22}N_8O_3S$ (394.45)	C H N	45.67 5.62 28.41	46.21 5.66 28.03
9c		Bu	-	53	>300	$C_{15}H_{22}N_8O_2S$ (378.45)	C H N	47.60 5.86 29.61	47.22 5.66 30.39
10	-	-	-	23	190	$C_{17}H_{16}N_8O_2S$ (396.43)	C H N	51.51 4.07 28.27	51.21 4.15 29.09
11a		-	-	12	218-220	$C_{17}H_{19}N_7O_4S_2$ (449.51)	C H N S	45.42 4.26 21.81 14.27	45.83 4.15 21.62 13.80
11b		-	-	56	Charring	$C_{12}H_{15}N_7O_3S_2$ (369.42)	C H N S	39.01 4.09 26.54 17.36	38.65 4.39 26.89 17.57
12a		-		68	200	$C_{22}H_{23}N_7O_6S$ (513.53)	C H N	51.45 4.51 19.09	52.02 4.77 18.72
12b		-		49	Charring	$C_{17}H_{19}N_7O_5S$ (433.44)	C H N	47.11 4.42 22.62	47.70 4.34 22.40
12c		-		18	>300	$C_{17}H_{19}N_7O_4S$ (417.44)	C H N	48.91 4.59 23.49	48.51 4.97 24.06
13a		-		64	248	$C_{21}H_{20}BrN_7O_6S$ (578.40)	C H N	43.61 3.49 16.95	43.77 4.05 16.55
13b		-		33	>300	$C_{16}H_{16}BrN_7O_5S$ (498.31)	C H N	38.56 3.24 19.68	39.09 3.29 20.10
13c		-		17	>300	$C_{16}H_{16}BrN_7O_4S$ (482.31)	C H N	39.84 3.34 20.33	40.21 3.63 20.52

Table 3. Antimicrobial activity of tested compounds against *Staphylococcus aureus* IFO 3060, *Bacillus subtilis* IFO 3007, *Escherichia coli* IFO 3301, *Pseudomonas aeuroginosa* IFO 3448, and *Candida albicans* IFO 0583**.**

Compd. No.	Diameter of Inhibition Zone (mm)				
	S. aureus	*B. subtilis*	*E. coli*	*P. aeuroginosa*	*C albicans*
3a	14	**20**	14	10	12
3b	11	**16**	12	10	8
4a	10	**22**	15	12	10
4b	10	**16**	12	10	10
5a	11	**18**	**16**	12	8
5b	12	**18**	12	10	14
6	10	12	**16**	12	8
7a	**24**	**26**	**19**	14	10
7b	**18**	**18**	11	12	10
7c	14	**16**	**20**	**16**	10
8a	**16**	12	**18**	12	10
8b	10	**18**	12	10	8
8c	**20**	**18**	14	8	10
9a	**15**	**20**	13	10	11
9c	**18**	12	12	8	12
12a	10	14	14	10	12
12b	11	11	12	10	14
12c	12	**18**	12	8	10
13a	14	**16**	**16**	12	8
13b	12	12	**18**	12	10
13c	11	12	10	8	11
Ampicillin	**26**	**30**	**17**	**16**	**NT**
Gentamicin	**21**	**25**	**26**	**25**	**NT**
Clotrimazole	**NT**	**NT**	**NT**	**NT**	**21**

NT: Not Tested

Not active (8 mm), Weak activity (8-12 mm), Moderate activity (12-15 mm), Strong activity (> 15 mm). Solvent: DMSO (8 mm).

Table 4. The Minimal Inhibitory Concentrations (MIC, µg/ml) of the most active compounds, the broad spectrum antibacterial drugs Ampicillin or Gentamicin against *Staphylococcus aureus* IFO 3060, *Bacillus subtilis* IFO 3007, *Escherichia coli* IFO 3301, and *Pseudomonas aeuroginosa* IFO 3448**.**

Compd. No.	Minimal Inhibitory Concentration (MIC, µg/ml)			
	S. aureus	*B. subtilis*	*E. coli*	*P. aeuroginosa*
3a	NT	**4.0**	NT	NT
4a	NT	**3.0**	NT	NT
5a	NT	8.0	NT	NT
7a	**2.0**	**2.0**	**2.0**	NT
7b	NT	4.0	NT	NT
7c	NT	NT	**2.0**	**2.0**
8a	NT	NT	4.0	NT
8c	**2.0**	4.0	NT	NT
9a	4.0	**2.0**	NT	NT
Ampicillin	2.0	0.5	2.0	2.0
Gentamicin	2.0	2.0	0.5	1.0

NT: Not Tested

Table 5. The activity of compounds **7a, 7c**, and **8c**, in the methyl green/DNA displacement assay.

DNA-active compounds	IC_{50}, µg/ml*
7a	61±2
7c	40±3
8c	22±1

*Values represent the concentration (mean ± SD, n=3 to 5 separate determinations) required for a 50 % decrease in the initial absorbance of the DNA- methyl green solution.

Table 6. Reverse docking data of compounds **7a**, **7c**, and **8c**.

PDB-ID	Target name	Energy score (kcal/mol)		
		Compd. No.**7a**	Compd. No. **7c**	Compd. No. **8c**
1APT	Penicillopepsin	-44.88	-37.43	-
1PPM	Penicillopepsin	-44.61	-	-45.75
1PPK	Penicillopepsin	-41.15	-	-41.82
1QIQ	Isopenicillin N Synthetase	-41.97	-	-
1UIM	3-dehydroquinate Synthase	-41.33	-	-
1EI1	DNA Gyrase B Subunit	-	-38.74	-
1PVG	DNA Topoisomerase II	-	-36.53	-
1IFX	NH(3)-dependent NAD(+) Synthetase	-	-	-41.68

DNA-binding assay

TLC plates (RP-18 F_{254} ; 0.25mm; Merck) were predeveloped with MeOH-H_2O (8:2). Test compounds were then applied (5 mg/ml in MeOH) at the origin, followed by the addition of DNA (1 mg/ml in H_2O and MeOH mixture) at the same position at the origin. The plates were then developed with the same solvent system and the position of the DNA was determined by spraying with anisaldehyde reagent. The reagent yields a blue colour reaction with DNA, and the intensity of the colour was proportional to the quantity of DNA added to the plate. Ethidium bromide was used as positive control.

Colourimetric assay for compounds that bind to DNA

DNA/methyl green (20 mg, Sigma, St. Louis, MO, USA) was suspended in 100 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 7.5 mM $MgSO_4$ and stirred at 37°C with a magnetic stirrer for 24 h. Unless otherwise indicated, samples to be tested were dissolved in EtOH in Eppendorff tubes. The solvent was removed under vacuum, and 200 µl of the DNA/methyl green solution was added to each tube. The absorption maximum for the DNA/methyl green complex is 642.5-645 nm. Samples were incubated in the dark at ambient temperature. After 24 h, the final absorbance of samples was determined. Readings were corrected for initial absorbance and normalized as a % of the untreated DNA/methyl green absorbance value. IC_{50} 's were determined for each compound as shown in **Table 5**.

Molecular docking procedure using Tarfisdock

The newly selected compounds were sketched and energy minimized by Schrodinger software and then submitted to the website http://www.dddc.ac.cn/tarfisdock [24].

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