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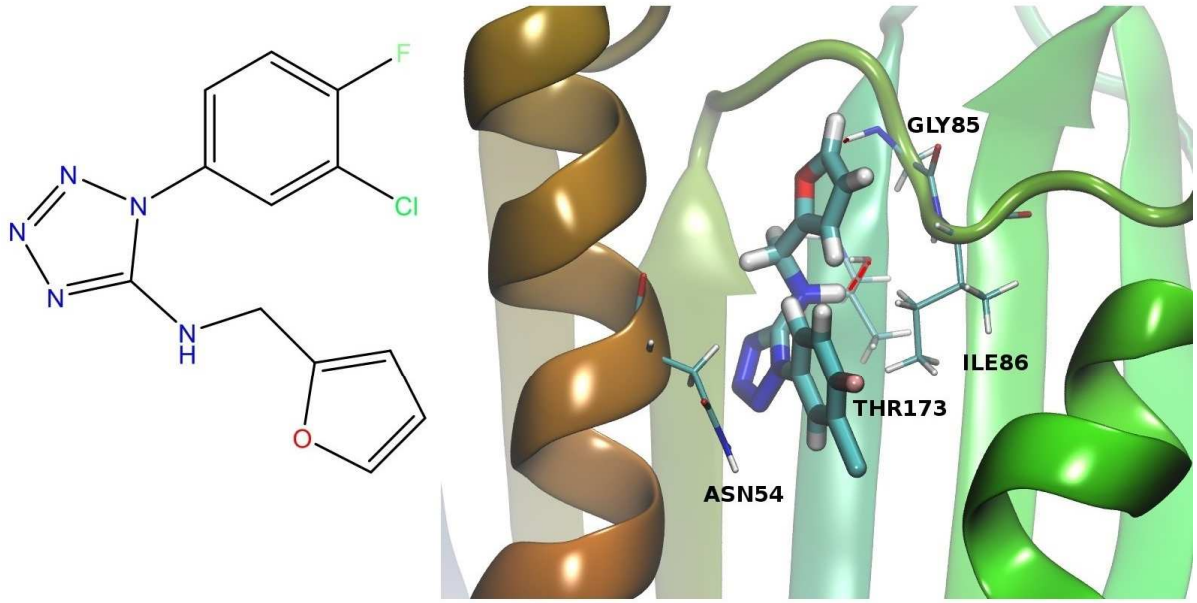

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Design and synthesis of novel 1*H*-Tetrazol-5-amine based potent antimicrobial agents: DNA topoisomerase IV and gyrase affinity evaluation supported by molecular docking studies.

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Keywords: 1H-Tetrazol-5-amine, Antimicrobial activity, Topoisomerase type IV, DNA gyrase, Cytotoxicity, Molecular docking

Abstract: A total of 14 of 1,5-disubstituted tetrazole derivatives were prepared by reacting appropriate thiourea and sodium azide in the presence of mercury (II) chloride and

triethylamine. All compounds were evaluated *in vitro* for their antimicrobial activity. Derivatives **10** and **11** showed the highest inhibition against Gram-positive and Gram-negative strains (standard and hospital strains). The observed minimal inhibitory concentrations values were in the range of 1 - 208 μM (0.25 − 64 μg/ml). Inhibitory activity of 1,5-tetrazole derivatives **10** and **11** against gyrase and topoisomerase IV isolated from *S. aureus* was studied. Evaluation was supported by molecular docking studies for all synthesized derivatives and reference ciprofloxacin. Moreover, selected tetrazoles (**2**, **3**, **5**, **6**, **8**, **9**, **10** and **11**) were evaluated for their cytotoxicity. All tested compounds are non-cytotoxic against *HaCaT* and A549 cells ($CC_{50} \leq 60$ μM).

1. Introduction

New classes of antimicrobials are needed due to the fact of increasing resistance of bacteria [1 – 3]. Different synthetic antimicrobial agents have been discovered and are being used in the clinical treatment in various communities, environments and hospital-acquired microbial infections [4 – 5]. In some cases presented results showed higher antibacterial activities against tested strains in comparison to reference drugs such as Fluconazole, Chlormycin or Norfloxacin [6 – 7].

After more than two decades of intensive studies it became clear that tetrazoles are one of the most promising class of compounds with medicinal inclination. The first tetrazole derivatives were synthesized in 1885, however till 1950 that type of compounds was not eye-catching for scientific community [8]. Wide range of synthetic techniques was developed in recent years and large number of reports presenting new facts related to chemistry of tetrazole scaffold and their applications was published [9 – 10]. Consequently, tetrazoles started to be more attractive for further studies due to interesting physicochemical properties and possibility of attachment of wide range of functional substituents to core arrangement. Great number of studies were concentrated to find new biologically active compounds possessing tetrazole as core moiety [11 – 17]. A few of highly effective drugs which active pharmaceutical ingredients contain the tetrazole ring are reported. Examples are Losartan, Valsartan, Irbesartan, Flomoxef and Cefonicid [18 – 22]. First three medications are used mainly to treat high blood pressure (hypertension), next two are semi synthetic cephalosporin antibiotics. Tetrazole ring is a common motif in all of mentioned medicines but more as a modification of structures core part. In position 1 and/or 5 of tetrazole ring are attached arrangements which are increasing solubility of compound (e.g. -sulfomethyl in Cefonicid). On the other hand this

positions can be occupied by phenyl, benzoil rings substituted by $–NO_2$, -Cl, -Br, -F to improve antimicrobial properties. Currently we can observe urgent call for development of new agents against Gram-positive, Gram-negative bacteria and yeasts. Since microbes are becoming multi-drug resistant it is very challenging to obtain new effective antibacterial agents.

It was found that incorporation of the tetrazole ring into a molecule of organic substrate relatively often leads, not only to an increase in the efficacy, but also to an enhance of the prolongation of drug action. As a rule, this is not accompanied by an increase in acute toxicity [23]. Nevertheless, the characteristic of these new molecules should show a low toxicity and known mechanism of action. According to the limited literature data, some tetrazole derivatives possess antimicrobial properties [24 – 26].

It could be stated that tetrazole based small molecules were not explored properly, especially as antimicrobial agents. It should be pointed that most of presented structures are complex and tetrazole is not dominating scaffold. Our studies are focused on small tetrazole based molecules and our major goal is to evaluate their potential in a struggle against multi-resistant bacterial strains. For the first time structural diversity of 1,5-disubstituted tetrazole derivatives have been presented and antimicrobial activity against Gram-positive and Gram-negative bacteria have been summarized followed by topoisomerase IV inhibition assay and suitable molecular docking studies.

2. Result and discussion

2.1 Chemistry

Methods for synthesis of 1,5-disubstituted tetrazoles (5-substituted amino) are divided into four main groups [27, 10]: (i) amino group or ring functionalization of 5-aminotetrazole, (ii) the nucleophilic substitution of a leaving group in the 5-position of tetrazole with amines, (iii) reactions of aminoguanidine derivatives with sodium nitrite, and (iv) various azide-mediated tetrazole ring constructions including addition of azide to carbodiimides, cyanamides, and nucleophilic substitution by azide ion on chloroformamidines, aminoiminomethanesulfonic acid, and di- and trisubstituted carboximidamides. Our approach falls under the category azide-mediated tetrazole ring construction. In our work the synthesis of 1,5-disubstituted tetrazoles generated by oxidative desulfurization of 1,3-disubstituted thioureas, by external nucleofile such as sodium azide lead to corresponding 5-

aminotetrazoles, respectively. Mercury (II) chloride was used for desulfurization. The reaction was carried out at room temperature in DMF and in the presence of triethylamine (Table 1).

[Table 1]

Table 1 Reaction scheme and structures of obtained 1,5-disubstituted tetrazoles **1** – **14**.

Thioureas obtained in reaction of corresponding amine (R_1: 2-amino-1,3-thiazole, 3-amino-1*H*-1,2,4-triazole, 4-amino-4*H*-1,2,4-triazole, furan-2-ylmethanamine, 2-(1*H*-indol-3-yl)ethanamine) and suitable isothicyanates (R_2) were used as staring material [28 – 31]. The structural diversity of compounds was generated by choosing various aryl (derivatives **1** – **6**, **8** – **11** and **14**) and alkyl (compounds **7**, **12**, **13**) isothiocyanates. All compounds were obtained in good or very good yields (range 48 – 86 %).

Structures of compounds were determined using different spectroscopic methods (^{1}H NMR, ^{13}C NMR and MS). Spectral data (NMR, MS) of all compounds were in full agreement with their presented structures.

The structure of **6** was determined by X-ray crystallography (Fig. 1).

[Figure 1]

Fig. 1. Crystal structure of compound (**6**) showing displacement ellipsoids at the 50% probability level.

1-(4-nitrophenyl)-N-(4*H*-1,2,4-triazol-4-yl)-1*H*-tetrazol-5-amine (**6**) crystallizes in the $P2_1$ space group (Fig. 1, Table 2), the asymmetric unit contains one calcium cation, which coordinates six water molecules and organic anion. Additionally, there is second anion present and two “free” water molecules.

[Table 2]

Table 2. Crystal data and structure refinement for 1-(4-nitrophenyl)-N-(4*H*-1,2,4-triazol-4-yl)-1*H*-tetrazol-5-amine (**6**).

2.2 Biological studies

2.2.1 Antimicrobial study

All obtained compounds were tested *in vitro* against a number of bacteria, including Gram-positive cocci and Gram-negative rods. Microorganisms used in this study have common applications in the antimicrobial tests for many substances like antibiotics, antiseptic drugs and in the search for new antimicrobial agents [31]. All tested compounds were

screened for their minimal inhibitory concentrations (MIC) [32]. The results revealed that 13 out of 14 investigated compounds exhibited high and broad antibacterial activity, especially against standard *Staphylococcus*, *Bacillus*, *Enterococcus*, *Micrococcus* strains (Table 3).

[Table 3]

Table 3. Activity of compounds against standard bacteria strains expressed by minimal inhibitory concentrations (μM and μg/ml).

Within this group, the observed MIC values were in the range 1 - 208 μM (0.25 − 64 μg/ml). Two compounds **10** and **11** were active against all tested Gram-positive and Gram-negative strains in the range 1 - 208 μM (0.25 − 64 μg/ml).

Derivative **11** was more potent to Ciprofloxacin (Cip*) against standard *E. faecalis, M. luteus, E. coli, P. vulgaris* 1 - 7 μM (0.25 − 64 μg/ml).

Next, the activity of three selected compounds (**7**, **10** and **11**) against hospital strains of *S. aureus, S. epidermidis*, *P. aeruginosa* and *E. coli* was assigned (Table 4).

For this three compounds the activity against Gram-positive strains was similar and was in the range 7 - 56 μM (2 − 16 μg/ml). Only compounds **10** and **11** were active against Gram-negative rods in range 7 - 111 μM (2 − 32 μg/ml).

[Table 4]

Table 4. Activity of compounds against clinical Gram-positive and Gram-negative bacteria strains expressed by minimal inhibitory concentrations (μM and μg/ml).

It is worth to comment that from three compounds selected for evaluation of activity against hospital strains only derivative **7** showed slightly decreased activity in comparison to suitable standard strains. In general, level of results against standard strains is very often unreachable against hospital strains. Therefore, it need to be emphasized that compounds **10** and **11** level of activity remained practically unchanged. Minimal inhibitory concentrations values were in the range 1 - 208 μM (0.25 − 64 μg/ml) for standard strains for both derivatives and 7 - 111 μM (2 − 32 μg/ml) for compound **10**, 7 - 54 μM (2 − 16 μg/ml) for compound **11** in case of hospital strains. Both derivatives showed better results against five from eight used hospital strains of *S. aureus* and five from eight used hospital strains of *S. epidermidis* than reference ciprofloxacin. In case of one from eight used Gram-negative hospital strains of *E. coli* ML 16 both compounds were more active than reference material.

The main idea of designed synthesis and microbiological evaluation was to authenticate that tetrazoles will be more effective antimicrobials than corresponding thioureas. It was recognized in recent studies that introduction of variable constituents to phenyl ring will result induction of antimicrobial activity. It is well known that N-arylthioureas are showing better antibacterial properties than N-alkylthioureas. Our previous studies showed that there is a tendency for functionalities in N-arylthioureas and they could be arranged in following order of their decreasing influence as follows: 3-chloro-4-fluorophenyl > 3-bromophenyl > 3,4-dichlorophenyl > 3-fluorophenyl > phenylethyl > benzyl > 4-chlorophenyl. Furthermore, substituent groups on different positions of the phenyl ring resulted in various degrees of effect. In most cases derivatives possessing weakly deactivating halogen substituents at *meta*- and/or *para*- position of the benzene ring were found as the most active. For most of Gram-positive bacteria, disubstituted derivatives were more active than monosubstituted halogen compounds, because of stronger electronegativity effect produced. That phenomenon was also noticed for 3-bromo- and 3-fluorophenyl derivatives. The presence of halogen atoms at *ortho*-position, as well as the introducing of electron-donating substituents on aromatic ring has reduced antibacterial activity [31].

Prompted by recent results and our experience with this class of compounds specific N-arylthioureas were selected as starting materials to evaluate if the same tendency will remain when replacing thiourea moiety with tetrazole ring.

The activity of obtained 1,5-disubstituted tetrazoles was compared to corresponding thioureas which were used to synthesis of cyclic tetrazoles. Only the thiourea which was used to synthesis of compound **2** possesses similar activity to the cyclic tetrazole derivative but only against Gram-positive strains. This compound was therefore inactive against Gram-negative rods. The other 1,5-disubstituted tetrazoles were more active against Gram-positive and Gram-negative strains of bacteria in comparison to used thioureas. Only compound **12** and its initial thiourea were inactive. So, the reaction of cyclisation of thioureas to 1,5-disubstituted tetrazoles was associated with the increase of antimicrobial activity.

There is a clear correlation between the type of substituents of tetrazole ring and the antimicrobial activity of tested compounds. Non-polar substituent in position 1 in tetrazole ring eliminates antimicrobial activity (compound **12**), but the introduction of benzene ring with electron-donating substituents in the same position increases antimicrobial activity. Comparison of halogen substituent connected to benzene ring shows that chloride substituent has the strongest influence on microbiological activity. The introduction of furan-2-

ylmethanamine substituent in position 5 in tetrazole ring escalates the antimicrobial activity against Gram-negative rods (compounds **10** and **11**).

2.2.2 Molecular docking studies

We used docking procedure to investigate binding modes of 14 different compounds and ciprofloxacin to DNA gyrase and topoisomerase IV. All investigates compounds preferred bounding at the ATP binding sites of gyrase and topoisomerase molecules. Estimated binding free energies for resulting conformers ranged from -3,25 to -7,02 kcal/mol (Table 5). Compounds **7** and **10** created hydrogen bonds with catalytic Asp81 of DNA gyrase (Fig. 2). This key interaction with Asp residue was also observed in our recent work in complexes of thiourea derivative [33] and was also reported by other group for a set of azaindole ureas analogs [34]. Ciprofloxacin and compound **11** showed different interacting pattern at ATP gyrase binding site. None of these molecules interacted with Asp81 residue, but they created two hydrogen bonds with the protein: Ciprofloxacin with Ile51, Arg144 residues and compound **11** with Gly85, Thr173 residues, respectively (Fig. 2).

[Table 5]

LC = number of members of the largest cluster calculated for 1000 docking runs using RMSD cutoff tolerance = 3 Å
BE = estimated free energy of binding by AutoDock4 energy function

Table 5. DNA gyrase and topoisomerase IV binding data based on docking results for compounds **1**-**14** and ciprofloxacin.

[Figure 2]

Fig. 2. Binding modes of compounds a) **7**, b) **10** c) **11** and d) ciprofloxacin to DNA gyrase. Left panel presents protein-ligand interaction scheme generated using PoseView server [35]. Black dashed lines indicate hydrogen bonds. Green solid lines show hydrophobic interactions. Right panel shows ligand position inside the binding site of the protein. Red dotted lines indicate hydrogen bonds. Figures were generated using VMD program [36].

2.2.3 Topoisomerase IV inhibition assay

Topoisomerase IV is a bacterial type II topoisomerase that is essential for proper chromosome segregation. It is the primary target of second-generation fluoroquinolones, such as Ciprofloxacin and Levofloxacin [37], that stimulate topoisomerase IV-mediated DNA cleavage both by increasing rates of DNA scission and by inhibiting relegation of cleaved DNA. As a result, quinolones inhibit the overall catalytic activity of topoisomerase IV

primarily by interfering with enzyme-ATP interactions [38]. Another type of bacterial type II topoisomerases is DNA gyrase. In general, it is supposed that in Gram-positive bacteria species, topoisomerase IV rather than DNA gyrase appears to be the primary target of most quinolone-based antibiotics. In this work the influence of 1,5-disubstituted tetrazole derivatives was tested for both topoisomerase IV and DNA gyrase.

To compare the inhibition of gyrase supercoiling caused by **11** and **10** compounds were titrated into supercoiling reactions.

Compound **11** was found to be an inhibitor of gyrase supercoiling with potencies higher to that of ciprofloxacin, with IC_{50} of 0.9 ± 0.1 for **11**, compared with IC_{50} of 3.5 ± 0.3 for ciprofloxacin (Table 6). This IC_{50} indicates the relative affinities of **11** for gyrase. A second compound, **10** was also tested but showed weaker inhibitory activity towards gyrase (Table 6). To determine whether the actions of tested compounds are gyrase specific, the effect of **10** and **11** on topoisomerase IV was tested. We have found that **11** inhibited topoisomerase IV decatenation more strongly than **10** did, with an IC_{50} of 2.6 ± 0.25 μg/ml vs. 11.9 ± 1.3 μg/ml for **10**, suggesting these compounds are active against topoisomerase IV (Table 6).

[Table 6]

*Concentration (μg/ml) of tested compound required to inhibit 50% of enzyme.

Table 6. Affinity of selected compounds towards bacterial type II topoisomerases, expressed as IC_{50} ± SEM (μg/ml).

Presented preliminary results showed that 1,5-disubstituted tetrazole derivatives were able to inhibit the activity of bacterial gyrase and topoisomerases IV from *S. aureus*. It can be stated that for studied compounds **10, 11** there is a clear correlation of minimal inhibitory concentration results and affinity towards bacterial type II topoisomerases. Compound **11** was approximately two times more active against tested strains compared to derivative **10**, which is reflected in stronger inhibition of topoisomerase IV decatenation and gyrase supercoiling.

Gyrase inhibitors, especially fluoroquinolones, are of key importance in antibacterial therapy. Our study has identified compound **11** as highly potent gyrase inhibitor which may serve as lead compound for drug development.

2.2.4 Cytotoxic activity in HaCaT and A549 cells

Cytotoxic effect of the selected derivatives **2**, **3**, **5**, **6**, **8**, **9**, **10** and **11** was measured in cell viability assessment in human immortal keratinocyte cell line from adult human skin (HaCaT) and human epithelial lung carcinoma cell line (A549).

In the present study, these compounds slightly affected the viability of the cells and only when used in high concentrations. Our results suggest, that all new synthesized 1,5-disubstituted tetrazoles were less toxic in human immortal keratinocyte cell line from adult human skin, and more toxic on human epithelial lung carcinoma cell line (Table 7). The relationship between cytotoxicity and antimicrobial activity was also established through the selectivity index (SI) and shown in table (Table 7). The highest SI value was observed for compound **11** (*M. luteus* and *P. vulgaris* – 84), subsequently for compound **10** with results in range 2.85 – 11.42. This information is promising for possible future applications of obtained 1,5-disubstiuted tetrazoles as e.g. the antimicrobial agents.

[Table 7]

The IC_{50} value is defined as the concentration of a compound that corresponds to a 50% growth inhibition. Data are expressed as mean ± SD. [a]Human immortal keratinocyte cell line from adult human skin (HaCaT). [b] Human epithelial lung carcinoma cell line (A549). [c] Value calculated using formula: SI = IC_{50} for normal cell line HaCaT / MIC. Cisplatin and Doxorubicin – positive control. For positive control calculation is not presented (-) since compounds were not evaluated for antimicrobial activity.

Table 7. Cytotoxicity results of selected tetrazoles.

3. Conclusions

In this paper, a new fourteen 1*H*-tetrazol-5-amine based compounds have been designed, synthesized and evaluated for antimicrobial activity. Results showed that all except one of investigated compounds exhibited high and broad antibacterial activity, especially against standard *Staphylococcus, Bacillus, Enterococcus, Micrococcus* strains, presenting minimal inhibitory concentration values in the range 1 - 208 μM (0.25 − 64 μg/ml). Suitable thioureas used as starting material for synthesis were less active [28 – 31]. Tetrazole derivatives **7**, **10** and **11** were found as most potent antimicrobial agents. The activity of those compounds against clinical Gram-positive strains was similar and was in the range 7 - 56 μM (2 − 16 μg/ml), only **10** and **11** were active against clinical Gram-negative rods in range 7 - 111 μM (2 − 32 μg/ml). It is worth to point out that derivative **11** was more potent to Ciprofloxacin against standard strains *E. faecalis, M. luteus, E. coli, P. vulgaris* 1 - 7 μM (0.25 − 64 μg/ml). All synthesized compounds were conducted to molecular docking experiment, however derivative **10** and **7** were selected for more intensive evaluation. It was found that compounds **7** and **10** created hydrogen bonds with catalytic Asp81 of DNA gyrase (Fig. 2). This key interaction with Asp residue was also observed in our team recent work in complexes of thiourea derivative [33] and was also reported by other group for a set of azaindole ureas

analogs [34]. Ciprofloxacin and compound **11** showed different interacting pattern at ATP gyrase binding site. None of these molecules interacted with Asp81 residue, but they created two hydrogen bonds with the protein: Ciprofloxacin with Ile51, Arg144 residues and compound **11** with Gly85, Thr173 residues, respectively (Fig. 2). Results of docking were in line with affinity of selected compounds towards bacterial type II topoisomerases. The most potent **10** and **11** were able to inhibit the activity of bacterial gyrase and topoisomerases IV from *S. aureus*. These two were in the group of compounds for which cytotoxic activity in HaCaT and A549 cells was measured. It was observed that studied tetrazoles were less toxic in human immortal keratinocyte cell line from adult human skin, and more toxic on human epithelial lung carcinoma cell line. The highest selectivity index value was observed for compound **11** (*M. luteus* and *P. vulgaris* – 84), subsequently for compound **10** with SI results in range 2.85 – 11.42.

Our studies revealed two most promising compounds after results from antimicrobial, molecular docking and cytotoxicity studies. We have focused on these two "lead" compounds and in our opinion there was no true need to conduct sophisticated and time consuming studies for rest of compounds at this moment (e.g. correlation between antimicrobial and topoisomerase IV and DNA Gyrase inhibitory activities). Nevertheless, we have decided to continue our research for separate group of tetrazoles with furan-2-ylmethyl moiety since we are aware of necessity of complex structure-activity relationship studies for large group of compounds possessing most active structural motif.

Gathering all presented results it can be stated that 1,5-disubstitued tetrazoles should be considered as promising antimicrobial agents. Our studies indicated that compound **11** possess significant antimicrobial activity and may serve as a lead compound in search for further drug discovery.

We will continue our research for new antimicrobial agents in group of tetrazole compounds possessing furan-2-ylmethyl moiety.

4. Experimental

4.1 Chemistry

4.1.1 General procedure

Procedure of purchasing reagents, solvents, recording of spectra and other chemistry related methodology was already presented in previous papers [35, 28-31, 33].

Triethylamine (2-3 drops) was added to a suspension of suitable thiourea derivative (1 mmol), sodium azide (3.75 mmol) and mercuric chloride (1.25 mmol) in 5 ml of dry DMF. The resulting mixture was stirred for maximum 6 h at room temperature or until TLC showed end of reaction. The suspension was filtered through paper filter, washing with $CHCl_3$. The filtrate was diluted with water, extracted three times with 15 ml of $CHCl_3$, the combined organic fractions were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (chloroform : methanol; 9.5 : 0.5).

*4.1.1.1 N-(1-(3,4-dichlorophenyl)-1H-tetrazol-5-yl)thiazol-2-amine (**1**).*

Mp. 196 - 197°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 12.70 (s, 1H, NH), 7.93 – 7.90 (m, 1H), 7.75 – 7.72 (m, 1H), 7.64 – 7.59 (t, *J* = 9.0 Hz, 1H), 7.25 – 7.24 (d, *J* = 3.0 Hz, 1H), 6.90 – 6.89 (d, *J* = 3.0 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ (ppm): 168.37, 158.33, 131.67, 131.61, 125.20, 124.84, 123.83, 120.11, 117.54, 108.56. HRMS (ESI) calcd for $C_{10}H_5Cl_2N_6S$ $[M - H]^-$: 310.9673; found 310.9680.

*4.1.1.2 N-(1-(3-chloro-4-fluorophenyl)-1H-tetrazol-5-yl)thiazol-2-amine (**2**).*

Mp. 234°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 12.74 (s, 1H, NH), 8.24 – 8.21 (m, 1H), 7.97 – 7.91 (m, 1H), 7.68 – 7.62 (t, *J* = 9.0 Hz, 1H), 7.33 – 7.31 (d, *J* = 6.0 Hz, 1H), 6.94 – 6.92 (d, *J* = 6.0 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ (ppm): 168.37, 156.08, 155.04, 131.65, 125.13, 124.84, 123.93, 120.36, 117.84, 108.53. HRMS (ESI) calcd for $C_{10}H_5N_6FClS$ $[M - H]^-$: 294.9977; found 294.9969.

*4.1.1.3 N-(1-(4-(trifluoromethyl)phenyl)-1H-tetrazol-5-yl)thiazol-2-amine (**3**).*

Mp. 205°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 12.70 (s, 1H, NH), 8.02 – 8.01 (d, *J* = 3.0 Hz, 1H), 7.81 – 7.77 (m, 1H), 7.57 – 7.55 (m, 1H), 7.32 – 7.30 (d, *J* = 6.0 Hz, 1H), 6.93 – 6.91 (d, *J* = 6.0 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ (ppm): 168.21, 156.03, 155.00, 131.49, 125.05, 124.72, 123.70, 119.97, 117.94, 117.38, 108.40. HRMS (ESI) calcd for $C_{11}H_6F_3N_6$ $[M - H]^-$: 310.4703; found 310.4700.

*4.1.1.4 1-(4-chloro-3-nitrophenyl)-N-(1H-1,2,4-triazol-3-yl)-1H-tetrazol-5-amine (**4**).*

Mp. 246 - 247°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.96 (s, 1H), 8.47 – 8.46 (d, *J* = 3.0 Hz, 1H), 8.03 – 7.99 (m, 1H), 7.80 – 7.77 (d, *J* = 9.0 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ (ppm): 161.65, 143.53, 142.01, 141.45, 124.84 (3C), 118.78 (2C). HRMS (ESI) calcd for $C_9H_5N_9ClO_2$ $[M - H]^-$: 306.0255; found 306.0246.

*4.1.1.5 1-(4-bromophenyl)-N-(4H-1,2,4-triazol-4-yl)-1H-tetrazol-5-amine (**5**).*

Mp. decomposition 201°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.79 (s, 2H), 7.88 – 7.84 (m, 2H), 7.77 – 7.74 (m, 2H). ^{13}C NMR (DMSO-d_6) δ (ppm): 143.65, 142.29, 141.60, 125.02 (3C), 118.91 (3C). HRMS (ESI) calcd for $C_9H_6BrN_8$ [M - H]$^-$: 305.9329; found 305.9332.

*4.1.1.6 1-(4-nitrophenyl)-N-(4H-1,2,4-triazol-4-yl)-1H-tetrazol-5-amine (**6**).*

Mp. decomposition 209°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.51 (s, 2H), 8.47 – 8.42 (m, 2H), 8.39 – 8.35 (m, 2H). ^{13}C NMR (DMSO-d_6) δ (ppm): 143.69, 142.21, 141.66, 125.10 (3C), 119.03 (3C). HRMS (ESI) calcd for $C_9H_6N_9O_2$ [M - H]$^-$: 272.0652; found 272.0644.

*4.1.1.7 5-((4H-1,2,4-triazol-4-yl)amino)-1H-tetrazole-1-carboxylic acid (**7**).*

Mp. decomposition 198°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.81 (s, 1H), 8.72 (s, 1H). ^{13}C NMR (DMSO-d_6) δ (ppm): 161.83, 143.86, 142.45, 141.88. HRMS (ESI) calcd for $C_4H_3N_8O_2$ [M - H]$^-$: 195.2519; found 195.2514.

*4.1.1.8 1-(3-chloro-4-fluorophenyl)-N-(4H-1,2,4-triazol-4-yl)-1H-tetrazol-5-amine (**8**).*

Mp. decomposition 211°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.44 – 8.39 (m, 3H), 8.11 – 8.05 (m, 1H), 7.56 – 7.50 (t, *J* = 9.0 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ (ppm): 160.13, 142.77, 132.68, 124.85, 122.49 (2C), 121.49, 120.18, 117.89. HRMS (ESI) calcd for $C_9H_5ClFN_8$ [M - H]$^-$: 279.0319; found 279.0310.

*4.1.1.9 1-(4-fluorophenyl)-N-(4H-1,2,4-triazol-4-yl)-1H-tetrazol-5-amine (**9**).*

Mp. decomposition 207°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.43 (s, 2H), 8.15 – 8.10 (m, 2H), 7.35 – 7.26 (m, 2H). ^{13}C NMR (DMSO-d_6) δ (ppm): 142.40 (3C), 121.51 (3C), 115.61 (3C). HRMS (ESI) calcd for $C_9H_6FN_8$ [M - H]$^-$: 245.0693; found 245.0699.

*4.1.1.10 1-(3-chloro-4-methylphenyl)-N-(furan-2-ylmethyl)-1H-tetrazol-5-amine (**10**).*

Mp. 196°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.96 (s, 1H, NH), 7.65 – 7.57 (m, 1H), 7.19 – 7.12 (m, 1H), 6.77 – 6.73 (t, *J* = 6.0 Hz, 1H), 6.39 – 6.38 (m, 1H), 6.26 – 6.24 (m, 1H), 4.28 – 4.26 (d, *J* = 6.0 Hz, 2H), 2.25 (s, 3H). ^{13}C NMR (DMSO-d_6) δ (ppm): 163.40, 156.82, 146.71, 144.03, 135.36, 129.90, 129.51, 128.02, 125.63, 108.58, 106.71, 73.10. HRMS (ESI) calcd for $C_{13}H_{11}ClN_5O$ [M - H]$^-$: 287.0133; found 287.0127.

*4.1.1.11 1-(3-chloro-4-fluorophenyl)-N-(furan-2-ylmethyl)-1H-tetrazol-5-amine (**11**).*

Mp. 205°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.25 – 8.11 (m, 2H), 8.00 – 7.99 (d, *J* = 3.0 Hz, 1H), 7.81 – 7.88 (m, 1H), 7.70 – 7.61 (m, 1H), 7.58 – 7.46 (m, 1H), 4.63 (s, 2H). ^{13}C NMR (DMSO-d_6) δ (ppm): 163.40, 157.15, 144.03, 135.81, 135.19, 129.51, 129.33, 128.02, 125.18, 108.58, 106.58, 72.96. HRMS (ESI) calcd for $C_{12}H_8Cl_2N_5O$ [M - H]$^+$: 293.6864; found 293.6871.

*4.1.1.12 N-(2-(1H-indol-3-yl)ethyl)-1-ethyl-1H-tetrazol-5-amine (**12**).*

Mp. 148°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 10.82 (s, NH), 7.56 – 6.97 (m, 5H), 4.13 – 4.06 (m, 2H), 3.30 – 3.25 (m, 2H), 3.23 – 2.98 (m, 2H), 1.13 – 1.08 (dt, *J* = 9.0 Hz, 3H). ^{13}C NMR (DMSO-d_6) δ (ppm): 155.59, 136.61, 127.44, 123.28, 121.41, 118.74, 112.08, 111.88, 110.38, 44.80, 38.71, 24.69, 14.42. HRMS (ESI) calcd for $C_{13}H_{16}N_6$ $[M + Na]^+$: 279.1324; found 279.1340.

*4.1.1.13 5-((2-(1H-indol-3-yl)ethyl)amino)-1H-tetrazole-1-carboxylic acid (**13**).*

Mp. 168 - 169°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 10.82 (s, 1H, NH), 7.57 – 7.54 (d, *J* = 9.0 Hz, 1H), 7.35 – 7.32 (d, *J* = 9.0 Hz, 1H), 7.17 – 6.95 (m, 4H), 3.49 – 3.42 (m, 2H), 2.98 – 2.93 (t, *J* = 6.0 Hz, 2H). ^{13}C NMR (DMSO-d_6) δ (ppm): 155.89, 136.95, 127.84, 123.78, 121.70, 118.63, 112.23, 111.67, 110.49. 45.68, 38.89, 15.21. HRMS (ESI) calcd for $C_{12}H_{12}N_6O_2$ $[M + H]^+$: 272.9306; found 272.9312.

*4.1.1.14 N-(2-(1H-indol-3-yl)ethyl)-1-(4-fluorophenyl)-1H-tetrazol-5-amine (**14**).*

Mp. 165°C. ^{1}H NMR (DMSO-d_6) δ (ppm): 10.79 (s, 1H, NH), 7.52 – 7.51 (d, *J* = 3.0 Hz, 2H), 7.32 – 7.29 (d, *J* = 9.0 Hz, 1H), 7.14 – 6.92 (m, 5H), 3.43 – 3.41 (m, 2H), 2.95 – 2.90 (t, *J* = 6.0 Hz, 2H). ^{13}C NMR (DMSO-d_6) δ (ppm): 155.99, 155.38, 136.45, 127.24, 123.11, 121.31, 119.05, 119.02, 119.00, 112.25, 112.22, 111.75, 110.54 (2C), 110.22, 38.51, 14.89. HRMS (ESI) calcd for $C_{17}H_{15}FN_6$ $[M + Na]^+$: 345.3291; found 345.3288.

4.2. Biological assays

4.2.1 - 2. In vitro evaluation of antimicrobial activity and Media, growth conditions and antimicrobial activity assays

Procedure, microorganisms and other conditions used in this studies were presented in previous papers [35, 28 - 31, 33].

4.2.3 - 4. Inhibition of bacterial S. aureus DNA Gyrase Supercoiling Assay and Inhibition of bacterial S. aureus Topoisomerase IV Decatenation Assay

Methodology of both assays was presented in previous paper [33].

4.2.5. Cytotoxic activity in HaCaT and A549 cells

4.2.5.1 - 2. Cell Culture: Conditions and Treatments and Cell Viability Assessment (Mitochondrial Function Assessment)

Description related to cell culture, suitable conditions and methodology of cell viability assessment was presented in previous paper [35].

4.3. Crystallography

The X-ray measurement of (**6**) was performed at 100 (2) K on a Bruker D8 VENTURE diffractometer with TRIUMPH monochromator and MoKα radiation (0.71073 Å). The crystals were positioned 40 mm from the CCD camera; 720 frames were measured at 0.5° intervals with a counting time of 30s. Data collection, cell refinement and data reduction were carried out with the Bruker SAINT software package [39]. The data were corrected for Lorentz and polarization effects, multi-scan method (SADABS) for absorption correction was applied. The structures were solved by direct methods [40] and refined using SHELXL Software Package [41]. The refinement was based on F^2 for all reflections except for those with very negative F^2. The weighted R factor, wR and all goodness-of-fit S values are based on F^2. The non-hydrogen atoms were refined anisotropically. The hydrogen atoms were located from a difference map and were refined isotropically. The atomic scattering factors were taken from the International Tables [42]. Selected crystal data are given in Table 2. CCDC 1529625 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html.

4.4. Molecular docking

We performed molecular docking of 14 compounds and ciprofloxacin to DNA gyrase and topoisomerase IV protein receptors. Structures of the ligands were constructed using Automated Topology Builder server (ATB Version 2.2) [43]. We have used the same protein models and the docking procedure reported in our recent studies [33]. Docking calculations and data analysis were conducted using AutoDock4 (v. 4.2) and AutoDockTools4 [44] respectively. For each receptor-ligand complex 1000 lowest energy conformers were obtained. Structural clustering (with RMSD 3 Å cutoff) was used to identify the most favorable ligand position. The central structure of the largest cluster was selected as final docked conformation (Table 5).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at …

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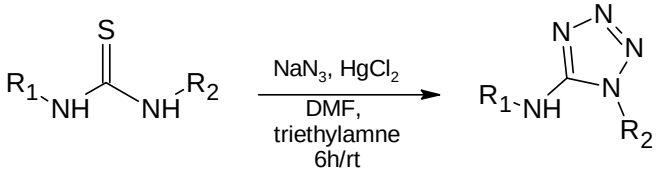

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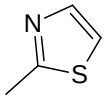
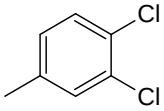
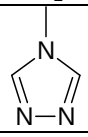
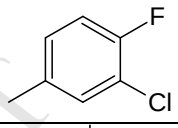
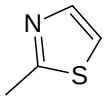
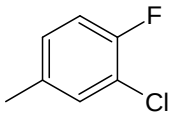
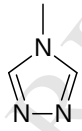
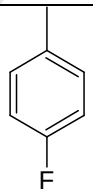
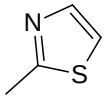
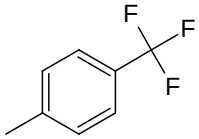
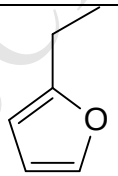
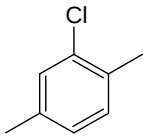
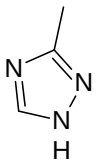
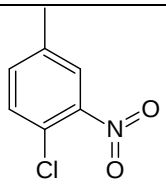
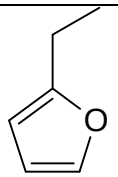
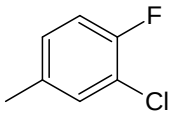
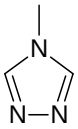
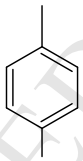
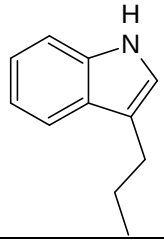
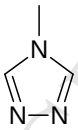
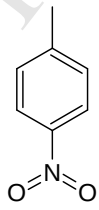
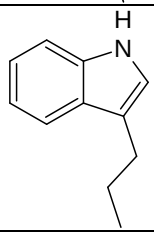
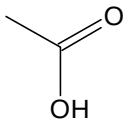
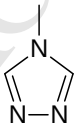
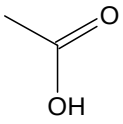
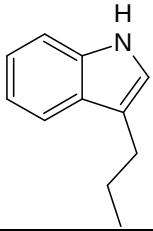
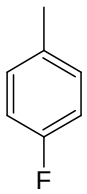
1			**8**		
2			**9**		
3			**10**		
4			**11**		
5			**12**		Ethyl
6			**13**		
7			**14**		

Compound	(**6**)
Empirical formula	$C_9H_{18}Ca_1N_9O_8$, $C_9H_6N_9O_2$, $2(H_2O)$
Formula weight	728.66
Space group	$P2_1$
Unit cell dimensions	
a [Å]	11.0311(5)
b [Å]	7.6811(3)
c [Å]	18.2762(8)
β [°]	102.3576(16)
Volume *V* [$Å^3$]	1512.68(11)
Z [molecules/cell]	2
$D_{calculated}$ [g/cm^{-3}]	1.600
Absorption coefficient [mm^{-1}]	0.298
θ range for data collection [°]	3.26-25.04
Limiting indices	-13 < = *h* = > 13
	-9 < = *k* = > 9
	-21 < = *l* = > 21
Reflections collected/unique	34811/5346
Data/parameters	5346/491
Goodness of Fit	1.090
Final *R* index ($I > 2\sigma$)	0.0417
wR^2	0.1219
Largest diff. Peak and hole [$Å^{-3}$]	0.758 and -0.473

Bacteria strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Cip.*
S. aureus NCTC 4163	103 (32)	108 (32)	51 (16)	208 (64)	52 (16)	7 (2)	20 (4)	14 (4)	65 (16)	14 (4)	7 (2)	>999 (>256)	59 (16)	50 (16)	2 (0.5)
S. aureus ATCC 25923	51 (16)	108 (32)	26 (8)	208 (64)	52 (16)	7 (2)	20 (4)	29 (8)	65 (16)	14 (4)	7 (2)	>999 (>256)	15 (4)	12 (4)	2 (0.5)
S. aureus ATCC 6538	51 (16)	108 (32)	51 (16)	208 (64)	52 (16)	7 (2)	20 (4)	14 (4)	33 (8)	7 (2)	7 (2)	>999 (>256)	15 (4)	25 (8)	2 (0.5)
S. epidermidis ATCC 12228	51 (16)	27 (8)	13 (4)	104 (32)	26 (8)	4 (1)	10 (2)	29 (8)	33 (8)	7 (2)	7 (2)	999 (256)	29 (8)	50 (16)	2 (0.5)
S. epidermidis ATCC 35984	26 (8)	14 (4)	6 (2)	52 (16)	13 (4)	4 (1)	5 (1)	14 (4)	16 (4)	7 (2)	3 (1)	999 (256)	15 (4)	12 (4)	2 (0.5)
B. subtilis ATCC 6633	6 (2)	14 (4)	26 (8)	52 (16)	26 (8)	29 (8)	5 (1)	57 (16)	65 (16)	7 (2)	3 (1)	999 (256)	15 (4)	12 (4)	1 (0.250)
B. cereus ATCC 11778	26 (8)	14 (4)	26 (8)	26 (8)	26 (8)	29 (8)	10 (2)	29 (8)	65 (16)	7 (2)	3 (1)	999 (256)	7 (2)	6 (2)	<1 (<0.25)
E. hirae ATCC 10541	205 (64)	27 (8)	51 (16)	208 (64)	52 (16)	29 (8)	10 (2)	29 (8)	65 (16)	28 (8)	14 (4)	999 (256)	29 (8)	25 (8)	3 (1)
E. faecalis ATCC 29212	103 (32)	54 (16)	51 (16)	208 (64)	52 (16)	234 (64)	10 (2)	114 (32)	8 (2)	56 (16)	7 (2)	999 (256)	59 (16)	50 (16)	12 (4)
M. luteus ATCC 10240	51 (16)	27 (8)	51 (16)	52 (16)	26 (8)	59 (16)	20 (4)	57 (16)	2 (0.5)	7 (2)	1 (0.25)	999 (256)	15 (4)	12 (4)	3 (1)
M. luteus ATCC 9341	26 (8)	14 (4)	6 (2)	52 (16)	26 (8)	59 (16)	20 (4)	57 (16)	2 (0.5)	14 (4)	1 (0.25)	999 (256)	15 (4)	12 (4)	6 (2)
E. coli ATCC 10538	821 (256)	>865 (>256)	411 (128)	417 (128)	52 (16)	117 (32)	20 (4)	57 (16)	16 (4)	14 (4)	7 (2)	999 (256)	29 (8)	25 (8)	12 (4)
P. vulgaris NCTC 4635	821 (256)	>865 (>256)	411 (128)	208 (64)	13 (4)	117 (32)	10 (2)	57 (16)	8 (2)	14 (4)	1 (0.25)	999 (256)	15 (4)	12 (4)	2 (0.5)
P. aeruginosa ATCC 15442	821 (256)	>865 (>256)	411 (128)	834 (256)	834 (256)	938 (256)	1304 (256)	914 (256)	1040 (256)	111 (32)	218 (64)	999 (256)	471 (128)	397 (128)	2 (0.5)
P. aeruginosa ATCC 27853	821 (256)	>865 (>256)	411 (128)	834 (256)	834 (256)	938 (256)	1304 (256)	>914 (>256)	1040 (256)	14 (4)	7 (2)	999 (256)	471 (128)	397 (128)	2 (0.5)
B. bronchiseptica ATCC 4617	410 (128)	>865 (>256)	205 (64)	417 (128)	209 (64)	117 (32)	652 (128)	>914 (>256)	130 (32)	14 (4)	7 (2)	999 (256)	29 (8)	12 (4)	2 (0.5)

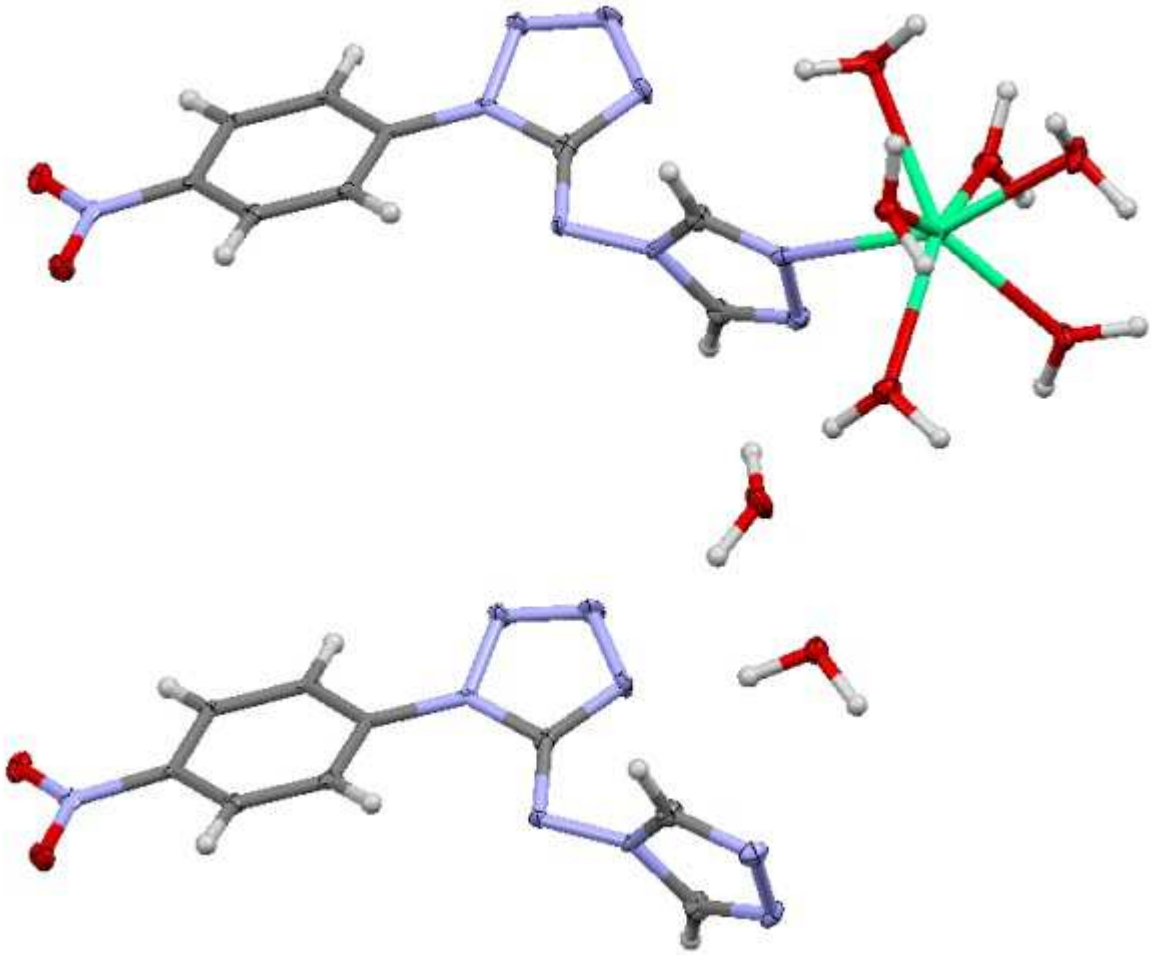
Bacteria strain	7	10	11	Ciprofloxacin
S. aureus 495	41 (8)	7 (2)	7 (2)	773 (256)
S. aureus 496	82 (16)	14 (4)	7 (2)	2 (0.5)
S. aureus 497	82 (16)	7 (2)	14 (4)	2 (0.5)
S. aureus 498	82 (16)	7 (2)	7 (2)	2 (0.5)
S. aureus 537	82 (16)	14 (4)	7 (2)	773 (256)
S. aureus 572	41 (8)	14 (4)	14 (4)	386 (128)
S. aureus 585	82 (16)	7 (2)	14 (4)	773 (256)
S. aureus 586	82 (16)	14 (4)	14 (4)	773 (256)
S. epidermidis 423	20 (4)	7 (2)	14 (4)	2 (0.5)
S. epidermidis 431	20 (4)	7 (2)	7 (2)	24 (8)
S. epidermidis 432	41 (8)	14 (4)	7 (2)	193 (64)
S. epidermidis 433	20 (4)	14 (4)	14 (4)	193 (64)
S. epidermidis 469	20 (4)	7 (2)	7 (2)	2 (0.5)
S. epidermidis 471	41 (8)	14 (4)	7 (2)	97 (32)
S. epidermidis 510	20 (4)	14 (4)	14 (4)	2 (0.5)
S. epidermidis 511	20 (4)	14 (4)	14 (4)	97 (32)
P. aeruginosa 6m	326 (64)	14 (4)	7 (2)	0.2 (0.06)
P. aeruginosa 7m	326 (64)	14 (4)	7 (2)	0.2 (0.06)
P. aeruginosa 10m	326 (64)	14 (4)	27 (8)	0.4 (0.12)
P. aeruginosa 11m	652 (128)	28 (8)	27 (8)	0.8 (0.24)
P. aeruginosa 12m	326 (64)	14 (4)	7 (2)	0.4 (0.12)
P. aeruginosa 16m	326 (64)	28 (8)	14 (4)	0.2 (0.06)
P. aeruginosa 18m	652 (128)	28 (8)	14 (4)	0.4 (0.12)
P. aeruginosa 31m	326 (64)	14 (4)	7 (2)	0.4 (0.12)
E. coli ML 5	326 (64)	14 (4)	7 (2)	0.2 (0.06)
E. coli ML 6	1304 (256)	111 (32)	54 (16)	0.2 (0.06)
E. coli ML 8	326 (64)	14 (4)	7 (2)	0.2 (0.06)
E. coli ML 9	326 (64)	28 (8)	14 (4)	0.2 (0.06)
E. coli ML 12	>1304 (>256)	111 (32)	54 (16)	48 (16)

E. coli ML 15	1304 (256)	111 (32)	54 (16)	0.2 (0.06)
E. coli ML 16	326 (256)	14 (4)	7 (2)	193 (64)
E. coli ML 17	326 (256)	14 (4)	7 (2)	0.2 (0.06)

Compound	LC gyrase	BE gyrase [kcal/mol]	LC topoisimerase	BE topoisomerase [kcal/mol]
1	274	-4.89	458	-4.34
2	477	-3.88	393	-4.21
3	371	-3.78	502	-4.44
4	266	-6.61	893	-5.63
5	514	-3.68	786	-3.83
6	1000	-5.68	855	-5.92
7	**651**	**-3.69**	**850**	**-3.6**
8	750	-3.69	375	-3.83
9	825	-3.25	974	-3.84
10	**196**	**-4.73**	**665**	**-4.94**
11	**249**	**-4.96**	**268**	**-5.1**
12	645	-5.62	620	-5.51
13	795	-5.77	628	-5.68
14	359	-6.1	291	-7.02
ciprofloxacin	**931**	**-6.13**	**700**	**-5.2**

	*IC_{50}	
Compounds	*S. aureus* DNA Gyrase	*S. aureus* Topoisomerase IV
10	22.8 ± 0.4	11.9 ± 1.3
11	0.9 ± 0.1	2.6 ± 0.2
Ciprofloxacin	3.5 ± 0.3	1.70 ± 0.15

Compound	Cytotoxic activity IC_{50} (μM)		Selectivity index (SI)						
			Gram-positive bacteria				Gram-negative bacteria		
	[b] A549	[a] HaCaT	*S. aureus* NCTC 4163	*B. subtilis* ATCC 6633	*E. hirae* ATCC 10541	*M. luteus* ATCC 10240	*E. coli* ATCC 0538	*P. vulgaris* NCTC 4635	*B. bronchiseptica* ATCC 4617
2	60 ± 1,2	60 ± 2,4	0.55	4.28	2.22	2.22	0.07	0.07	0.07
3	76 ± 2,1	80 ± 1,8	1.56	3.07	1.56	1.56	0.19	0.19	0.39
5	62 ± 1,8	65 ± 2,2	1.25	2.5	1.25	2.5	1.25	5.0	0.31
6	60 ± 1,3	65 ± 0,9	9.28	2.24	2.24	1.10	0.55	0.55	0.55
8	60 ± 2,4	62 ± 3,6	4.42	1.08	2.13	1.08	1.08	1.08	0.07
9	80 ± 2,2	84 ± 2,1	1.29	1.29	2.89	1.47	1.47	1.47	0.09
10	78 ± 0,8	80 ± 2,5	5.71	11.42	2.85	11.42	11.42	11.42	11.42
11	80 ± 1,7	84 ± 2,7	12	28	6	84	12	84	12
Cisplatin	1.95 ± 0.8	2.84 ± 1.1	-	-	-	-	-	-	-
Doxorubicin	0.63 ± 0.2	1.09 ± 0.2	-	-	-	-	-	-	-



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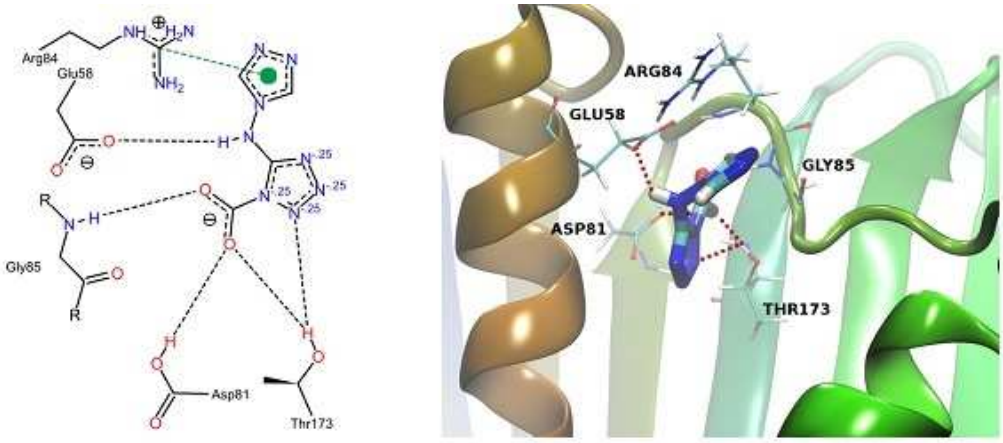
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ARG84
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ASP81
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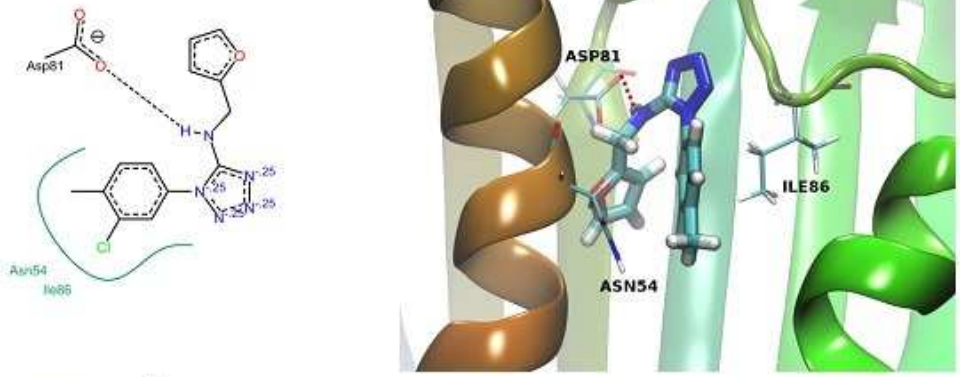
b)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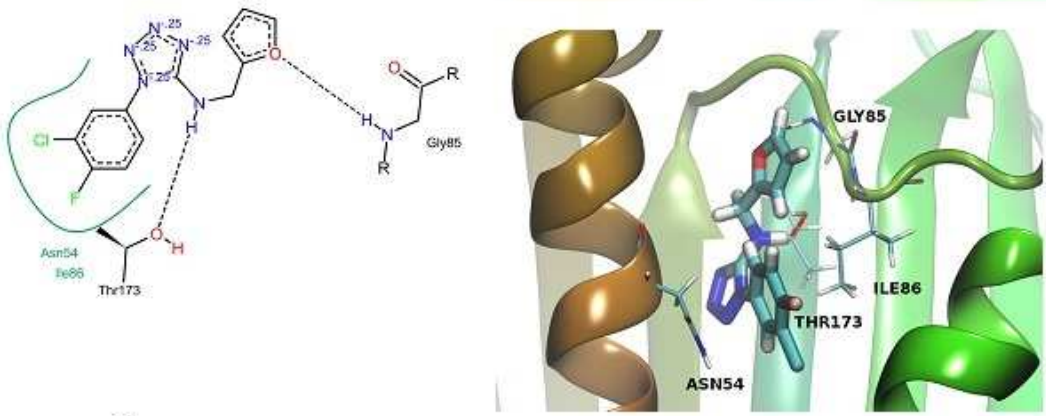
Asn54
Ile86
ASP81
ILE86
ASN54

c)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Thr173
Gly85
GLY85
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THR173
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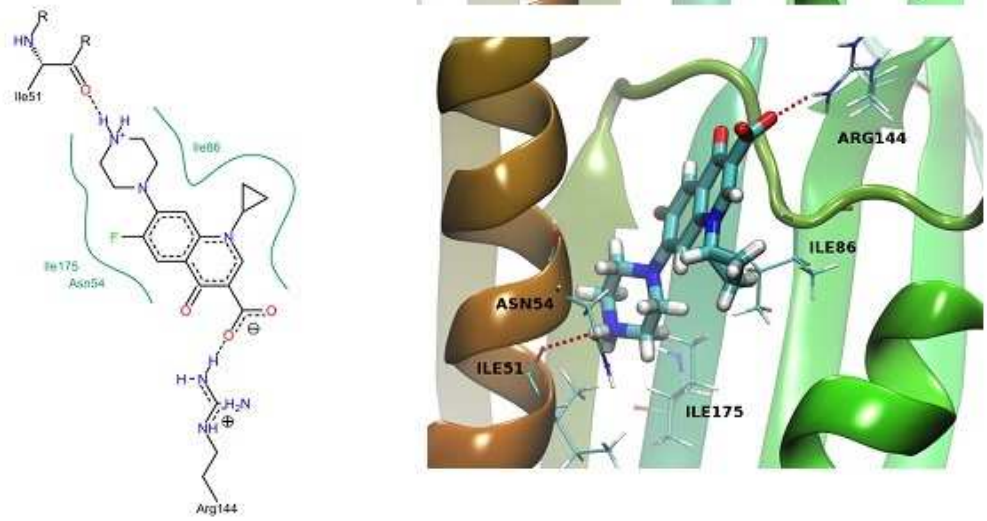
d)


Ile51
Ile86
Ile175
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F
Arg144
ARG144
ILE86
ASN54
ILE51
ILE175

- New fourteen 1,5-disubstituted tetrazole derivatives were synthesized.
- Results showed that compounds exhibited high and broad antibacterial activity.
- Tetrazole derivatives **7**, **10** and **11** were found as most potent antimicrobial agents.
- Minimal inhibitory concentrations of compound **10** and **11** were in the range 1 - 208 μM.