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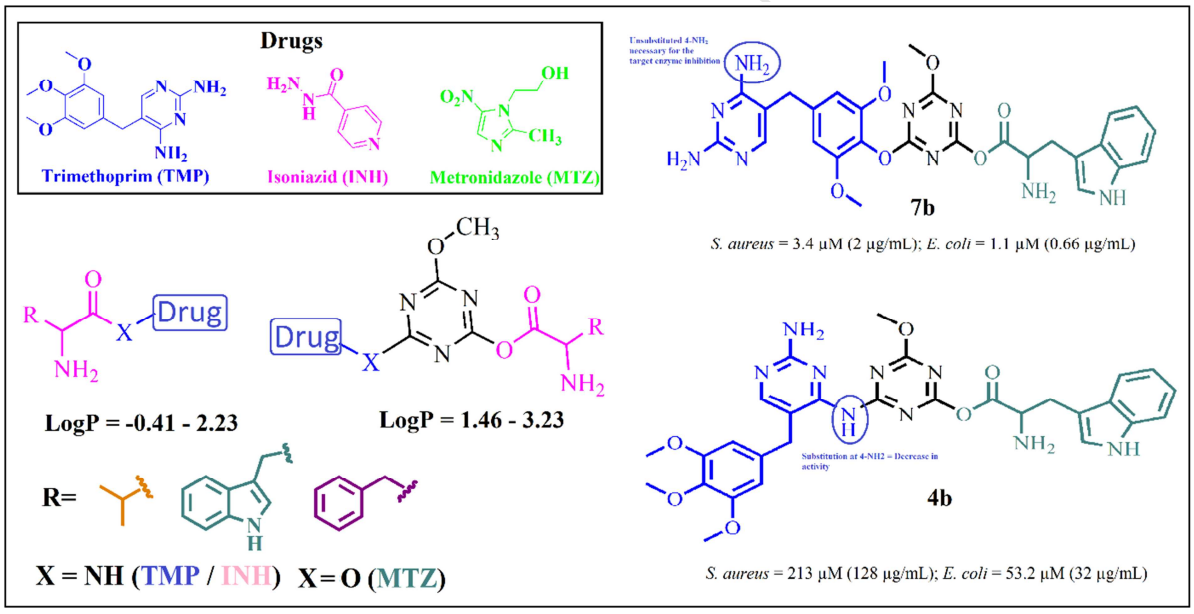
Graphical Abstract



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Amino Acid Conjugated Antimicrobial Drugs: Synthesis, Lipophilicity-Activity Relationship, Antibacterial and Urease Inhibition Activity

Atta Ullah [1], Fatima Iftikhar [1], Muhammad Arfan [2], Syeda Tayyaba Batool Kazmi [3], Muhammad Naveed Anjum [4], Ihsan-ul Haq [3], Muhammad Ayaz [1], Sadia Farooq [1], Umer Rashid [1,5,*]

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Abstract

Present work describes the *in vitro* antibacterial evaluation of some new amino acid conjugated antimicrobial drugs. Structural modification was attempted on the three existing antimicrobial pharmaceuticals namely trimethoprim, metronidazole, isoniazid. Twenty one compounds from seven series of conjugates of these drugs were synthesized by coupling with some selected Boc-protected amino acids. The effect of structural features and lipophilicity on the antibacterial activity was investigated. The synthesized compounds were evaluated against five standard American type culture collection (ATCC) i.e. *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* strains of bacteria. Our results identified a close relationship between the lipophilicity and the activity. Triazine skeleton proved beneficial for the increase in hydrophobicity and potency. Compounds with greater hydrophobicity have shown excellent activities against Gram-negative strains of bacteria than Gram-positive. 4-amino unsubstituted trimethoprim-triazine derivative **7b** have shown superior activity with MIC = 3.4 µM (2 µg/mL) for *S. aureus* and 1.1 µM (0.66 µg/mL) for *E. coli*. The synthesized compounds were also evaluated for their urease inhibition study. Microbial urease from *Bacillus pasteurii* was chosen for this study. Triazine derivative **7a** showed excellent inhibition with IC_{50}= 6.23 $\pm$ 0.09 µM. Docking studies on the crystal structure of *B. pasteurii* urease (PDB ID 4UBP) were carried out.

Key words: Structural modification; amino acid conjugates; trimethoprim; metronidazole; isoniazid, Lipophilicity-Activity Relationship

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Introduction

An antimicrobial drug is a substance that acts against all kinds of the microbial organisms including bacteria, fungi, protozoa and viruses. In the history of chemotherapy, antimicrobials are most successful forms that have contributed a lot to control the human ailment. The modern antibiotic era is associated with the idea of Paul Ehrlich's "magic bullets" concept and with the serendipitous discovery of penicillin by Alexander Fleming in 1929. The antibiotic discoveries in this era has propelled the drug research strategies and thousands of antibiotics were discovered and used in the clinical practices. In fact, the antibiotics used today were originated in this era of drug discovery between 1950 and 1970. Indeed after this golden era, there is a considerable gap in the identification of new classes of the compounds. Unfortunately, the discovery efforts were declined due to emergence of antibiotic resistance of pathogens over the last three decades [1-4].

To combat the wave of resistance, novel drug discovery approaches has been emerged. These approaches are strengthened due to advancement in the high-throughput pharmacological screening and synthesis of diverse chemical libraries, rational drug design due to improvement in protein structure determination and revolution in computing. Today, drug discovery scientists are concerned with the discovery of new synthetic compounds as well as the chemical modification of existing pharmaceuticals [4].

Bacterial ureases are the key determinants of pathogenic symptoms in animals as well as in human beings [5]. Urease is recognized by researchers as basic cause of pathologies stimulated by a well-known bacteria, *Helicobacter pylori* (HP). It permits HP to uphold at low pH all through colonization, thus playing pivotal role in producing peptic as well as gastric ulcers. For

the eradication of *H. pylori*, a combination of a proton pump inhibitor (e.g. omeprazole) and two antibiotics (Clarithromycin plus amoxicillin or metronidazole) are used as first-line therapy [6].

A very important approach in today's drug discovery process is the growing popularity of the amino acids or peptide based drugs as therapeutic agents. Amino acids are critical to life, and have many functions in metabolism. Structurally, amino acid are an important organic compound self-possessed of amine ($-NH_2$) and carboxylic (-COOH) functional group along with side chain particular to each amino acid [7]. The essential elements of an amino acid are carbon, hydrogen, oxygen and nitrogen, though other elements are found in the side-chains of several amino acids. Due to structural diversity, they provide a balance between hydrophobicity and hydrophilicity which is necessary for the cell membrane permeability and solubility. There are many drug discovery initiatives where amino acids/peptides are successfully conjugated with the biologically active cores and shown stability, enhanced potency, selectivity, permeability, solubility and reduced toxicity [8-17].

From all the above facts, we anticipated that amino acid conjugated antibacterial drugs may result in new leads possessing good pharmacological activities. We have already reported a successful example of structural modification of trimethoprim (**1**, **TMP**, **Figure 1**) that was resulted in enhanced antibacterial potential [5]. This time, we tried to synthesize its conjugates with amino acids. The other two drugs we chose were isoniazid (**2**) and metronidazole (**3**). Isoniazid (INH) which is a lead antibacterial drug compound use to treat active tuberculosis (T.B) infections and other skin problem. It is also used to stop the growth of bacteria. Metronidazole (**3**, MTZ) belongs to nitroimidazole class of antibiotics and is used to treat a wide variety of bacterial infections and diseases. Inspired by the structural diversity, activity profile of these three clinically available drugs, we hypothesized that the structural modification of these

drugs may result in a new leads possessing good pharmacological activities. The amino acids chosen are valine (**4**), tryptophan (**5**) and phenylalanine (**6**) due to their structural diversity. Tryptophan, a derivative of alanine, have a bicyclic indole ring linked at β-carbon atom of the side chain. Due to this indole ring, the amino acid has both polar and lipophilic properties and thus interacts with hydrophobic bilayer and with polar head groups via amine function. In contrast to tryptophan, valine is hydrophobic and non-polar amino acid with aliphatic isopropyl group. While, phenylalanine contains hydrophobic phenyl ring at β-position. Taking into consideration the aforesaid, we envisaged that the coupling of these drugs with N-protected amino acids may resulted in enhanced efficacy.

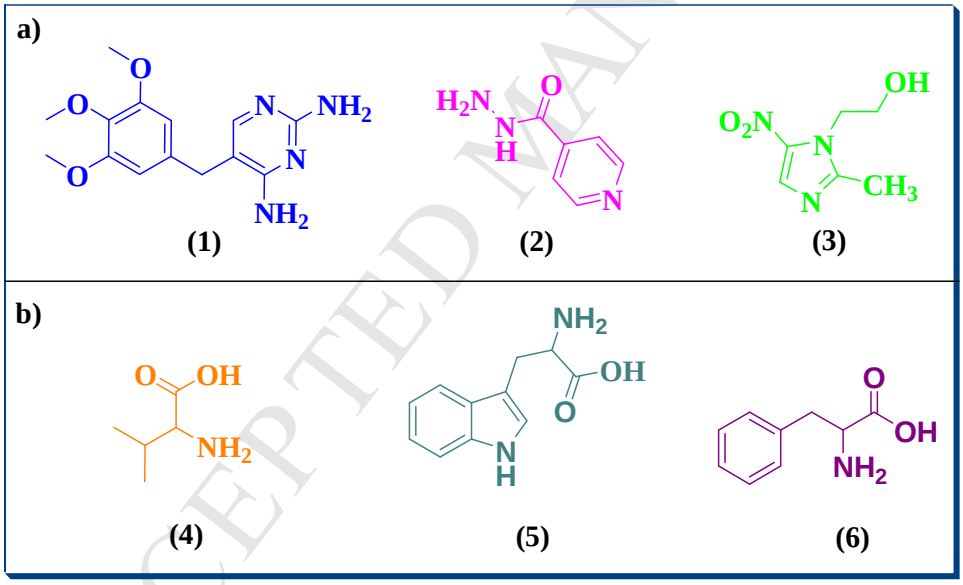

Figure 1: Structures of the starting materials used in current study. (a) Commercially available drugs (**1-3**); (b) Some selected amino acids (**4-6**)

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2. Results and Discussion

2.1. Design strategy based on structure-property relationship

Options for the treatment of Gram-negative microorganisms such as *E. coli*, *P. aeruginosa* are limited due to multi-drug resistant or focus of the research for the development of drugs to combat Gram-positive bacteria [18]. The study design of our current research is based on influence of the lipophilicities on the uptake of target compounds by Gram-positive and negative bacteria. The main goal of this study is to design the antibacterials that can; 1) penetrate to the site of drug action, (2) interact with molecular targets within bacterium. The difference in the composition of the outer cell envelop of the Gram-positive and negative bacteria hampers the uptake of certain compounds [19-20]. The relative hydrophobicity/hydrophilicity and molecular weight has a certain role in their uptake. We employed lipophilicity-activity and structure activity relationship (SAR) strategies. The overall strategy is shown in **Figure 2**. Our computational analysis have shown that the logP values have been increased to some extent by the formation of amide or ester of the drugs (**Figure 2a)**. Trimethoprim has four nitrogen atoms but also contains three methoxy groups and a bridged methylene. The calculated LogP value for trimethoprim is 1.28, whereas, for isoniazid and metronidazole -0.71 and -0.15, respectively. To get our goal, we considered the introduction of triazine nucleus between drugs and amino acid. Our computed logP values suggests that the introduction of triazine skeleton resulted in stronger hydrophobicity (**Figure 2b).** Encouraged with these computational data, we proceed for the synthesis and *in vitro* antibacterial study against some selected strains.

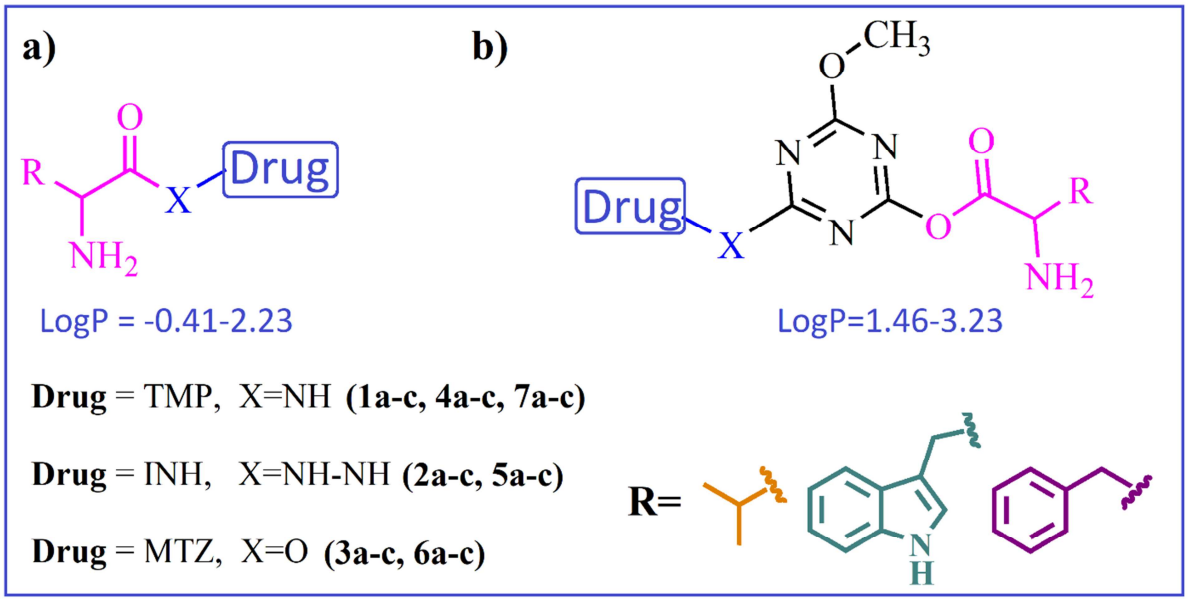

Figure 2: Overall strategy for the design of target antibacterials

2.2. Chemistry

Boc-protected amino acids (**8-10**) were obtained by the reaction of amino acids (**4-6**) with commercially available di-*tert*-butylcarbonate (**7**, *t*-BuOCO)$_2$O) in 10% NaOH in dioxane (**Scheme 1**). The ^{1}H NMR spectrum of all the Boc-protected amino acids showed a nine protons (three methyl groups) singlet in the range of 1.48-1.49 ppm.

The coupling of N-protected Boc-amino acids with the already existing antimicrobial drugs was carried out by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). 1-Hydroxy-benzotriazole (HOBt) was used to avoid racemization and triethylamine (Et_3N) was used to maintain the pH. To obtain the final products, Boc group was de-protected by using trifluoroacetic acid (TFA, CF_3COOH) in dichloromethane (DCM, 10 mL / g of the compound. The coupling of trimethoprim (TMP, **1**) and isoniazid (INH, **2**) is shown in **Schemes 1**.

The coupling of TMP (**1**) with *Boc*-protected amino acids (**8-10**) resulted in the formation of amide at 4-position of the pyrimidine ring as major products (**1a-c**). The greater susceptibility of 4-amino group in 2,4-di amino pyrimidines is due to the fact that carbon bearing amino group at 2-position is directly adjacent to two N-atoms of the pyrimidine ring, i.e. under the effect of two electron-withdrawing groups. While, in case of C-4, it experiences this effect from only one side. This is an evident from the shielded protons of 4-NH_2 group in the ^{1}H NMR spectrum of trimethoprim as compared to the more de-shielded protons of amino group at 2-psoition. In the ^{1}H NMR spectra of compounds **1a-c** and **4a-c**, broad singlets in the range of 7.97-8.03 were assigned to 4-NH_2 protons. While, in the ^{1}H NMR spectra of 4-amino unsubstituted compounds **7a-c,** peaks at 7.42-7.46 were assigned to 2-NH_2 protons. Furthermore, in trimethoprim, there is an additional electron-donating group at C-5, which further enhances the nucleophilicity at 4-position. Therefore, as per our expectations the 4-amino substituted products were found to be the major product in every case. These results are also consistent with our previously reported results [5].

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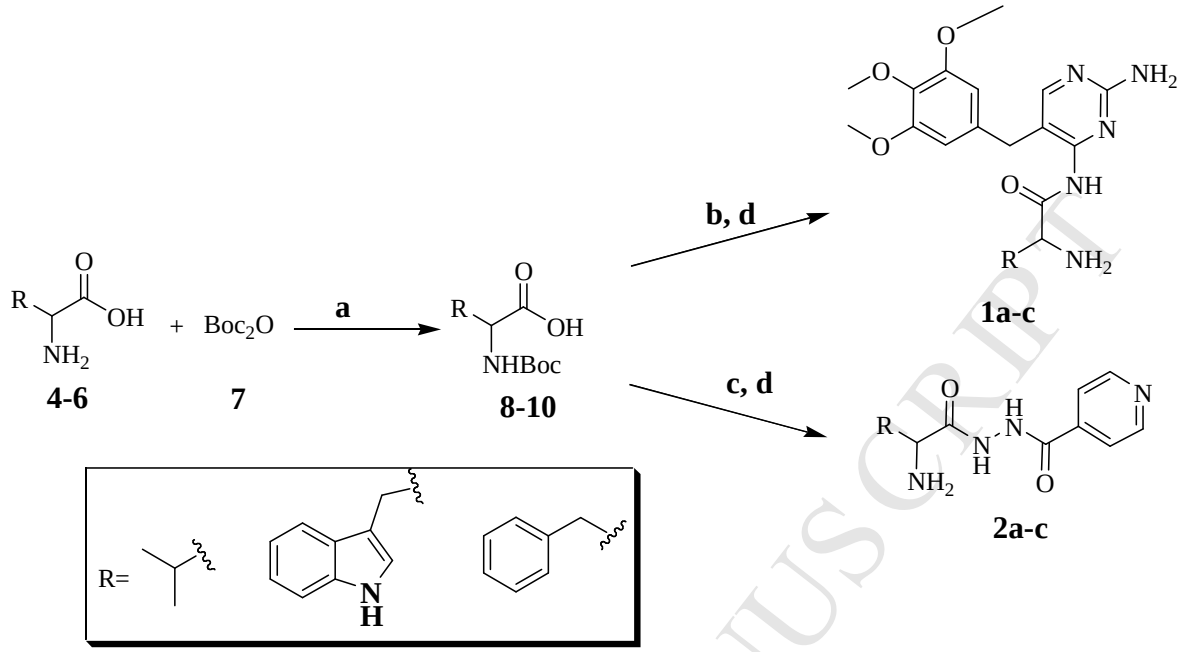

152

153 **Scheme 1:** Synthesis of target compounds **1a-c** and **2a-c**. Reagents and conditions: **a)** Dioxane, NaOH;

154 **b)** TMP (**1**), HOBt, EDC, Et_3N, $CHCl_3$; **c)** INH (**2**), HOBt, EDC, Et_3N, $CHCl_3$; **d)** CF_3COOH, DCM

155 Metronidazole is an important 5-nitroimidazole derivative. Mao et al reported the salicylic acid
156 derivatives by coupling with metronidazole showed promising inhibition of *H. pylori* urease
157 [21]. Keeping in view this activity, we decided to synthesize some derivatives by the reaction of
158 carboxylic hydroxyl group of N-protected amino acid and pendant hydroxyl group of
159 metronidazole. A two-step reaction involves first the formation of metronidazole tosylate (**12**) by
160 the reaction of metronidazole (**3**) with *p*-toluenesulfonyl (**11**). Treatment of **12** with N-protected
161 amino acids (**8-10**) in DMF using K_2CO_3 as base followed by deprotection of N-Boc using
162 trifluoroacetic acid in DCM yield our desired products (**3a-c**) (**Scheme 2**).

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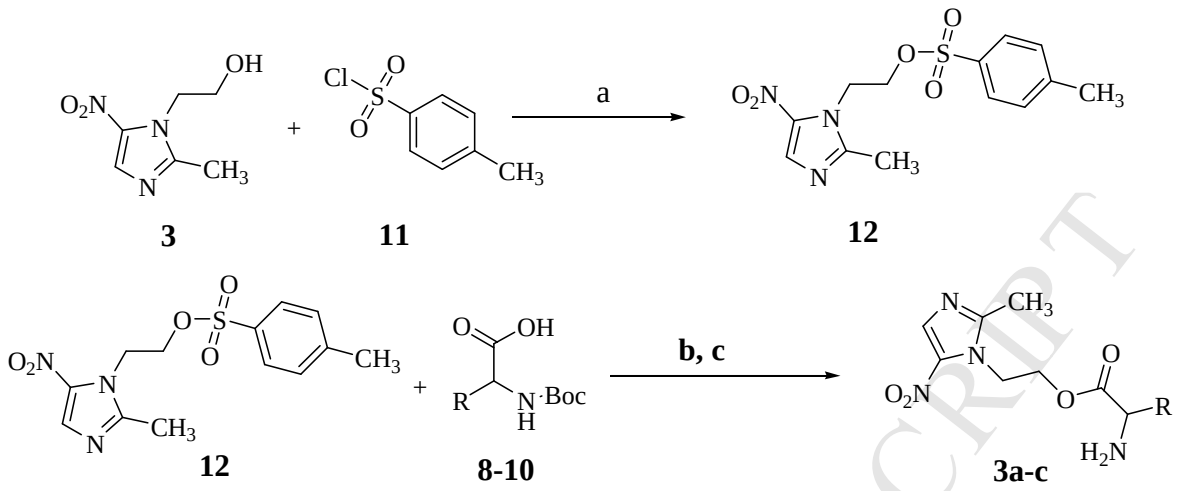
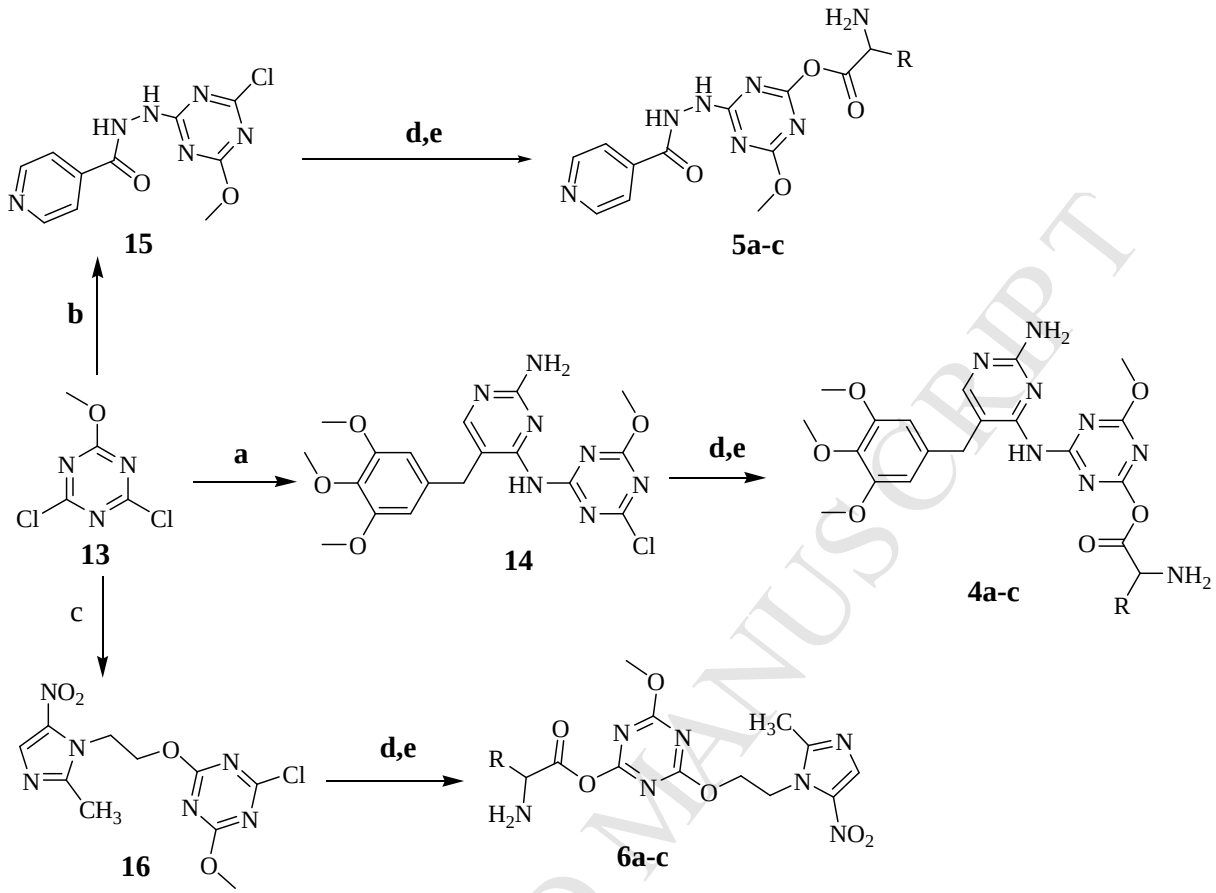


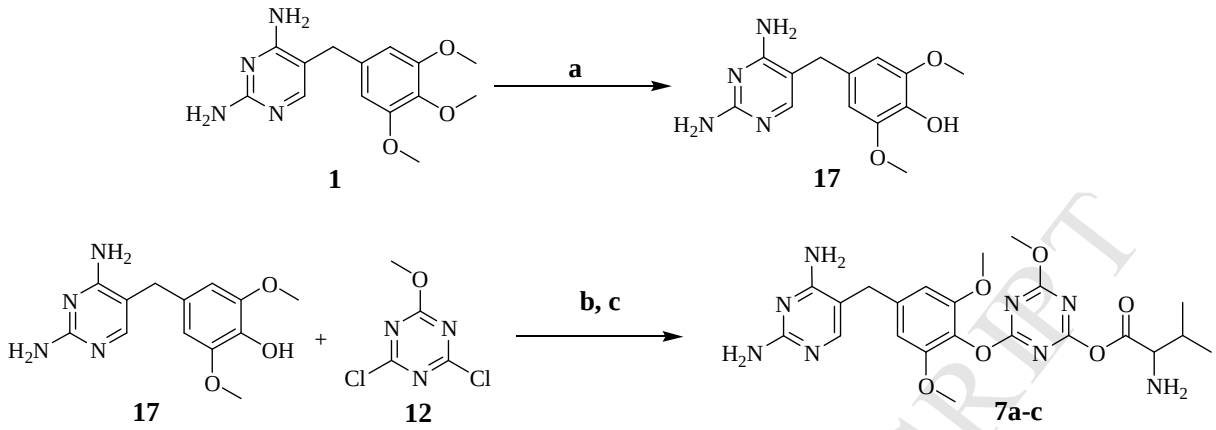
Scheme 2: Synthesis of target compounds **3a-c.** Reagents and conditions: a) DCM, TEA; b) K_2CO_3, DMF; c) CF_3COOH, DCM

Our computed logP values indicate the introduction of triazine nucleus enhances the hydrophobicity. Hence in another attempt, we synthesized di-substituted triazine hybrids of all the three studied drugs and amino acid. Triazine scaffolds play important role in exerting specific biological effects due to favorable drug-target interactions [22]. Here, we tried commercially available 2,4-dichloro-6-methoxy-1,3,5-triazine (**13**) to react with the drugs and N-protected amino acids *via* two-step protocol followed by deprotection to produce hybrids **(4a-c, 5a-c and 6a-c Scheme 3)**. In first substitution step, nucleophilic attack of the drugs was carried out by stirring the reactants at 0 ^{o}C to yield products **14-16 (Scheme 3)**. In second step same mixture was refluxed with Boc-protected amino acids and finally deprotection step with TFA in DCM yielded required products (**Scheme 3**).



Scheme 3: Synthesis of target compounds **4a-c**, **5a-c** and **6a-c**. Reagents and conditions: **a)** Compound **1**, K_2CO_3, THF, 0 ^oC, **b)** Compound **2**, K_2CO_3, THF, 0 ^oC, **c)** Compound **3**, K_2CO_3, THF, 0 ^oC, **d)** Compounds **8-10**, K_2CO_3, THF, reflux, **e)** CF_3COOH, DCM

As discussed in our previous report, unsubstituted 4-NH_2 is necessary for the target enzyme inhibition [5]. In current study, we have observed some inhibition and moderate activity from our synthesized products in **Scheme 1** (**1a-c**) and **Scheme 3** (**4a-c**). We again here tried the substitution at trimethoxy ring. Compounds **7a-c** were synthesized by hydrolyzing the methoxy group using 48% HBr to obtain **17 (Scheme 4)**. Further two step protocol was employed as in schemes **3** to obtain triazine derivatives **7a-c (Scheme 4)**.



Scheme 4: Synthesis of target compounds **7a-c.** Reagents and conditions: a) 48% HBr; b) Compounds **8-10**, K_2CO_3, THF, reflux, **e)** CF_3COOH, DCM

2.3. *In vitro* antibacterial studies

Targeted conjugates were evaluated for their antibacterial activity against two Gram-positive i.e. *S. aureus* (ATCC- 6538), *B. subtilis* (ATCC-6633) and three Gram-negative i.e. *E. coli* (ATCC-25922), *S. typhi* (ATCC-14028) and *P. aeruginosa* (ATCC-15442) strains of bacteria. Minimum inhibitory concentration (MIC) values of these compounds were calculated using standard methods. Antibacterial activity of the synthesized compounds was compared with trimethoprim, ciprofloxacin and roxithromycin.

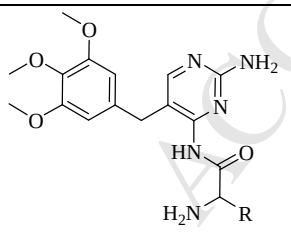
2.3.1. Antibacterial potency of trimethoprim derivatives

Bioassay data of synthesized trimethoprim derivatives **1a-c**, **4a-c** and **7a-c** is shown in **Table 1**. It revealed that among the direct conjugates, conjugates of the trimethoprim **1a-c** and triazine derivatives (**4a-c**) where 4-NH_2 is blocked showed moderate to poor activity. These results are contrary to our previously reported results where the substitution at 4-NH_2 group resulted in the complete loss in antibacterial activity [5]. Compound **1a** has shown some activity against Gram-

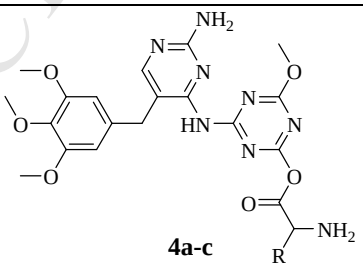
positive bacteria (MIC= 82 µM for *S. aureus* and 68.3 µM for *B. subtilis*). Conjugation with tryptophan and phenylalanine increased the inhibitory potency against Gram-negative bacteria and decrease the anti-Gram-positive activity. On the other hand, triazine derivatives **4a-c** anti-Gram-negative inhibition is dominant. **4c** showed good inhibition against *P. aeruginosa* with MIC value of 28.4 µM (16 µg/mL). This results showed that there is no permeability problem for the compounds. The moderate activity may be due to blocked 4-NH_2.

To further study of SAR of trimethoprim derivatives, we proceed for our previously reported strategy and trimethoxyphenyl ring was further used for investigations. Here, the effect of unsubstituted 4-NH_2 on antibacterial potency was explored. Excellent inhibition was observed for all types of bacterial strains under study. Superior activity was shown by compound **7b** not only against Gram-negative bacteria but also for Gram-positive strains. The MIC for *E. coli* = 1.1 µM (0.66 µg/mL); for *S. typhi* 2.3 µM (1.33 µg/ mL) and for *P. aeruginosa* 1.4 µM (0.83 µg/mL). The MIC value for Gram-positive *S. aureus* was found 3.4 µM (2 µg/ml) and for *B. subtilis* 6.8 µM (4 µg/mL). In the tested concentrations, MTZ and INH did not show any inhibition. The results are shown in **Table 1**.

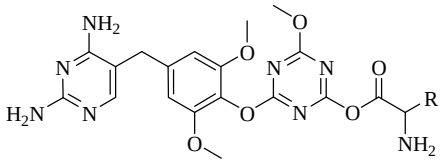
Table 1: SAR and antibacterial activity of trimethoprim derivatives



1a-c

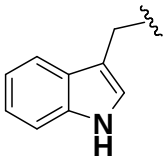
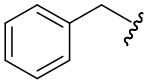
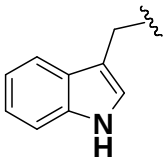
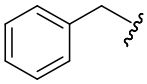
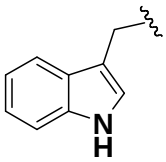
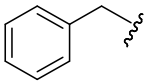


4a-c



7a-c

Compound No.	R	LogP [a]	Antibacterial activity MIC in µM (µg/mL) [b]				
			Gram Positive		*Gram Negative*		
			Sa	*Bs*	*Ec*	*St*	*Pa*

1a		1.30	82.2 (32)	68.3 (26.6)	164.4 (64)	218.9 (85.3)	136.9 (53.3)
1b		2.23	134.3 (64)	223.7 (106.6)	67.1 (32)	55.8 (26.6)	67.1 (32)
1c		2.14	243.8 (106.6)	243.8 (106.6)	73.1 (32)	97.4 (42.6)	73.1 (32)
4a		2.68	165.7 (85.3)	124.4 (64)	62.2 (32)	82.8 (42.6)	51.7 (26.6)
4b		3.23	212.8 (128)	212.8 (128)	53.2 (32)	53.2 (32)	53.2 (32)
4c		3.20	303.2 (170.6)	227.5 (128)	47.3 (26.6)	56.8 (32)	28.4 (16)
7a		2.19	**10.6 (5.3)**	**7.9 (4)**	**6.6 (3.3)**	**15.9 (8)**	**6.6 (3.3)**
7b		1.98	**3.4 (2)**	**6.8 (4)**	**1.1 (0.66)**	**2.3 (1.33)**	**1.4 (0.83)**
7c		2.64	**12 (6.6)**	**19.3 (10.6)**	**3.6 (2)**	**7.3 (4)**	**6 (3.3)**
Trimethoprim			22.7 (6.6)	NP	55.1 (16)	NP [d]	NP
Ciprofloxacin			30.2 (10)	30.2 (10)	30.2 (10)	NP	30.2 (10)
Roxithromycin			0.6 (0.5)	0.6 (0.5)	0.6 (0.5)	NP	0.3 (0.25)
Metronidazole			NI [e]	NI	NI	NI	NI
Isoniazid			NI	NI	NI	NI	NI

[a] LogP values were calculated via VCCL online software; [b] The reported MIC values in µM. MIC value in µg/ml are shown in parenthesis. These MIC values are an average of at least three individual measurements; [c] The values shown in bold are the better inhibitors than the standard drugs used; [d] NP= Not performed; [e] NI= No inhibition shown in tested concentration; *S. aureus* (ATCC- 6538), *B. subtilis* (ATCC-6633), *E. coli* (ATCC-25922), *S. typhi* (ATCC-14028) and *P. aeruginosa* (ATCC-15442).

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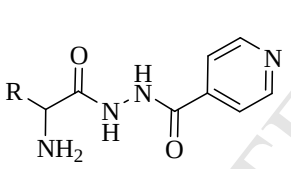
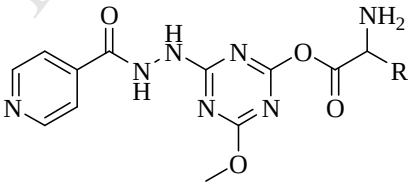
2.3.2. Antibacterial potency of isoniazid derivatives

Except compound **2b**, the SAR analogues having isoniazid (**2a** and **2c**) were found inactive and showed no inhibitory efficacy in the tested concentration domain. This inactivity is probably due to cell impermeability. Tryptophan conjugate (**2b**) demonstrated some weak inhibition against Gram-positive strains. However, addition of triazine skeleton (**5a-c**) was helpful for improving activity. Compound **5a** showed low MIC values for tested Gram-negative strains with MIC value of 59 µM (21.3 µg/mL) against *E. coli*. Compound **5b** exhibited low MIC values for both types of strains. It showed MIC value of 47.5 µM (21.3 µg/mL) and 71 µM (32 µg/mL) against *S. aureus* and *B. subtilis*. While, MIC values for *E. coli*, *S. typhi* and *P. aeruginosa* are 36 µM, 36 µM and 60 µM respectively (**Table 2**).

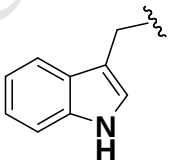
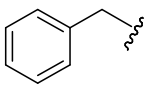
Table 2: SAR and antibacterial activity of isoniazid derivatives

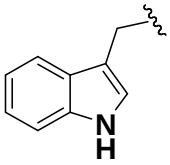
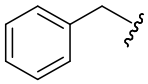


2a-c



5a-c

Compound No.	R	LogP [b]	Antibacterial activity MIC in µM (µg/mL) [a]				
			Gram Positive		*Gram Negative*		
			Sa	***Bs***	***Ec***	***St***	***Pa***
2a		-0.41	NI	NI	NI	NI	NI
2b		0.64	98.9 (32)	98.9 (32)	527.7 (170.6)	792 (256)	660 (213.3)
2c		0.05	NI	NI	NI	NI	NI

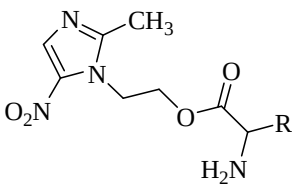
5a		1.58	117.8 (42.6)	177 (64)	59 (21.3)	74 (32)	74 (26.6)
5b		2.23	47.5 (21.3)	71 (32)	36 (16)	36 (16)	60 (26.6)
5c		2.50	104 (42.6)	156 (64)	52 (21.3)	78 (32)	65 (26.6)

[a] The reported MIC values in µM. MIC value in µg/ml are shown in parenthesis. These MIC values are an average of at least three individual measurements;

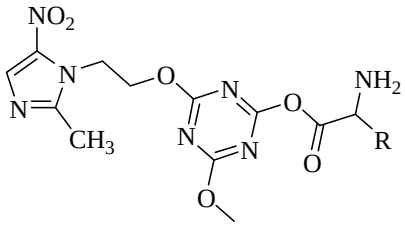
2.3.3. Antibacterial potency of metronidazole derivatives

Metronidazole derivatives **3a-c** demonstrated variable inhibitory efficacy against all the bacterial strains. Compound **3a** exhibited poor inhibition against Gram-negative strains, while compound **3b** exhibited moderate anti-*E. coli* with MIC value of 74.4 µM (26.6 µg/mL). Compound **3c** showed good anti-*S. aureus* inhibitory potency with MIC value of 50.3 µM (16 µg/mL). Here, again the addition of triazine skeleton proved beneficial for the increase in potency. Depending upon the structural features, the target compounds **6a-c** demonstrated weak to excellent inhibitory potential. Compound **6a** showed weak inhibition for both types of the tested strains. Low MIC value was shown by compound **6b** and **6c** against both the tested strains. Compound **6b** with MIC = 2 µM (1 µg/mL) against *E. coli* also inhibited *S. typhi* and *P. aeruginosa* with MIC of 6.8 µM (3.3 µg/mL) and 4 µM (2 µg/mL) respectively. Excitingly, it also exhibited remarkable activity against *S. aureus* and *B. subtilis* (MIC = 16.5 µM and 13 µM respectively) superior to trimethoprim and ciprofloxacin.

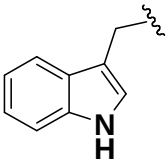
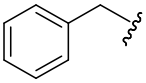
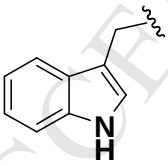
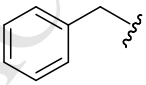
252 **Table 3**: SAR and antibacterial activity of metronidazole derivatives



3a-c



6a-c

Compound No.	R	LogP [b]	Antibacterial activity MIC in µM (µg/mL)[a]				
			Gram Positive		*Gram Negative*		
			Sa	***Bs***	***Ec***	***St***	***Pa***
3a		0.45	118.5 (32)	158 (42.6)	473 (128)	631.6 (170.6)	474 (128)
3b		1.74	179 (64)	239 (85.3)	74.4 (26.6)	89.5 (32)	119.2 (42.6)
3c		1.04	50.3 (16)	83.6 (26.6)	201 (64)	201 (64)	268 (85.3)
6a		1.46	81 (32)	81 (32)	67.3 (26.6)	81 (32)	54 (21.3)
6b		1.93	**16.5 (8)**	**13 (6.3)**	**2.0 (1.0)**	**6.8 (3.3)**	**4.0 (2.0)**
6c		2.13	24 (10.6)	18 (8)	7.4 (3.3)	18 (8)	**9 (4)**

253 [a] The reported MIC values in µM. MIC value in µg/ml are shown in parenthesis. These MIC values are an average of at least three individual

254 measurements;

255

256

2.4. Lipophilicity-activity relationship

It has been reported that the main cause of the failure of the antibiotic drugs is lack of their penetration due to a formidable barrier i.e. cell wall. As discussed the difference in the composition of the outer cell envelop of the Gram-positive and negative bacteria hampers the uptake of certain compounds. There are a number of examples in which the tested drug is effective against Gram-negative bacteria but at the same time ineffective against Gram-positive strain. A number of cheminformatics investigations have been performed on small to large data bases to analyze the effect of physiochemical properties on the antibacterial activity [19, 23-24]. Amongst the physiochemical properties that determine the pharmacokinetic properties of the drugs, hydrophobicity/hydrophilicity is important characteristic to improve the pharmacokinetic properties and thus in turn penetration into the site of action.

In present research, we tried to investigate the influence of the lipophilicities on the uptake of target compounds by Gram-positive and negative bacteria. The computed logP values are presented in **Tables 1-3**. The logP values of all the synthesized compounds lies between -0.41 to 3.23. In general, it can be concluded that the antibacterial activities increases with increase in the hydrophobicity. The logP values of trimethoprim derivatives **1a-c** showed greater lipophilicity and the logP values of **1b** and **1c** is 2.23 and 2.14, respectively. However, valine conjugate **1a** showed comparatively low logP value (1.30) and effective against Gram-positive strains. The logP values of the triazine derivatives (**4a-c** and **7a-c**) of trimethoprim followed the order of **4b** > **4c** > **4a** > **7c** > **7a** > **7b**. Among all the target compounds 4-NH_2 substituted derivatives **4b** and **4c** showed highest values of logP. Compounds **7a-c** with logP values in the range of 1.98-2.64 showed high inhibition potency for both tested strains.

Isoniazid derivatives (**2a-c**) possessed the lowest logP values and are strongly hydrophilic. This hydrophilicity is likely to the reason of their inactivity due to cell impermeability. However, with introduction of triazine nucleus (**5a-c**) their logP values increases in the range of 1.58-2.5. The logP values of the metronidazole derivatives (**3a-c** and **6a-b**) followed the order **6c** > **6b** > **3b** > **6a** > **3c** > **3a**. In comparison to first series of metronidazole derivatives (**3a-c**), triazine derivatives (**6a-c**) showed better hydrophobicity and also bioactivity. Compound **6b** and **6c** (logP= 1.93 and 2.13) inhibit both types of strains equally. In conclusion, triazine derivatives have highest logP values. It can be seen from **Figure 3** that for most active compounds, our studied cell permeability parameter logP lies in the range of 1.98-2.68. The MIC values in this range of LogP values lies between 0.6 – 10 μg/mL. These compounds have shown excellent activities against Gram-negative strains of bacteria than Gram-positive. However in few cases, compounds falling under the same range of logP have also shown excellent activity against Gram-positive species (e.g. *S. aureus*). This high activity or in other words high cell permeability may be due to other physiochemical parameters such as polar surface area or high molecular weight.

ACCEPTED MANUSCRIPT

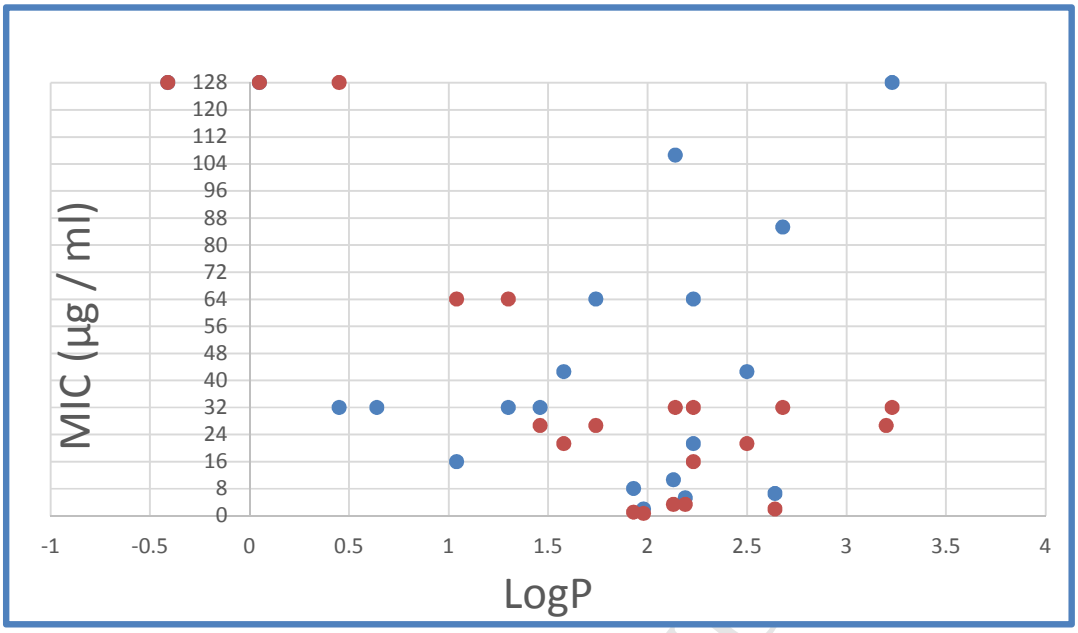


Figure 3: Distribution of logP values in relationship with antibacterial activity. For most active compounds, logP lies in the range of 1.98-2.68. Excellent activity= 0.6 – 10 µg / mL; moderate activity= 11-32 µg / mL; > 32 µg / mL poor activity (Blue dot for *S. aureus* and red for *E. coli*.)

2.4. *In vitro* urease inhibition activity against *B. pasteurii*

The structures of all ureases exhibit common similarities regardless of their source. Due to these similarities, inhibition of one urease isozyme will likely consider as inhibition of other isozyme [25]. Therefore, we carried out our studies on *Bacillus pasteurii*. The synthesized conjugates of drugs were screened for their urease inhibition according to Weatherburn indophenols method by the production of ammonia [26]. Trimethoprim derivatives (**1a-c**) displayed the IC_{50} value in the range of 31.34-52.41 µM. While, isoniazid derivatives (**2a-c**) showed potency in the range of 21.11-48.6 µM. Metronidazole derivatives (**3a-c**) showed better inhibitor activity in the range of 13.61-29.53 µM. Metronidazole conjugate with tryptophan (**3b**) was emerged as the most potent compound of this series with IC_{50} value of 13.61 µM. Derivatives **4a-c** showed good urease inhibition with IC_{50} value in the range of 11.97-19.29 µM. Isoniazid derivatives **5a-5c** showed

moderate inhibition of *B. pasteurii* urease. The metronidazole derivatives **6a-c** exhibited good inhibition wit IC_{50} value in the range of 11.43-19.51 µM. Excellent urease inhibition was demonstrated by trimethoprim derivatives **7a-c**. The IC_{50} value lies between 6.23-11.51 µM. Compound **7a** with IC_{50} of 6.23 ± 0.09 µM emerged as the most potent compound.

Table 4: In vitro urease inhibition activity against *B. pasteurii*

Compound No.	IC_{50} (µM± S.E.M) or %age inhibition	Compound No.	IC_{50} (µM± S.E.M) or %age inhibition
1a	52.41 ± 0.42	**5a**	29.31 ± 0.89
1b	35.10 ± 0.39	**5b**	37.29 ± 0.28
1c	31.34 ± 0.88	**5c**	30.98 ± 1.09
2a	48.6 ± 1.02	**6a**	19.51 ± 0.65
2b	37.01 ± 0.97	**6b**	16.32 ± 0.69
2c	21.11 ± 0.18	**6c**	11.43 ± 0.33
3a	29.53 ± 0.33	**7a**	**6.23 ± 0.09**
3b	**13.61 ± 0.14** [c]	**7b**	**11.51 ± 0.17**
3c	27.44 ± 0.56	**7c**	**8.33 ± 0.11**
4a	**19.29 ± 0.35**	Metronidazole	13%
4b	**11.97 ± 0.11**	Thiourea	**21± 0.10**
4c	**13.75 ± 0.42**		

2.7. Docking studies on Bacillus *pasteurii* urease (BPU)

Docking study of the synthesized compounds was carried out by using AutoDock 4.2. Crystal structure of *B. pasteurii* urease was retrieved from protein data bank (PDB ID 4UBP, 1.55 Å resolution) with acetohydroxamic acid (HEA) as co-crystalized ligand [27]. The reliability of the docking was checked by performing the re-docking of co-crystalized ligand acetohydroxamic

acid (HEA). The root mean square deviation (RMSD) between co-crystallized and re-docked conformation is 1.14 Å.

The bioactivity data presented in **Table 4** indicated that among all the synthesized compounds TMP-triazine derivatives (**4a-c** and **7a-c**) showed good to excellent activities. The visual inspection of the lowest energy docked pose of the TMP derivative **compound 4b** (IC_{50} = 11.97 ± 0.11 µM) showed that it coordinates with *bi*-nickel center **(**Ni798 and Ni799) *via* its 2,4-diaminopyrimidine ring of TMP (**Figure 4a**). Carbamylated Lys490 (KCX490, a non-standard bridging residue) forms hydrogen bond interaction with 2-NH_2 group of pyrimidine ring. The guanidinium group of Arg339 forms hydrogen bond donor interactions with one of the methoxy oxygen of the TMP and also with nitrogen atom of triazine ring. Gly280 forms bifurcated hydrogen bond interactions with both the amino groups of TMP pyrimidine ring. Cyst322 forms a π-sulphur interaction with its sulphur atom and π-system of triazine ring. Imidazole ring of His249 is observed to form a π-π T-shaped interactions with pyrimidine ring of TMP (**Figure 4b**).

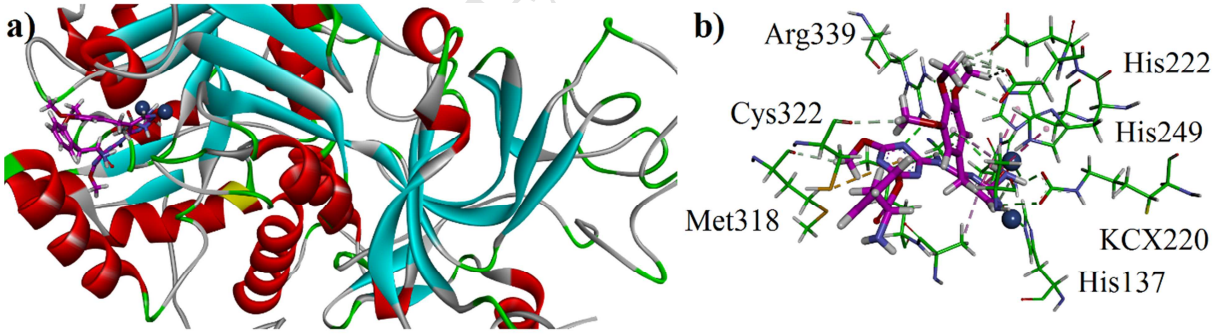


Figure 4: a) Depiction of the lowest energy docking-poses of the **compound 4b** into the binding site of *B. pasteurii* urease (PDB ID 4UBP). A ribbon model of the enzyme is presented; (b) Close-up depiction of the docking pose of **compound 4b** showing different types of ligand-enzyme interactions in the binding site of 4UBP. The key residues are represented as green stick model. Ni atoms (798 and 799) are shown in blue ball model.

Compound **7a** emerged as the most potent inhibitor of the BPU with IC_{50} value of 6.23 ± 0.09 µM. The visual inspection of the lowest energy docked pose of the most active compound **7a** showed that it coordinates with *bi*-nickel center **(**Ni798 and Ni799) *via* its 2,4-diaminopyrimidine ring of TMP (**Figure 5a**). A difference in the activity of **4b** and **7a** is the unsubstituted 4-NH_2 on pyrimidine ring. In **7a**, stronger ligation with Ni *bi*-center and surrounding residues was observed than substituted on (4b). 4-NH_2 group is involved in the hydrogen bonding (HB) with Gly280 and Asp363. While, 2-NH2 group establishes HB interactions with His137 and His139. Ring nitrogen (N-1) forms HB with His249 and His275. His324 forms a HB interactions with carbonyl oxygen of valine. Apart from HB interactions, many π-CH interactions also stabilize the ligand-enzyme complex (**Figure 5b**).

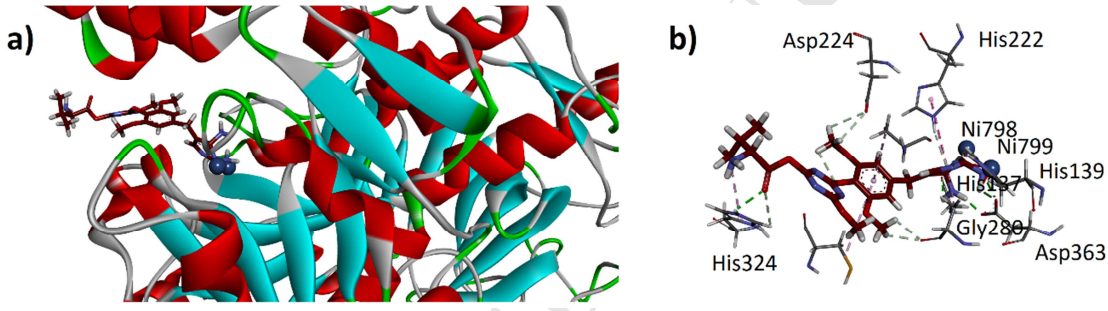


Figure 5: a) Depiction of the lowest energy docking-poses of the most active **compound 7a** into the binding site of *B. pasteurii* urease (PDB ID 4UBP). A ribbon model of the enzyme is presented; (b) Close-up depiction of the docking pose of **compound 7a** showing different types of ligand-enzyme interactions

3. Conclusion

In summary, a series of some novel derivatives of three existing antimicrobial pharmaceuticals namely trimethoprim, metronidazole and isoniazid were synthesized by coupling with some selected Boc-protected amino acids. These derivatives were assayed for their antibacterial activity against *S. aureus*, *B. subtilis*, *E. coli*, *S. typhi* and *P. aeruginosa*. The results of the study revealed that the lipophilicity is an important physio-chemical characteristic for antibacterial

activity. In general high inhibition was demonstrated by the compounds having logP lies in the range of 1.98-2.68. With few exceptions, compounds with greater hydrophobicity are more active against Gram-negative strains. Compounds **7a-c** and **6b** showed good activity against all the tested strains. Urease inhibition activity against *Bacillus pasteurii* was also evaluated. These findings suggest that these compounds are a good starting point for the rational development of new antibacterials.

4. Material and methods

All the reagents and solvents were purchased from standard commercial vendors and were used without any further purification. Amino acids, $(BOC)_2O$ and EDC were purchased from Merck. Trimethoprim, metronidazole and isoniazid was taken from Islamabad Pharmaceutical Products, Islamabad. ^{1}H and ^{13}C-NMR spectra were recorded in deuterated solvents on a Bruker spectrometer at 300 and 75 MHz respectively using tetramethylsilane (TMS) as internal reference. Chemical shifts are given in δ scale (ppm). Melting points were determined in open capillaries using Gallenkamp melting point apparatus (MP-D). The progress of all the reactions was monitored by TLC on 2.0 x 5.0 cm aluminum sheets pre-coated with silica gel 60F254 with a layer thickness of 0.25 mm (Merck). LC-MS spectra were obtained using Agilent technologies 1200 series high performance liquid chromatography comprising of G1315 DAD (diode array detector) and ion trap LCMS G2445D SL. Elemental analyses were conducted using a LECO-932 CHNS Analyzer (LECO Corporation, USA).

4.1. Synthesis of Boc-N-Protected Amino acids (8-10)

Amino group of amino acids (**4-6**) was protected with Boc group by following procedure:

The solution of amino acid (**4-6**) in 75 mL of dioxane, 40 mL of H_2O and 408 mL solution of IM NaOH was stirred and cooled in an ice bath. $(BOC)_2O$ (9g, 41.14 mmol) was added to solution and stirred for one hour at room temperature. The solution was concentrated under vacuum to about 30-40 mL. Cooled in an ice water bath, covered with layer of ethyl acetate (25mL) and acidified with dilute solution of $KHSO_4$ to pH 2-3. The aqueous layer was extracted with ethyl acetate and washed with distilled H_2O, brine and dried over $MgSO_4$. The solvent was removed under reduced pressure and residue was chromatographed on a silica gel column using methanol 2 % solution in chloroform as an eluent to afford pure product in 82 % yield.

4.1.1. 2-(tert-butoxycarbonyl)-3-methylbutanoic acid (8)

White powder, ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.98 (d, 6H, J=6.9 Hz, CH_3), 1.48 (s, 9H, 3 x CH_3), 2.41 (m, 1H, CH), 5.09 (d, 1H, CH), 7.95 (*br*s, 1H, NHBoc), 11.28 (br s, 1H, OH).

4.1.2. 2-(tert-butoxycarbonyl)-3-(1H-indol-3-yl)propanoic acid (9)

White powder, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.49 (s, 9H, 3 x CH_3), 3.43(m, 2H, *CH_2*), 4.96 (t, 1H,*J*=8.4 Hz,*CH*), 7.20 (m, 2H, *Ar-H*), 7.33 (s, 1H, *Ar-H*), 7.45 (m, 2H, *Ar-H*), 7.98 (*br*s, 1H, NHBoc), 10.60 (brs, 1H, NH-indole), 11.30 (brs, 1H, OH).

4.1.3. 2-(tert-butoxycarbonyl)-3-phenylpropanoic acid (10)

White powder, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (s, 9H, 3 x CH_3), 3.24 (dd, 1H, *CH_2*), 3.73 (dd, 1H, *CH_2*), 4.93 (dd, 1H, *CH*), 7.14 (m, 5H, *Ar-H*), 7.97 (*br* s, 1H, NH), 11.29 (brs, 1H, OH).

4.2. Procedure for the deprotection of Boc

A fully protected amino acid (0.86 g, 3.32 mmol) was dissolved by stirring in CH_2C1_2 (7 mL) then at 0^o under N_2, CF_3COOH (8 equiv. 2.05mL, 26.56 mmol) was added drop wise by

maintaining 0 ^{o}C temp of ice bath. The mixture was allowed to stir at room temperature for 6 hours. The organic solvent was evaporated and the residue was dried under vacuum.

4.3. General procedure for the coupling of N-Boc amino acids and antimicrobial drugs

A mixture of Boc-protected amino acid (4 mmol) and HOBt (0.62 g, 4 mmol) in $CHCl_3$ was stirred and cooled to 0 ^{o}C (ice bath). Then, antibacterial drugs (**1-3**) (4 mmol) triethylamine (4 mmol) and EDC (4 mmol) were added. The mixture was allowed to warm to room temperature and stirring continued overnight. The mixture was diluted with $CHCI_3$ and washed with 1 N HC1 (thoroughly), aq. sat. $NaHCO_3$ and NaCl solution. The organic phase was dried $MgSO_4$ and evaporated. The crude residues were purified by column chromatography using n-hexane/ ethyl acetate resulted in 71-80 % yield.

4.3.1. N-(5-(3,4,5-trimethoxybenzyl)-2-aminopyrimidin-4-yl)-2-amino-3-methylbutanamide (1a)

1a was synthesized according to the general procedure by using N-Boc-valine (4 mmol) and trimethoprim (4 mmol). Deprotection of N-Boc according to procedure in **section 4.2** resulted in the pure product **1a**. White solid; R_f = 0.51 ($CHCl_3$/MeOH 6:1); Yield 74%; mp. 183-185 ^{o}C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.98 (d, 6H, *J*=6.6 Hz, CH_3), 2.42 (m, 1H, CH), 3.63 (s, 2H, CH_2), 3.81 (s, 9H, O-CH_3), 5.09 (d, 1H, *J*=6.6 Hz, CH), 6.59 (brs, 2H, NH_2), 6.76 (s, 2H, *Ar-H*), 7.66 (s, 1H, *Ar-H*), 8.01 (brs, 2H, NH_2), 8.73 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 18.3 (2C), 34.3, 36.4, 57.3 (3C), 63.1, 109.3 (2C), 111.4, 128.9, 140.2, 151.6 (2C), 156.0, 160.4, 162.6, 171.8.; LC-MS: *m/z*=390.2 $[M+H]^+$; Anal. Calcd for $C_{19}H_{27}N_5O_4$: C, 58.60; H, 6.99; N, 17.98; Found: C, 58.63; H, 6.97; N, 17.95.

4.3.2. N-(5-(3,4,5-trimethoxybenzyl)-2-aminopyrimidin-4-yl)-2-amino-3-(1H-indol-3-yl)propanamide (1b)

1b was synthesized according to the general procedure by using N-Boc tryptophan (4 mmol) and trimethoprim (4 mmol). After deprotection pure product was obtained in 71% yield. White solid; R_f = 0.54 ($CHCl_3$/MeOH 4:1) ; mp. 216-218 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.43 (m, 2H, *CH_2*), 3.69 (s, 2H, *CH_2*), 3.85 (s, 9H, 3 x OCH_3), 4.96 (t, 1H, *J*=8.4 Hz,*CH*), 6.59 (brs, 2H, NH_2), 6.77 (s, 2H, *Ar-H*), 7.21 (m, 2H, *Ar-H*), 7.31 (s, 1H, *Ar-H*), 7.43 (m, 2H, *Ar-H*), 7.65 (s, 1H, *Ar-H*), 8.03 (brs, 2H, NH_2), 8.75 (*br* s, 1H, NH), 10.63 (*br* s, 1H, NH-indole); ^{13}C-NMR (75 MHz, DMSO-d_6): 32.4, 36.4, 56.8, 57.4 (3C), 109.1 (2C), 109.8, 111.0, 112.4, 117.7, 121.4 (2C), 122.0, 124.1, 126.6, 129.1, 134.2, 138.1, 152.5, 155.8, 160.5, 162.6, 172.0.; LC-MS: *m/z*= 477.2 $[M+H]^+$; Anal. Calcd for $C_{25}H_{28}N_6O_4$: C, 63.01; H, 5.92; N, 17.64; Found: C, 63.03; H, 5.90; N, 17.62.

4.3.3. N-(5-(3,4,5-trimethoxybenzyl)-2-aminopyrimidin-4-yl)-2-amino-3-phenyl propanamide (1c)

White solid; R_f = 0.53 ($CHCl_3$/MeOH 4:1); Yield 74%; mp. 203-205 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.42 (m, 2H, *CH_2*), 3.68 (s, 2H, *CH_2*), 3.81 (s, 9H, O-CH_3), 4.97 (t, 1H, *J*=8.4 Hz, *CH*), 6.69 (brs, 2H, NH_2), 6.78 (s, 2H, *Ar-H*), 7.14 (m, 5H, *Ar-H*), 7.65 (s, 1H, *Ar-H*), 8.03 (brs, 2H, NH_2), 8.76 (*br* s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): 36.3, 38.4, 55.9 (3C), 57.3, 107.7, 109.2, 111.3, 124.9, 126.4, 129.1(2C), 130.7 137.4, 142.5, 151.5 (2C), 155.9 (2C), 160.4, 162.4, 172.0.; LC-MS: *m/z*= 438.2 $[M+H]^+$; Anal. Calcd for $C_{23}H_{27}N_5O_4$: C, 63.14; H, 6.22; N, 16.01; Found: C, 63.12; H, 6.20; N, 16.03.

4.3.4. N'-(2-amino-3-methylbutanoyl)isonicotinohydrazide (2a)

White solid; R_f = 0.57 ($CHCl_3$/MeOH 4:1); Yield 79%; mp. 146-148 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.98 (d, 6H, *J* = 6.6 Hz, CH_3), 2.40 (m, 1H, CH), 5.07 (d, 1H, *J* = 6.6 Hz, CH), 6.61 (brs, 2H, NH_2), 7.68 (d, 2H, *J* = 6.0 Hz, *Ar-H*), 8.73 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 9.22 (*br* s, 1H, NH), 9.64 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 18.4 (2C), 34.2, 62.9, 121.3, 122.5, 141.4, 147.6, 148.1, 162.4, 171.0.; LC-MS: *m/z*= 237.1 $[M+H]^+$; Anal. Calcd for $C_{11}H_{16}N_4O_2$: C, 55.92; H, 6.83; N, 23.71; Found: C, 55.98; H, 6.81; N, 23.69.

4.3.5. N'-(2-amino-3-(1H-indol-3-yl)propanoyl)isonicotinohydrazide (2b)

White solid; R_f = 0.51 ($CHCl_3$/MeOH 4:1); Yield 73%; mp. 189-191 ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.04 (dd, 1H, *J*=16.8, 9.6 Hz, C*H*H), 3.48 (dd, 1H, *J*=16.5, 10.5 Hz, CH*H*), 4.98 (dd, 1H, *J*=10.2, 9.9 Hz, *CH*), 6.58 (brs, 2H, NH_2), 7.19 (m, 2H, *Ar-H*), 7.29 (s, 1H, *Ar-H*), 7.40 (m, 2H, *Ar-H*), 7.67 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 8.74 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 9.22 (*br* s, 1H, NH), 9.63 (*br* s, 1H, NH), 10.61 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 32.4, 56.7, 109.6, 112.4, 121.1, 121.2, 122.1 (2C), 124.1, 126.5 (2C), 134.3, 141.4, 147.5 (2C), 162.5, 172.0.; LC-MS: *m/z*= 324.1 $[M+H]^+$;Anal. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66; Found: C, 63.18; H, 5.32; N, 21.63.

4.3.6. N'-(2-amino-3-phenylpropanoyl)isonicotinohydrazide (2c)

White solid; R_f = 0.47 ($CHCl_3$/MeOH 4:1); Yield 80%; mp. 171-173 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.12 (dd, 1H,*J*=16.8, 9.6 Hz, C*H*H), 3.64 (dd, 1H, *J*=16.5, 9.6 Hz, CH*H*), 4.86 (dd, 1H, *J*=10.2, 9.9 Hz, C*H*), 6.71 (brs, 2H, NH_2), 7.16 (m, 5H, *Ar-H*), 7.66 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 8.73 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 9.23 (*br* s, 1H, NH), 9.65 (*br*s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 38.3, 55.9, 121.1 (2C), 124.8, 126.2 (2C), 128.9 (2C), 137.3, 141.3, 147.4, 148.7,

162.4, 172.5.; LC-MS: *m/z*= 285.1 $[M+H]^+$; Anal. Calcd for $C_{15}H_{16}N_4O_2$: C, 63.37; H, 5.67; N, 19.71; Found: C, 63.32; H, 5.65; N, 19.74.

4.4. General procedure for the synthesis of metronidazole derivatives (3a-c)

In the first step, the tosylate of metronidazole (MTZ) was synthesized by using a reported procedure [28]. 20 mmol (3.42 g) of MTZ was allowed to react with 20 mmol (3.8 g) of 4-methylbenzenesulfonyl chloride (**11**, tosyl chloride) in 20 mL of DCM as a solvent. Few drops of TEA (as a base) was added by constant stirring at 0 ^oC. After the completion of reaction, the tosylate of MTZ (MTZ-OTs) thus formed was extracted with ethyl acetate (2 x 30 mL). The organic layer was combined and washed with saturated $NaHCO_3$ and dried over anhydrous sodium sulphate.

In the second step, a mixture of MTZ-OTs i.e. 5 mmol and DHPM carbohydrazides (5 mmol) was dissolved in 20 ml of DMF as a solvent) and K_2CO_3 (8 mmol) was added in round bottom flask, and stirred at 75-80 ^oC. The reaction condition was monitored by TLC. After completion of the reaction, the reaction mixture was poured into ice and extracted with chloroform. The organic layer was separated and dried over anhydrous sodium sulphate. Finally, the product was purified by using silica gel chromatography ($CHCl_3$-MeOH).

4.4.1. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 2-amino-3-methylbutanoate (3a)

Off white solid; R_f = 0.49 ($CHCl_3$/MeOH 6:1); Yield 83%; mp. 127-129 ^oC; ^1H NMR (300 MHz, DMSO-d_6): δ 0.96 (d, 6H, *J*= 6.6 Hz, CH_3), 2.42 (m, 1H, CH), 2.73 (s, 3H, *CH_3*), 4.47 (t, 2H, *J* = 4.8 Hz, CH_2), 5.10 (d, 1H, CH), 5.46 (t, 2H, *J*=4.8 Hz, CH_2), 6.59 (brs, 2H, NH_2), 7.85 (s, 1H, *Ar-H*); ^{13}C-NMR (75 MHz, DMSO-d_6): 12.0, 17.9 (2C), 34.3 (2C), 62.6, 63.2, 129.1, 140.4,

150.4, 171.3.; LC-MS: *m/z*= 271.1 $[M+H]^+$; Anal. Calcd for $C_{11}H_{18}N_4O_4$: C, 48.88; H, 6.71; N, 20.73; Found: C, 48.82; H, 6.73; N, 20.75.

4.4.2. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 2-amino-3-(1H-indol-3-yl)propanoate (3b)

Off white solid; R_f = 0.45 (n-hexane/ethyl acetate 3:1); Yield 78%; mp. 143-145 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.74 (s, 3H, *CH*$_3$), 3.10 (dd, 1H, *J*=17.1, 7.5 Hz, C*H*H), 3.50 (dd, 1H, *J*=17.1, 12.3 Hz, C*H*H), 4.47 (t, 2H, *J*=4.8 Hz, CH_2), 4.96 (dd, 1H, *J*=12.3, 7.8 Hz, *CH*), 5.44 (t, 2H, *J*=4.8 Hz, CH_2), 6.61 (brs, 2H, NH_2), 7.23 (m, 2H, *Ar-H*), 7.28 (s, 1H, *Ar-H*), 7.38 (m, 2H, *Ar-H*), 7.84 (s, 1H, *Ar-H*), 10.58 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 12.2, 31.9, 36.3, 55.8, 63.0, 109.5, 111.6, 119.0, 120.4 (2C), 124.0, 126.3, 129.0, 134.7, 140.4, 150.5, 172.6.; LC-MS: *m/z*= 358.1 $[M+H]^+$; Anal. Calcd for $C_{17}H_{19}N_5O_4$: C, 57.14; H, 5.36; N, 19.60; Found: C, 57.11; H, 5.38; N, 19.58.

4.4.3. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 2-amino-3-phenylpropanoate (3c)

Off white solid; R_f = 0.53 (n-hexane/ethyl acetate 3:1); Yield 87%; mp. 115-117 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.72 (s, 3H, *CH*$_3$), 3.10 (dd, 1H, *J*=17.1, 7.5 Hz, C*H*H), 3.49 (dd, 1H, *J*=17.1, 12.3 Hz, C*H*H), 4.46 (t, 2H, *J*=4.5 Hz, CH_2), 4.96 (dd, 1H, *J*=12.3, 7.8 Hz, *CH*), 5.47 (t, 2H, *J*=4.5 Hz, CH_2), 6.70 (brs, 2H, NH_2), 7.15 (m, 5H, *Ar-H*), 7.86 (s, 1H, *Ar-H*); ^{13}C-NMR (75 MHz, DMSO-d_6): 11.9, 36.0, 37.7, 56.3. 62.8, 124.7, 125.9, 128.3 (2C), 129.6, 137.0, 140.3 (2C), 149.8, 172.1.; LC-MS: *m/z*= 319.1 $[M+H]^+$; Anal. Calcd for $C_{15}H_{18}N_4O_4$: C, 56.60; H, 5.70; N, 17.60; Found: C, 56.57; H, 5.68; N, 17.62.

4.5. General procedure for the synthesis of di-substituted triazine derivatives (4a-c) (5a-c) (6a-c)

In the first step mono-substituted triazine (**13**) was synthesized by using following procedure. A solution of triazine (**12,** 2 mmol) and K_2CO_3 (4 mmol) was stirred in anhydrous THF at 0 ^oC under nitrogen. Antimicrobial drugs (**1-3**) were then added drop-wise and the reaction mixture was allowed to warm at room temperature. The progress of the reaction was monitored by TLC. After the completion of the first step, a solution of N-Boc protected amino acids (**8-10**) in THF were added drop-wise. After the completion of reaction the mixture, the crude residues were purified by column chromatography using n-hexane / ethyl acetate. In the final step the di-substituted product was de-protected by using procedure described in **section 4.2**.

4.5.1. 4-(5-(3,4,5-trimethoxybenzyl)-2-aminopyrimidin-4-ylamino)-6-methoxy-1,3,5-triazin-2-yl 2-amino-3-methylbutanoate (4a)

4a was synthesized according to the general procedure by using TMP, triazine and valine. White solid; R_f = 0.47 (n-hexane/ethyl acetate 5:1); Yield 65%; mp. 210-212 ^oC; ^1H NMR (300 MHz, DMSO-d_6): δ 0.97 (d, 6H, *J*=6.9 Hz, CH_3), 2.44 (m, 1H, CH), 3.64 (s, 2H, *CH_2*), 3.77 (s, 9H, 3 x OCH_3), 3.96 (s, 3H, OCH_3), 5.01 (d, 1H, *J*=6.9 Hz, CH), 6.63 (brs, 2H, NH_2), 6.80 (s, 2H, *Ar-H*), 7.64 (s, 1H, *Ar-H*), 7.97 (brs, 2H, NH_2), 8.63 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 18.7 (2C), 33.9, 36.5, 56.1 (2C), 57.0, 62.9, 104.8, 106.8, 110.0 (2C), 111.8, 128.7, 140.2, 151.8, 155.9 (2C), 160.4, 168.1, 169.5, 171.2, 180.9; LC-MS: *m/z*= 515.2 $[M+H]^+$; Anal. Calcd for $C_{23}H_{30}N_8O_6$: C, 53.69; H, 5.88; N, 21.78; Found: 53.76; H, 5.85; N, 21.76.

4.5.2. 4-(5-(3,4,5-trimethoxybenzyl)-2-aminopyrimidin-4-ylamino)-6-methoxy-1,3,5-triazin-2-yl 2-amino-3-(1H-indol-2-yl)propanoate (4b)

4b was synthesized according to the general procedure by using TMP, triazine and tryptophan. White solid; R_f = 0.45 (n-hexane/ethyl acetate 5:1); Yield 61%; mp. 217-219 ^oC; ^1H NMR (300

MHz, DMSO-d_6): δ 3.42(m, 2H, C*H*$_2$), 3.69 (s, 2H, *CH*$_2$), 3.78 (s, 9H, 3 x OCH_3), 3.82 (s, 3H, OCH_3), 4.99 (t, 1H, *J*=8.7 Hz, *CH*), 6.63 (brs, 2H, NH_2), 6.78 (s, 2H, *Ar-H*), 7.18 (m, 2H, *Ar-H*), 7.33 (s, 1H, *Ar-H*), 7.38 (m, 2H, *Ar-H*), 7.66 (s, 1H, *Ar-H*), 7.98 (brs, 2H, NH_2), 8.62 (*br* s, 1H, NH), 10.55 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 31.9, 36.7, 56.2 (2C), 57.2, 58.1 (2C), 109.5 (2C), 109.9, 111.1, 112.4, 121.6, 122.1, 124.3, 125.9, 129.0, 134.2, 139.4 (2C), 152.5 (2C), 155.8, 160.5, 162.6, 168.4, 169.6, 171.0, 180.8.; LC-MS: *m/z*= 602.2 $[M+H]^+$; Anal. Calcd for $C_{29}H_{31}N_9O_6$: C, 57.90; H, 5.19; N, 20.95; Found: C, 57.92; H, 5.17; N, 20.97.

4.5.3. 4-(5-(3,4,5-trimethoxybenzyl)-2-aminopyrimidin-4-ylamino)-6-methoxy-1,3,5-triazin-2-yl 2-amino-3-phenylpropanoate (4c)

4c was synthesized according to the general procedure by using TMP, triazine and phenylalanine. White solid; R_f = 0.51 (n-hexane/ethyl acetate 5:1); Yield 70%; mp. 199-201 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.14(dd, 1H, *J*=16.8, 9.6 Hz, C*H*H), 3.45 (dd, 1H, *J*=16.5, 10.5 Hz, C*H*H), 3.68 (s, 2H, *CH*$_2$), 3.77 (s, 9H, 3 x OCH_3), 3.81 (s, 3H, OCH_3), 4.94 (dd, 1H, *J*=10.2, 9.9 Hz, C*H*), 6.65 (brs, 2H, NH_2), 6.79 (s, 2H, *Ar-H*), 7.17 (m, 5H, *Ar-H*), 7.64 (s, 1H, *Ar-H*), 7.97 (brs, 2H, NH_2), 8.61 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 36.4, 38.6, 56.4 (3C), 57.1, 60.34 (2C), 108.8, 111.4 (2C), 125.1, 126.4, 129.3 (2C), 137.4, 139.3 (2C), 151.5, 155.9 (2C), 160.4, 162.4, 168.1, 169.8, 171.3, 180.4.;LC-MS: *m/z*= 563.2 $[M+H]^+$; Anal. Calcd for $C_{27}H_{30}N_8O_6$: C, 57.64; H, 5.37; N, 19.92; O, 17.06; Found: C, 57.68; H, 5.34; N, 19.95.

4.5.4. 4□methoxy□6□[(pyridin□4□yl)hydrazido]□1,3,5□triazin□2□yl 2□amino□3□methylbutanoate (5a)

5a was synthesized according to the general procedure by using INH, triazine and valine. White solid; R_f = 0.41 (n-hexane/ethyl acetate 4:1); Yield 71%; mp. 129-131 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.10 (d, 6H, *J*=6.6 Hz, CH_3), 2.38 (m, 1H, CH), 3.80 (s, 3H, OCH_3), 4.78 (*br* s, 1H, NH), 5.04 (d, 1H, *J*=6.6 Hz, CH), 6.63 (brs, 2H, NH_2), 7.65 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 8.72 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 9.64 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 18.6 (2C), 34.0, 37.6, 55.2, 62.7, 121.1, 141.6, 147.9, 162.1, 169.1, 172.4, 172.5, 173.3, 181.1; LC-MS: *m/z*=362.2 $[M+H]^+$; Anal. Calcd for $C_{15}H_{19}N_7O_4C$, 49.86; H, 5.30; N, 27.13; Found:C, 49.82; H, 5.28; N, 27.16.

4.5.5. 4□methoxy□6□[(pyridin□4□yl)hydrazido]□1,3,5□triazin□2□yl 2□amino□3□(1H□indol□3□yl)propanoate (5b)

5b was synthesized according to the general procedure by using INH, triazine and tryptophan. Milky white solid; R_f = 5.0 (n-hexane/ethyl acetate 6:1); Yield 70 %; mp. 163-165 ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.08(dd, 1H,*J*=16.8, 9.6 Hz, C*H*H), 3.43 (dd, 1H, *J*=16.5, 10.5 Hz, CH*H*), 3.81 (s, 3H, CH_3O), 4.98 (dd, 1H, *J*=10.2, 9.9 Hz, *CH*), 4.80 (*br* s, 1H, NH), 6.36 (brs, 2H, NH_2), 7.20 (m, 2H, *Ar-H*), 7.32 (s, 1H, *Ar-H*), 7.38 (m, 2H, *Ar-H*), 7.67 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 8.74 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 9.60 (*br* s, 1H, NH), 10.64 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 37.5, 55.4, 62.5, 109.6, 112.4, 121.1, 121.2, 122.1, 124.1, 126.5, 134.3, 141.4, 147.5 (2C), 162.5, 171.1, 166.8, 172.0, 172.3, 173.6, 181.3; LC-MS: *m/z*= 449.2 $[M+H]^+$; Anal. Calcd for: $C_{21}H_{20}N_8O_4C$, 56.25; H, 4.50; N, 24.99; Found:C, 56.27; H, 4.52; N, 24.97.

4.5.6. 4□methoxy□6□[(pyridin□4□yl)hydrazido]□1,3,5□triazin□2□yl 2□amino□3□phenyl-propanoate (5c)

5c was synthesized according to the general procedure by using INH, triazine and phenylalanine. Milky white solid; R_f = 0.52 (n-hexane/ethyl acetate 6:1); Yield 74 %; mp. 181-183 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.14 (dd, 1H,*J*=16.8, 9.6 Hz, C*H*H), 3.74(dd, 1H, *J*=16.5, 9.6 Hz, CH*H*), 3.82 (s, 3H, OCH_3), 4.96 (dd, 1H, *J*=10.2, 9.9 Hz, C*H*), 4.77 (*br* s, 1H, NH), 6.56 (brs, 2H, NH_2), 7.11 (m, 5H, *Ar-H*), 7.63 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 8.75 (d, 2H, *J*= 6.0 Hz, *Ar-H*), 9.62 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 37.4, 55.4, 62.2, 124.1, 124.7, 127.7, 127.1, 128.8, 129.9, 130.8, 134.3, 141.8 (2C), 151.3, 166.9, 172.1, 172.8, 177.2, 181.09; LC-MS: *m/z*=410.2 $[M+H]^+$; Anal. Calcd for $C_{19}H_{19}N_7O_4C$, 55.74; H, 4.68; N, 23.95; Found: C, 55.70; H, 4.66; N, 23.97.

4.5.7. 4-methoxy-6-(2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethoxy)-1,3,5-triazin-2-yl 2-amino -3-methylbutanoate (6a)

6a was synthesized according to the general procedure by using MTZ, triazine and valine. Light yellow solid; R_f = 0.56 (n-hexane/ethyl acetate 4:1); Yield 78 %; mp. 121-123 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.00 (d, 6H, *J*=6.6 Hz, CH_3), 2.44 (m, 1H, CH), 2.72 (s, 3H, *CH_3*), 3.81 (s, 3H, OCH_3), 4.48 (t, 2H, *J*= 4.8 Hz, CH_2), 5.10 (d, 1H, *J*= 6.6 Hz, CH), 5.45 (t, 2H, *J*=4.8 Hz, CH_2), 6.61 (brs, 2H, NH_2), 7.84 (s, 1H, *Ar-H*); ^{13}C-NMR (75 MHz, DMSO-d_6): 12.4, 17.6 (2C), 34.1, 38.1, 57.2, 62.7, 73.1, 129.0, 140.8, 150.6, 169.3, 170.5, 172.9, 181.1; LC-MS: *m/z*=396.1 $[M+H]^+$; Anal. Calcd for $C_{15}H_{21}N_7O_6$: C, 45.57; H, 5.35; N, 24.80; Found: C, 45.53; H, 5.37; N, 24.78.

4.5.8. 4-methoxy-6-(2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethoxy)-1,3,5-triazin-2-yl 2-amino-3-(1H-indol-3-yl)propanoate (6b)

6b was synthesized according to the general procedure by usingMTZ, triazine and tryptophan. Yellow solid;R_f = 0.51 (n-hexane/ethyl acetate 5:1); Yield 73%. m.p. 148-150 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.73 (s, 3H, *CH*$_3$), 3.13(dd, 1H, *J*=17.1, 7.5 Hz, C*H*H), 3.47(dd, 1H, *J*=17.1, 12.3 Hz, C*H*H), 3.80 (s, 3H, OCH_3), 4.45 (t, 2H, *J*=4.8 Hz, CH_2), 4.94 (dd, 1H, *J*=12.3, 7.8 Hz, *CH*), 5.41 (t, 2H, *J*=4.8 Hz, CH_2), 6.63 (brs, 2H, NH_2), 7.26 (m, 2H, *Ar-H*), 7.30 (s, 1H, *Ar-H*), 7.37 (m, 2H, *Ar-H*), 7.82(s, 1H, *Ar-H*), 10.56 (*br* s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 12.3, 32.01, 39.5, 57.3, 62.8, 73.4, 109.1, 111.8, 119.05, 120.3, 123.8, 126.1, 127.8, 129.2, 134.4, 140.6, 150.7, 170.1, 172.6, 172.8, 181.2; LC-MS: *m/z*=483.2 $[M+H]^+$; Anal.Calcd for $C_{21}H_{22}N_8O_6$ C, 52.28; H, 4.60; N, 23.23 Found: C, 52.32; H, 4.58; N, 23.25.

4.5.9. 4-methoxy-6-(2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethoxy)-1,3,5-triazin-2-yl 2-amino-3-phenylpropanoate (6c)

6c was synthesized according to the general procedure by using MTZ, triazine and phenylalanine. Light yellow solid; R_f = 0.58 (n-hexane/ethyl acetate 5:1); Yield 83%; mp. 131-133 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.71 (s, 3H, *CH*$_3$), 3.14 (dd, 1H, *J*=17.1, 7.5 Hz, C*H*H), 3.45 (dd, 1H, *J*=17.1, 12.3 Hz, C*H*H), 3.82 (s, 3H, OCH_3), 4.44 (t, 2H, *J*=4.5 Hz, CH_2), 4.93 (dd, 1H, *J*=12.3, 7.8 Hz, *CH*), 5.44 (t, 2H, *J*=4.5 Hz, CH_2), 6.72 (brs, 2H, NH_2), 7.16 (m, 5H, *Ar-H*), 7.84 (s, 1H, *Ar-H*); ^{13}C-NMR (75 MHz, DMSO-d_6): 11.7, 36.02, 37.4, 56.6, 57.4, 62.9, 68.8, 124.5, 126.10, 128.1, 129.7, 137.1, 140.5, 150.02, 170.3 (2C), 172.4, 173.0, 181.1; LC-MS: *m/z*=444.16 $[M+H]^+$; Anal. Calcd for $C_{19}H_{21}N_7O_6$ C, 51.47; H, 4.77; N, 22.11; Found: C, 51.43; H, 4.79; N, 22.09.

4.6. General Procedure for the synthesis of trimethoprim derivatives (7a-c)

For the synthesis of **7a-c**, first of all we synthesized hydroxy TMP (**16**) according to our previously reported procedure [16].

5.8 g of trimethoprim (20mmol) was dissolved in 40 % HBr at room temperature. The mixture was then stirred at 100 ºC. After completion of reaction (TLC), the mixture was quenched addition of 50 % NaOH aq. (5-10 mL). After cooling to room temperature, it was placed at 4 ºC overnight to form crystals. The crystals were filtered and washed with ice-cold water (ca. 20 mL). After the crystals were dissolved in boiling water, the solution was neutralized to ca. pH 7 with 28% aqueous ammonia and placed at 4 ºC overnight for recrystallization. White crystals were filtered, washed with ice-cold water (*ca.* 100 mL), affording compound **16**. Its spectral data is in agreement with our previously reported for the same compound [16].

4.6.1. 4-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)-6-methoxy-1,3,5-triazin-2-yl 2-amino-3-methylbutanoate (7a)

7a was synthesized according to the general procedure by using TMP (4 mmol), triazine (4 mmol) and valine (4 mmol). Deprotection of N-Boc according to procedure in **section 4.2** resulted in the pure product **7a**. White solid; R_f = 0.48 (n-hexane/ethyl acetate 4:1); Yield 70 %; mp. 223-225 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.99 (d, 6H, *J*=6.9 Hz, CH_3), 2.43 (m, 1H, CH), 3.64 (s, 2H, *CH_2*), 3.83 (s, 6H, OCH_3), 3.90 (s, 3H, OCH_3), 5.06 (d, 1H, *J*=6.9 Hz, CH), 6.55 (brs, 2H, NH_2), 6.81 (s, 2H, *Ar-H*), 7.46 (*br* s, 2H, NH_2), 7.63 (s, 1H, *Ar-H*), 7.91 (brs, 2H, NH_2); ^{13}C-NMR (75 MHz, DMSO-d_6): 18.0 (2C), 34.6, 36.7, 56.9, 57.4, 63.1, 107.5, 108.9, 111.6, 128.3, 140.2, 151.6, 156.0, 158.1, 160.4, 162.6, 171.8, 173.3, 177.7, 181.5, 183.6. .LC-MS: *m/z*=501.21 $[M+H]^+$; Anal. Calcd for $C_{22}H_{28}N_8O_6C$, 52.79; H, 5.64; N, 22.39; Found: C, 52.75; H, 5.62; N, 22.41.

4.6.2. 4-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)-6-methoxy-1,3,5-triazin-2-yl 2-amino-3-(1H-indol-3-yl)propanoate (7b)

7b was synthesized according to the general procedure by using TMP (4 mmol), triazine (4 mmol) and tryptophan (4 mmol). After deprotection pure product was obtained in 68 % yield. White solid; R_f = 0.43 (n-hexane/ethyl acetate 4:1); mp.. 234-236 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.48 (m, 2H, *CH*$_2$), 3.72 (s, 2H, *CH*$_2$), 3.83 (s, 6H, 3 x OCH_3), 3.94 (s, 3H, OCH_3), 4.96 (t, 1H, *J*=8.4 Hz,*CH*), 6.61 (brs, 2H, NH_2), 6.71 (s, 2H, *Ar-H*), 7.25 (m, 2H, *Ar-H*), 7.33 (s, 1H, *Ar-H*), 7.42 (brs, 2H, NH_2), 7.47 (m, 2H, *Ar-H*), 7.63 (s, 1H, *Ar-H*), 7.91 (*br* s, 2H, NH_2), 10.65 (*br* s, 1H, NH-indole); ^{13}C-NMR (75 MHz, DMSO-d_6): 32.6, 36.1, 55.9, 56.8 (2C), 57.2, 106.3, 109.1, 109.6, 111.3, 112.6, 121.5, 122.2, 124.5, 126.1, 126.8, 129.3, 134.8, 152.3, 156.07, 160.2, 161.3, 162.9, 170.7, 172.1, 172.4, 173.6, 181.3; LC-MS: *m/z*= 588.2 $[M+H]^+$; Anal. Calcd for $C_{28}H_{29}N_9O_6$. C, 57.23; H, 4.97; N, 21.45; Found: C, 57.28; H, 4.95; N, 21.47.

4.6.3. 4-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)-6-methoxy-1,3,5-triazin-2-yl 2-amino-3-phenylpropanoate (7c)

7c was synthesized according to the general procedure by using TMP (4 mmol), triazine (4 mmol) and phenylalanine (4 mmol). White solid; R_f = 0.42 (n-hexane/ethyl acetate 5:1); Yield 74% ; mp. 217-219 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): 3.49 (m, 2H, *CH*$_2$), 3.73 (s, 2H, *CH*$_2$), 3.79 (s, 6H, OCH_3), 3.92 (s, 3H, OCH_3), 4.96 (t, 1H, *J*=8.4 Hz, *CH*), 6.60 (brs, 2H, NH_2), 6.69 (s, 2H, *Ar-H*), 7.18 (m, 5H, *Ar-H*), 7.46 (brs, 2H, NH_2), 7.60 (s, 1H, *Ar-H*), 7.89 (*br* s, 2H, NH_2); ^{13}C-NMR (75 MHz, DMSO-d_6): 33.4, 39.1, 51.2, 55.7, 56.1, 56.9, 105.9, 108.3, 109.6, 125.3, 126.9, 129.4, 137.4, 151.5, 152.3, 156.2, 156.8, 160.2, 160.5, 161.3, 162.6, 162.9, 170.9, 171.9,

172.7, 173.3, 178.6, 181.8. LC-MS: *m/z* = 549.21 $[M+H]^+$; Anal. Calcd for $C_{26}H_{28}N_8O_6C$, 56.93; H, 5.14; N, 20.43 Found: C, 56.95; H, 5.12; N, 20.41.

4.7. Antibacterial Screening:

Antibacterial and urease inhibition activity was performed at Department of Pharmacy, Quaid-i-Azam University Islamabad, Pakistan. Antibacterial activity was carried out against standard ATCC (American type culture collection) according to our previously reported procedure [28]. The standard bacterial strains; *Staphylococcus aureus* (ATCC- 6538), *Escherichia coli* (ATCC-25922), *Bacillus subtilis* (ATCC-6633) and *Pseudomonas aeruginosa* (ATCC-15442) and *S. typhi* (ATCC-14028) were used for the determination of antibacterial activity. All bacterial strains were cultured on nutrient agar and incubation was done at 37°C for 24 hours. While stock culture was preserved at 4°C.

4.7.1. Stock solutions

The 4 mg precisely weighed test samples were dissolved in 1 ml of DMSO. Stock solution of standard antibiotic i.e. Trimethoprim, Ciprofloxacin and Roxithromycin (4mg/mL) were also prepared in DMSO. Inoculum preparation. To 10 ml of sterile nutrient broth, a sterile loop full from a colony of bacteria was added. Incubation was carried out for 24 hours at 37°C and turbidity of the inoculum was adjusted by using McFarland 0.5 turbidity standard.

4.7.2. Procedure

The samples were tested in order to determine their minimum inhibitory concentration for 90% of organisms (MIC_{90}). Stock solution of each sample was serially diluted in 96-well microtiter plate with nutrient broth to obtain a final concentration ranging from 256 μg/mL to 0.16 μg/mL. A standardized inoculum for each bacterial strain was prepared so as to give inoculum density of

approximately 5 × 104 CFU/ml in each well. Accurately measured 195 µL of bacterial culture was dispensed in each well containing sample with the aid of micropipette. DMSO (1% final concentration) was used as negative control. Trimethoprim, Ciprofloxacin and Roxithromycin were used as reference or positive control. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the growth of bacterial strain. The assay was performed as triplicate analysis.

4.8. Urease inhibition assay

The synthesized conjugates were tested for their antibacterial potential using our previously reported indophenol's method [27]. Thiourea was used as standard inhibitor. Reaction mixture comprising of 25 µl of *B. pasteurii* urease, 55 µL of buffer containing 100 mM urea and 5 µL of test compounds were incubated at 30 ^{o}C for 20 minutes. 45 µL of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL alkali reagent (0.5% w/v NaOH and 0.1% v/v NOCl) were added to each tube. The increasing absorbance was measured at 630 nm using microplate reader (ELX-800 BioTek, USA). The results (change in absorbance per min.) were processed by using the Soft-Max Pro s4.5 software (Molecular Devices, USA).

4.9. Computational studies

Molecular docking studies were carried out using X-ray crystal structure of *B. pasteurii urease* (PDB code 4UBP) co-crystalized with acetohydroxamic acid (HEA). The structures of the compounds were drawn using Marvin Sketch 16.5.2 [29]. These ligands were optimized using "prepare ligands" in AutoDock 4.2. For enzyme downloaded from PDB, solvation parameters and Kollman charges for all the atoms were assigned. AutoDock Tools (ADT) were used to

create PDBQT file for both ligand and enzyme. A grid parameter file was generated using ADT. A cubic grid box of 40 Å (x, y, z) with a spacing of 0.375 Å was created. The grid map was created and centered in the active site region where E2020 (native ligand) embedded (X= 29.211182; Y= 71.746364; Z= 74.805909). To evaluate the lowest binding energy, docking studies were carried out using AutDock and a Lamarckian genetic algorithm (LGA) [30]. The maximum number of energy evaluations of (LGA) run was 2500,000 and the maximum number of evaluations were set to 27,000. Other parameters were set to default values of AutoDock 4.2. The view of the docking results and analysis of their surface with graphical representations were done using AutoDock and discovery studio visualizer [31]. LogP values were calculated via online software from Virtual Computational Chemistry Laboratory [32]

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Highlights

- 21 new compounds from 7 series of amino acids conjugates of three commercially available antimicrobial drugs were synthesized.
- *In vitro* antibacterial activity against five ATCC bacterial strains was assessed.
- Trimethoprim conjugates showed excellent in vitro activity against both strains.
- A close relationship between the lipophilicity and the activity was identified.
- In vitro *Bacillus pasteurii* urease inhibition was also studied.

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