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A new efficient domino approach for the synthesis of coumarin-pyrazolines as antimicrobial agents targeting bacterial D-alanine-D-alanine ligase

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Abstract

Inhibition of D-alanine-D-alanine ligase (Ddl) prevents bacterial growth, which makes this enzyme an attractive and viable target in the urgent search of novel effective antimicrobial drugs. In this work, a series of novel coumarin linked pyrazoline inhibitors of D-alanine-D-alanine ligase were synthesized and evaluated as inhibitors of *Escherichia coli* DdlB ligase in order to target resistant strain of bacteria by using environmentally benevolent β-cyclodextrin as a supramolecular catalyst *via* one-pot four component synthesis in water as a green reaction media. All the newly synthesized compounds have been characterized by elemental analysis and various spectroscopic methods. The new procedure has noteworthy advantages including easy work-up, short reaction times, high yields of products and column free synthesis. The synthesized compounds were evaluated *in vitro* for their antimicrobial activity. Among the synthesized compounds, namely 3-(5-(4-hydroxy-3-methoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one (**5f)** was found to be the most potent D-Alanine-D-Alanine ligase enzyme inhibitor with IC_{50} value 106 μM and the compound 3-(5-(p-tolyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one (**5g)** was found to be the second most potent inhibitor of the DdlB

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enzyme with IC_{50} value 111 μM against the standard D-Cycloserine. In addition, SAR study provides the evidence that -OH, $-CH_3$ and $–OCH_3$ group at 4- and 3-position of the coumarins linked to pyrazolines scaffold is increasing the enzymatic inhibition, followed by the molecular docking study of most active compounds **5a, 5g,** and **5j** against DdlB enzyme of E. coli exhibited good binding properties. This work thus highlights the coumarin linked pyrazoline motif as a very promising tool for the development of novel antimicrobial compounds acting through an interesting bactericidal mechanism of action.

1. Introduction

Over the last decades, the principles of green chemistry have been successfully embraced by the scientific community and now the awareness of environmental issues regarding chemical processes is considered mandatory. Several contributions to the green chemistry issue can be found in the literature, covering different aspects of this topic.[1-5] In this scenario, chemists and chemical engineers are expected to develop safe, sustainable and eco-friendly processes to attend all social demands as well as economic development, whilst providing environmental protection and preservation of the natural resources for future generations. Multicomponent reactions (MCRs) represent highly suitable synthetic tools to provide molecular keys, which fulfilling these criteria. In MCRs, three or more precursor components are combined to one reaction product, containing moieties of all precursors.[6] The "one-pot strategy" is considered the most efficient way to accomplish the synthesis of pharmaceutically relevant structures. In contrast to conventional multi-step reactions, it allows a fast way to build up substance libraries with the advantage of pot, step, and atom economy.[7] They also offer an eloquent tool for the one-pot synthesis of the distinct and complex molecule as well as small and drug-like heterocycles.

Microbial infections are becoming the most pressing issue for global health and the economy.[8] In recent years, the treatment of bacterial infections has become a major challenge in the realm of conventional antibiotic therapy.[9] The emergence of bacterial resistance to established antibiotics, as well as hospital-acquired infections, causes a growing concern for the global community.[10] Thus, increasing the resistance of microorganisms to currently available antimicrobial drugs is the major cause of morbidity and mortality throughout the world.[11] In an era of growing antibiotic resistance, the search for effective molecules with novel mechanisms of action is a priority.[12] Today, the bacterial cell wall and the enzymes involved in peptidoglycan

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biosynthesis, a major cell wall structural component, constitute validated targets of many antimicrobial agents.[13] Searching for molecules acting on still unexploited or ill-exploited targets represents, therefore, an important strategy for maintaining our capacity to effectively fight bacteria and avoiding returning to the pre-antibiotic era. Coumarins represent an important family of precious structural units, largely distributed in a wide range of natural products and pharmaceutical candidates.[11–17] The functionalization of naturally occurring skeletons has gained significant attention, as interesting and unexpected biological properties would be generated.[18-20] Some synthetic analogs of 3-substituted coumarin derivatives **(Fig. 1)** were reported in the literature possess antibacterial and anticonvulsant activities respectively.[21] In addition, a lot of coumarin compounds as medicinal candidates for drugs with strong pharmacological activity, low toxicity and side effects, fewer drug resistance, high bioavailability, broad spectrum, better curative effects, etc., to treat various types of diseases are being actively studied.[22] Alternatively, the literature survey reveals that pyrazoline shows an integral architectural concept in heterocyclic chemistry, the importance of pyrazoline ring a scaffold for new anti-neoplastic agents was widely investigated,[23,24] which also represents a common motif in many pharmaceutically active compounds and demonstrating a wide range of activities.

It was also envisaged that coumarin and pyrazoline pharmacophores if linked together, would generate novel molecular templates which are likely to exhibit interesting biological properties in animal models. In particular, those pyrazoline pharmacophores linked to coumarin have been reported to possess antitumor, antimalarial and anticancer properties.[25] Against this background, the efforts are concentrated on establishing coumarin scaffold integrated with pyrazoline frame-work to describe the relevance pharmacological activity. Based on these interesting biological activity profiles of coumarins and pyrazolines analogs, we are inspired and made an effort to synthesize some new number of coumarin integrated pyrazolines analogs as potent antibacterial agents targeting D-alanine-D-alanine ligase (Ddl) in bacteria. Molecular docking of the drug molecule with the receptor (target) offers important information about drug-receptor interactions and is commonly employed to identify the binding orientation of drug molecules to their protein targets in order to predict the affinity and activity.[26] Expectedly, the additive effect of this combination might produce a synergistic effect in enhancing the bioactivity of the coumarin-pyrazoline derivatives.

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The captivating framework of coumarin linked pyrazoline increased the thirst of chemists in preparing these compounds by numerous methods.[25] Many of these methods, however, engage synthetic problems associated with difficult separation processes and expensive, hardly available, or non-recoverable catalysts, expensive reagents, or long reaction time. Therefore, there is a need to develop a new efficient and green method for the synthesis of these heterocyclic compounds. Hence, in a quest for a new easy and eco-friendly procedure for the synthesis of coumarins linked to pyrazoline, we planned our strategy to exploit cyclodextrins as catalysts an aqueous medium. Cyclodextrin-mediated organic reactions an aqueous medium are very useful both from economical and environmental point of view. Cyclodextrins apart from being nontoxic are considered to be metabolically safe. Cyclodextrins are cyclic oligosaccharides of D-(+)-glucopyranosyl units linked by α-1,4-glycosidic bonds with a hydrophilic outer surface and a hydrophobic central cavity, of different sizes, and are able to form complexes with the hydrophobic guest in water.[27] They have substrate selective binding ability and catalyze a wide range of chemical reactions through noncovalent bonding, forming reversible host-guest complexes just like enzymes. There are several examples in organic chemistry of reactions catalyzed by cyclodextrins. They have been used to catalyze oxidations,[28] reductions,[29,30] ring openings,[31] protections,[32] deprotections,[33,34] and even cycloadditions.[35,36] In all of these examples, the cyclodextrins have always been used in a catalytic amount and always recovered and reused.

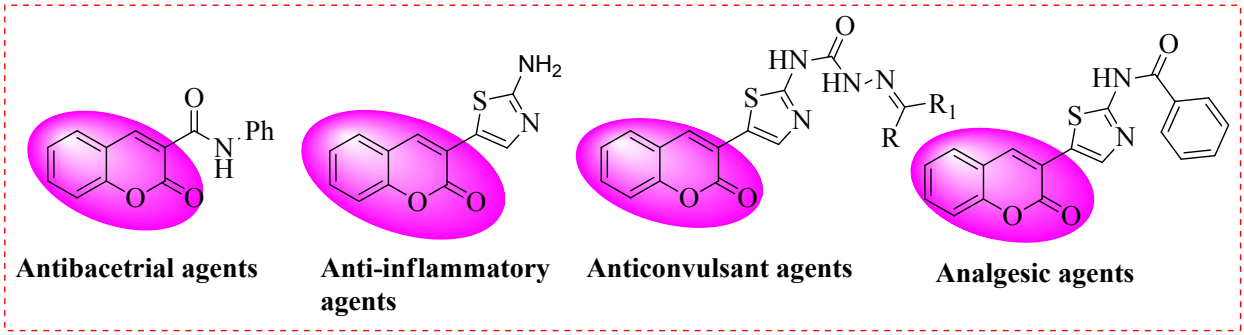

Figure 1. Representative examples of pharmacologically active compounds bearing coumarin-annulated scaffolds

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2. Result and discussion

In our initial study toward the development of this methodology, a model reaction between salicylaldehyde, **1** (1 mol), ethyl acetoacetate **2** (1 mol), hydrazine hydrate **3** (1 mol), and benzaldehyde **4** (1 mol) **(Scheme 2)**, using water as solvent, was investigated in detail by varying the catalyst, in order to develop optimized conditions **(Table 1)**. Then, under the optimized reaction conditions, we carried out one-pot reaction between salicylaldehyde, ethyl acetoacetate, hydrazine hydrate, and benzaldehyde in water (in the absence of cyclodextrin) and in water (in the presence of cyclodextrin) to compare the reported with the new strategy and to investigate the effects of time and yield **(Scheme 2).** In all the instances, the reaction did not proceed with as expected, improvement of yields which resulted in **(Table 1,** entry 1**)**.

Then the screening was initiated by using the main cyclodextrins (α-CD, β-CD, and γ-CD) to determine their catalytic efficiency. The catalytic activity of CD was established on the basis that coumarin linked pyrazoline formation was not observed in the absence of cyclodextrin in water at 100 °C for 7-8 hr or longer time **(Table 1**, entry 1**)**. The α-CD and γ-CD did not show any catalytic activity in this transformation, maybe the small or large cavity is not able to form inclusion complexes with the substrates. Superbly good results were obtained with β-CD as catalyst **(Table 1**, entry 3**)**. Of course, β-CD is able to include in its cavity of all substrates, but lowering the amount of β-CD to 5 mol% gave identical results to the former reactions. By increasing the catalyst of mol% gives an excellent yield of product, which showed that cyclodextrins play an essential role to catalyze the reaction. Hence β-CD was chosen as a catalyst for this transformation. The results changed using 15 mol% of cyclodextrin; high yields of products were observed, in fact, when the quantity of catalyst was increased to over 20 mol%, the yield was constant, This behavior could be due to the formation of host-guest complexes in which the reactants are hosted inside its lipophilic cavity by means of non-covalent interactions, and it provides an indirect proof that the β-CD behaves as an effective chemical reactor. In this way, the substrates are first solubilized in the aqueous medium, where they would be otherwise insoluble. Second, the large dimensions of the β-CD cavity would allow the formation of complexes, which could be either homo- or heterocomplexes. In particular, the formation of heterocomplexes.

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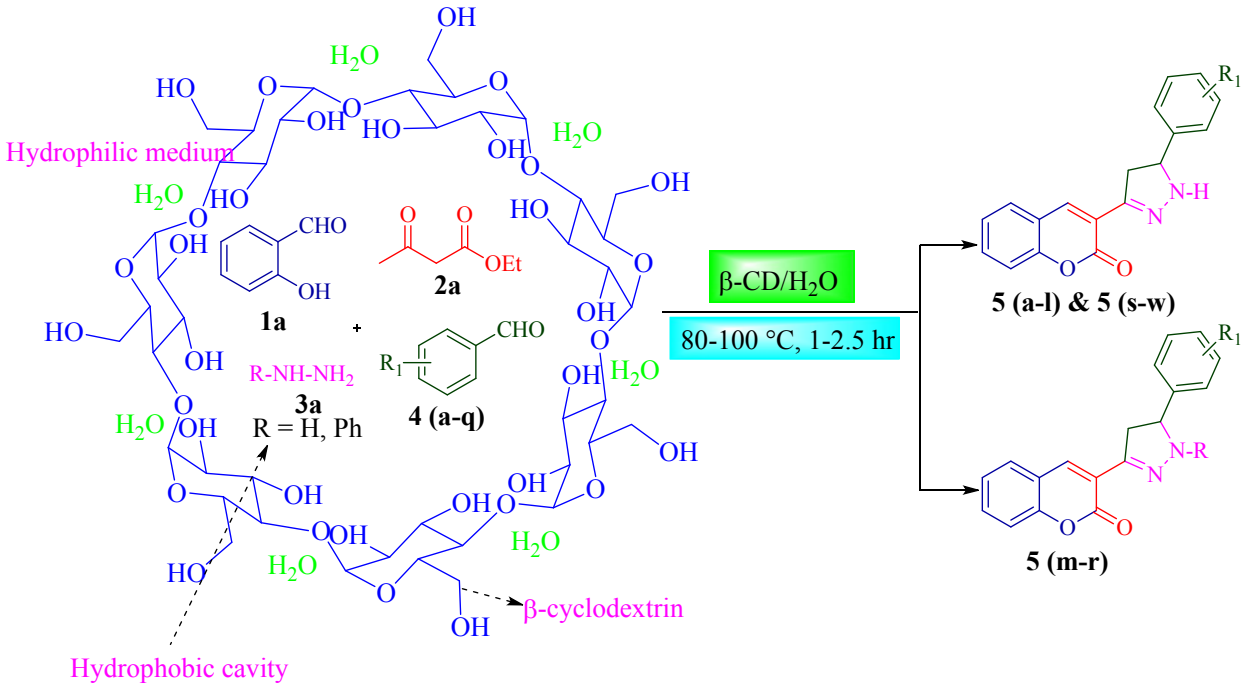
We also screened different solvents such as EtOH, CH_3OH, 1,4-Dioxane, EtOH: H_2O, DMSO, THF, CH_3CN, and H_2O with cyclodextrin a catalyst **(Table 1**, entries 5-11**)**. After several optimizations, we found promising results with water as a solvent due to better solubility of β-cyclodextrin in water. Subsequently, to verify the general procedure of reaction variously substituted aldehyde and substituted hydrazine hydrate were tested under optimized reaction conditions, the results have been summarized in **(Table 2**).



Scheme 1. General scheme for the synthesis of 3-(5-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-2*H*-chromen-2-one and 3-(1,5-diphenyl-4,5-dihydro-1H-pyrazol-3-yl)-2*H*-chromen-2-one derivatives

Temperature has an imperative role on the product yield. To our surprise, at lower temperature furnished the product in trace to poor yield, but by increasing the temperature upto 80-100 ^oC desired product form in excellent yields within much shorter reaction time. The reaction was very clean with no side product formation.

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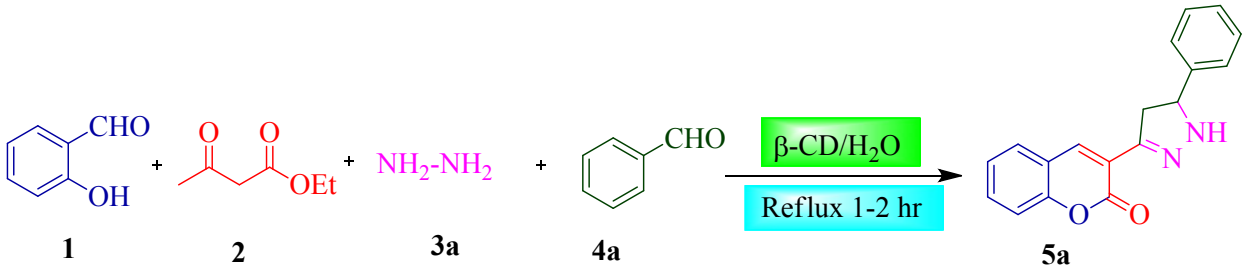

Scheme 2. Standard model reaction

The catalyst reusability was studied four times including the use of a fresh catalyst for the synthesis of compound **(5a)** and there was an inevitable loss of catalyst during the recovery process. Besides this, no significant loss in catalytic activity was observed **(Fig. 2)** the catalyst was reused the next batch without any treatment.

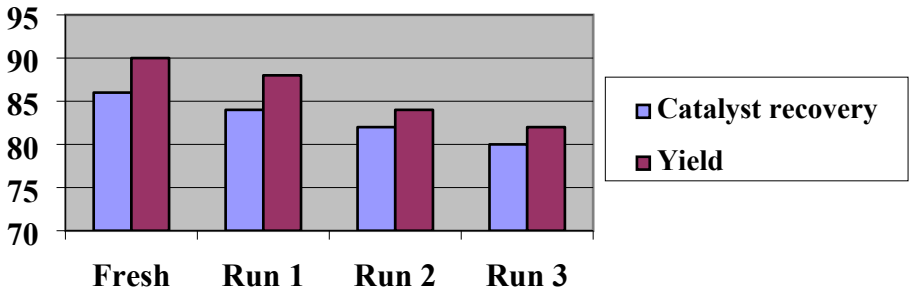

Figure 2. Reuse and recovery of β-CD and its effect on yield

Table 1. Summary of different catalyst and solvent used for the synthesis of **5a**

Entry	Catalyst	Solvent	Time (hr)	Yield[a] (%)
1.	-	Water	7-8	NR[b]
2.	α-CD	Water	7	41
3.	β-CD	Water	1	90
4.	γ-CD	Water	5	45
5.	β-CD	EtOH	6	43
6.	β-CD	MeOH	7	20
7.	β-CD	1,4-Dioxane	7	40
8.	β-CD	DMSO	7-8	34
9.	β-CD	EtOH:H_2O	5	56
10.	β-CD	THF	7	41
11.	β-CD	CH_3CN	5	39

[a]Isolated Yield of product,[b]No Reaction

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Table 2. Synthesis of compound coumarins linked to pyrazolines **5 (a-w)**

Entry	Products	-R[1]	-R	Time[a]	Yield[b] (%)	M. P. °C (Found)	M. P. °C (Reported)[37]
1.	**5a**	Ph	H	1.00	97	189-191	-
2.	**5b**	3,4-di- OCH_3	H	1.05	97	149-152	-
3.	**5c**	3,4,5-tri- OCH_3	H	1.05	96	171-174	-
4.	**5d**	4-OCH_3	H	1.45	92	182-185	183-185
5.	**5e**	2-OCH_3-Napthaldehyde	H	1.00	86	146-148	-
6.	**5f**	4-OH,3-OCH_3	H	1.19	83	139-142	-
7.	**5g**	4-CH_3	H	1.26	83	169-171	165-167
8.	**5h**	4-Cl	H	1.20	82	176-179	175-177
9.	**5i**	2,6-di-Cl	H	1.10	92	156-159	158-160
10.	**5j**	2-Thiophenealdehyde	H	1.30	83	128-131	-
11.	**5k**	Valeraldehyde	H	1.49	96	140-142	-
12.	**5l**	Butyraldehyde	H	1.30	84	146-149	-
13.	**5m**	Ph	Ph	1.00	81	132-135	-
14.	**5n**	4-CH_3	Ph	1.45	83	139-141	-
15.	**5o**	4-OH	Ph	1.45	83	107-110	-
16.	**5p**	N,N-di-CH_3	Ph	1.15	92	169-172	-
17.	**5q**	3-Br	Ph	1.45	79	152-155	-
18.	**5r**	Valeraldehyde	Ph	1.35	93	183-186	-
19.	**5s**	4-NO_2	H	1.00	81	196-198	199-201
20.	**5t**	N,N-di-CH_3	H	1.45	96	109-111	-
21.	**5u**	3-CH_3	H	1.00	86	132-135	-
22.	**5v**	4-F	H	1.10	86	176-178	174-176
23.	**5w**	4-OH	H	1.15	87	139-142	-

[a]Time in hr., [b]Isolated yield of products

3. Pharmacology

In Vitro Antimicrobial Activity

All the synthesized compounds were screened for their *in vitro* antifungal and antibacterial activity. The antibacterial activity was evaluated against three human pathogenic bacterial strains: *Escherichia coli* (NCIM-2256), *Bacillus subtilis* (NCIM-2063) and *Staphylococcus aureus* (NCIM-2901). The antifungal activity was evaluated against seven human pathogenic fungal strains: *Candida albicans* (NCIM-3471), *Candida glabrata* (NCYC-388), *Fusarium oxysporum* (NCIM-1332), *Aspergillus fumigates* (NCIM-902), *Aspergillus flavus* (NCIM-539), *Aspergillus niger* (NCIM-1196) and *Cryptococcus neoformans* (NCIM-576), which were often encountered clinically. Miconazole was used as standard drug. Minimum

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inhibitory concentration (MIC) values were determined as per CLSI guidelines.[38-40] Dimethyl sulfoxide (DMSO) was used a solvent control.

In Vitro Antifungal Activity

Antifungal activity was determined as per CLSI (formerly, NCCLS) guidelines.[38-40] The synthesized compounds **5 (a-r)** and the standard drug Miconazole were dissolved in DMSO solvent. The medium yeast nitrogen base was dissolved in phosphate buffer pH 7 and it was autoclaved at 110 °C for 10 min. With each set, a growth control without the antifungal agent and solvent control DMSO were included. The fungal strains were freshly sub cultured on to Sabouraud dextrose agar (SDA) and incubated at 25 °C for 72 hr. The fungal cells were suspended in sterile distilled water and diluted to get 10^5 cells/mL. 10 µL of the standardized suspension was inoculated onto the control plates and the media incorporated with the antifungal agents. The inoculated plates were incubated at 25 °C for 48 hr. The readings were taken at the end of 48 hr and 72 hr. The MIC was the lowest concentration of drug preventing the growth of macroscopically visible colonies on drug-containing plates when there was visible growth on the drug-free control plates.

In Vitro Antibacterial Activity

All the synthesized compounds **5 (a–r)** were screened for their *in vitro* antibacterial activity. Minimum inhibitory concentration (MIC) values were determined using the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). *In vitro* antibacterial activities of the synthesized compounds, **5 (a–r)** were tested in Nutrient Broth (NB) for bacteria by the two-fold serial dilution method. Seeded broth (broth containing microbial spores) was prepared in NB from 24 hr old bacterial cultures on nutrient agar (Hi-media) at 37± 1 °C. The bacterial suspension was adjusted with sterile saline to a concentration of 1×10^{-4}–10^{-5} C.F.U. The synthesized compounds and standard drug Ampicillin were prepared by two-fold serial dilutions to obtain the required concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, and 3.13 µg/mL. The tubes were incubated in BOD incubators at 37± 1 °C for bacteria. The MICs were recorded by visual observations after 24 hr (for bacteria) of incubation.[38-40]

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D-alanine-D-alanine ligase (DdlB) enzyme inhibition study

The D-Ala-adding activity of DdlB ligase was monitored by the detection of orthophosphate generated during the reaction based on the colorimetric malachite green method described by Walsh, A. et al.[41] Assays were performed at 37 °C in a mixture (final volume: 50 µL) containing 38.5 mMHepes, pH 8.0, 3.25 mM$MgCl_2$, 6.5 mM $(NH_4)_2SO_4$, 700 µM D-Ala, 500 µM ATP, purified DdlB (diluted in 20 mM Hepes, pH 7.2, and 1 mM dithiothreitol) and the test compound (IC_{50} values were determined for a range of inhibitor concentrations). All the compounds were soluble in the assay mixture containing 5 % DMSO. After 30 min of incubation, 100 µL Biomol reagent was added. After 5 min, absorbance was read at 650 nm. Residual activity was calculated with respect to a similar assay without inhibitor. To exclude possible non-specific (promiscuous) inhibitors, representative compounds **5 (a-r)** were also tested in the presence of Tween (0.003 %), Triton X-114 (0.005 %), and SDS (420 µM), as described by McGovern, S. L. et al.[42,43] No significant differences were found when compared to measurements without detergents.

***In –vitro* Antibacterial activity and D-alanine assay**

All the synthesized compounds **5 (a-r)** were screened for their *in vitro* antibacterial activity against *Staphylococcus aureus (NCIM-2901), Escherichia coli* 1411 (standard laboratory strains) and *Escherichia coli* SM1411 (an *acrAB* deficient derivative of 1411). Minimum inhibitory concentration (MIC) values were determined using the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). Dimethyl sulfoxide (DMSO) was used a solvent control. The results of the *in vitro* antibacterial activity are presented in (**Table 3)**. D-Alanine-d-alanine ligase (Ddl) is one of the key enzymes in peptidoglycan biosynthesis and is an important target for drug discovery. Inhibition of DdlB essential enzymes in either Gram-positive or Gram-negative organisms leads to the loss of cell shape and integrity followed by bacterial death. The synthesized derivatives **5 (a-r)** were tested for their inhibitory activity on DdlB from *E.coli*. The results are presented as IC_{50} values.

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Table 3. *In-vitro* antibacterial activity of the synthesized compounds **5 (a-r)**

Compd.	IC_{50} (μM)	MIC[a] (μg/ml)		
		E. coli 1411	*E. coli* SM1411	*S. aureus* NCIM-2901
5a	178	54	54	110
5b	129	35	32	58
5c	155	48	46	**50**
5d	220	55	54	120
5e	335	58	>60	166
5f	**106**	**14**	**14**	**32**
5g	**111**	**16**	**18**	**40**
5h	**124**	**30**	**28**	**36**
5i	**115**	**20**	**20**	**24**
5j	230	68	64	112
5k	245	>70	>70	158
5l	268	>70	>70	176
5m	>400	>70	>70	>200
5n	>400	>70	>70	>200
5o	348	>70	>70	185
5p	>400	>70	>70	>200
5q	>400	>70	>70	>200
5r	288	>70	>70	155
D-Cycloserine	276	16	16	32

[a] *Values are the average of three readings; E.coli 1411: Escherichia coli* 1411; *E.coli* SM1411 *Escherichia coli* SM 1411; *S. aureus* NCIM-2901: *Staphylococcus aureus (NCIM-2901); MIC: Minimum inhibitory concentration.*

From the results of *in-vitro* antibacterial activity data, it reveals that the compound **5f** was found to be the most potent antibacterial compound among the synthesized series against the tested pathogens. The compounds **5f** has shown MIC values 14 μg/mL, 14 μg/mL and 32 μg/mL against *E.coli 1411, E.coli* SM1411 and *S. aureus* NCIM-2901 respectively. From the MIC values of compound **5f,** it can be observed that it is more potent than that of standard drug D-cycloserine against the selected pathogens. The compound **5g** was found to be the second most active antibacterial compound among the synthesized series **5 (a-r).** The compounds **5g** has shown MIC values 16 μg/mL, 18 μg/mL and 40 μg/mL against *E.coli* 1411*, E.coli* SM1411 and *S. aureus* NCIM-2901 respectively. The compounds **5i** and **5h** were also found to be good antibacterial compounds among the synthesized series. The other active compounds are **5a**, **5b**, **5c**, **5d**, **5e**, **5j**, **5k**, **5l** and **5o**. The compounds **5m**, **5n**, **5p,** and **5q** were almost inactive against selected pathogens.

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The synthesized compounds were also screened for their D-alanine inhibitory activity. The synthesized derivatives **5 (a-r)** were tested for their inhibitory activity on DdlB from *E.coli*. The result of D-alanine enzyme assay reveals that most of the synthesized compounds have good D-alanine enzyme inhibitory activity. The synthesized compound **5f** was found as the most potent D-alanine enzyme inhibitor. The compound **5g** was found too be the second most potent inhibitor of the DdlB enzyme with IC_{50} value 111 μM. The compounds **5a**, **5b**, **5c**, **5d**, **5f**, **5g, 5h, 5i, 5j, 5k,** and **5l** were found to be better DdlB enzyme inhibitors than that of standard drug D-cycloserine.

Structure-activity relationship (SAR) revealed that the derivatives with substitution on the 'NH' group of the pyrazole heterocycle were less active antibacterial compounds than those with no substitution on the 'NH' group of the pyrazole heterocycle. The compounds **5 (m-r)** consists of substitution on the 'NH' group of the pyrazole heterocycle and are less active antibacterial compounds than compounds **5 (a-l)** with no substitution on the 'NH' group of the pyrazole heterocycle. The most potent enzyme inhibitor compound **5f** consists of the 4-hydroxy-3-methoxy group on the phenyl ring and no substitution on the 'NH' group of the pyrazole heterocycle. The compound **5g** was found to be the second most potent inhibitor of the DdlB enzyme with "CH_3" group on the phenyl ring and no substitution on the 'NH' group of the pyrazole heterocycle. The compound **5a** with no substitution on the phenyl ring was found to be less active than compounds with substitution on the phenyl ring. When the phenyl ring was replaced with the thiophene heterocycle there was a decrease in antibacterial potency of the synthesized compound **5j.** When the phenyl ring was replaced with the acyclic compounds there was a decrease in antibacterial potency of the synthesized compounds **5p** and **5q**.

In-vitro antifungal activity

The newly synthesized derivatives **5 (a-r)** were screened for their *in vitro* antifungal activity against different yeast and filamentous fungal pathogens. Minimum inhibitory concentration (MIC) values for *in vitro* antifungal activity were determined the method recommended the National Committee for Clinical Laboratory Standards (NCCLS). Miconazole was used as standard drug. Dimethyl sulfoxide (DMSO) was used a solvent control. The MIC (μg/mL) of all the tested compounds and that of the reference drug Miconazole has been listed in the **(Table 4)**. Results from the (**Table 4**) indicated that all the synthesized compounds have shown good to moderate antifungal activity against tested fungal strains.

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Table 4. *In-vitro* antifungal activity of the synthesized compounds **5 (a-r)**.

Compd.	MIC[a]µg/mL						
	Candida albicans	***Candida glabrata***	***Fusarium oxysporum***	***Aspergillus fumigates***	***Aspergillus flavus***	***Aspergillus niger***	***Cryptococcus neoformans***
5a	38	42	36	46	29	30	28
5b	**28**	**34**	**33**	**40**	**16**	**16**	**27**
5c	**28**	**32**	**30**	**38**	**18**	**14**	**16**
5d	**35**	**34**	**34**	**42**	**25**	**18**	**22**
5e	**25**	**28**	**30**	**34**	**14**	**18**	**20**
5f	48	44	39	49	33	36	31
5g	**24**	**25**	**28**	**32**	**14**	**14**	**16**
5h	**38**	**38**	**36**	**45**	26	26	28
5i	**36**	**33**	**35**	46	23	26	22
5j	**20**	**20**	**22**	**34**	**12**	**14**	**12**
5k	46	41	38	46	28	28	33
5l	40	40	42	46	29	32	32
5m	42	42	48	51	31	32	29
5n	69	58	64	72	52	54	48
5o	60	57	55	58	42	39	38
5p	52	48	49	54	36	33	38
5q	58	55	48	57	37	38	35
5r	60	62	58	60	44	40	42
Miconazole	**25**	**25**	**25**	**35**	**12**	**12**	**12**

Among the synthesized compounds the compound **5g** was found to be equipotent that of standard drug Miconazole against the selected pathogens. The compound **5g** has shown MIC values 24 µg/mL, 25 µg/mL, 28 µg/mL, 32 µg/mL, 14 µg/mL, 14 µg/mL and 16 µg/mL against *Candida albicans, Candida glabrata, Fusarium oxysporum, Aspergillus fumigates, Aspergillus flavus, Aspergillus niger* and *Cryptococcus neoformans* respectively. The compound **5j** with thiophene nucleus in the structure was found to be more potent than the standard drug Miconazole against *Candida albicans, Candida glabrata, Fusarium oxysporum,* and *Aspergillus fumigates*. The compound **5j** has shown MIC values 20 µg/mL, 20 µg/mL, 22 µg/mL, 34 µg/mL, 12 µg/mL, 14 µg/mL and 12 µg/mL against *Candida albicans, Candida glabrata, Fusarium oxysporum, Aspergillus fumigates, Aspergillus flavus, Aspergillus niger,* and *Cryptococcus neoformans* respectively.

Structure-activity relationship (SAR) revealed that the derivatives with substitution on the 'NH' group of the pyrazole heterocycle were less active antibacterial compounds than those with

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no substitution on the 'NH' group of the pyrazole heterocycle. The compounds **5 (m-r)** consists of substitution on the 'NH' group of the pyrazole heterocycle and are less active antibacterial compounds than compounds **5 (a-l)** with no substitution on the 'NH' group of the pyrazole heterocycle. The compound **5i** with di-chloro substitution on phenyl ring was found to be more potent than compound **5h** with p-chloro substitution on the phenyl ring. The compound **5c** with 3,4,5, tri-methoxy substitution on phenyl ring was found to be more potent antifungal compound than the compound **5b** with 3,4-dimethoxy substitution on the phenyl ring. The compound **5e** i.e. 3-(5-(3-methoxynaphthalen-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one was also found a good antifungal agent among the synthesized series.

Molecular docking

Docking is an effective and reliable approach to simulate the probable binding mode of ligands and proteins. Bacterial growth is inhibited if the enzyme D-alanine ligase (Ddl) is been inhibited by the designed molecule. D-Alanine ligase (Ddl) enzyme inhibition is an attractive and tenable target in the search for novel and effective antimicrobial drugs.[44] Recently, chromene derivatives have been reported to inhibit E. coli DdlB enzyme.[45] Hence, the designed and synthesized compounds were appraised for their inhibition effect using D-alanine-D-alanine ligase (DdlB) enzyme assay study. Molecular docking studies into a homology model of the E. coli DdlB enzyme (PDB entry: 2I80) were performed using Glide v. 6.8 (Schrodinger, LLC, New York, NY, USA, 2015) to predict its antifungal and antibacterial activity mode of action, respectively.

Molecular Docking Study into E. coli DdlB Enzyme

Molecular docking study was performed in Maestro 9.1 using Glide v. 6.8 (Schrodinger LLC). All compounds were built using Maestro build panel and optimized to lower energy conformers using Ligprep v3.5 (Schrodinger, Inc., New York, NY, USA). The coordinates for E. coli DdlB enzyme were taken from RCSB Protein Data Bank and prepared for docking using 'protein preparation wizard' in Maestro v10.3. The bond orders and formal charges were added for hetero-groups and hydrogens were added to all atoms in the structure. Side chains that are not close to the binding cavity and do not participate in salt bridges were neutralized and termini were capped by adding ACE and NMA residue. After preparation, the structure was refined to optimize the hydrogen bond network using the OPLS_2005 force field. The minimization was

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terminated when the energy converged or the RMSD reached a maximum cutoff of 0.30 Å. The extra precision (XP) docking mode for all compounds was performed on the generated grid of protein structure.[47] The final evaluation of ligand-protein binding was done with Glide score.[46]

Inhibition of Ddl in bacteria by drugs hinders the growth of bacteria, which makes this enzyme an irresistible and important target for the discovery of novel antimicrobial drug. Hence, molecular docking was performed against E. coli DdlB (PDB entry: 2I80). The docking results indicated that the compounds were held in the active pocket by the combination of various hydrogen and hydrophobic interactions with DdlB enzyme. The docking results revealed that the highest binding compound to DdlB enzyme was compound **5g** with a G-Score of -9.086 when compared with standard D-Cycloserine.

All compounds with highest docking scores showed hydrogen bonding with amino acid residue THR 23 and a Pi-Pi stacking interaction with the amino acid residue PHE 313 which helped in the smooth attachment of the molecule into the pocket of DdlB enzyme. Also, compound **5g** with the highest G-Score showed additional hydrogen bonding and Pi-Pi interaction with the amino acid residue HIP 96 which increases the chances of good activity of the compound.

< Figure 3. The docking pose of compound **5g** into the active pocket of DdlB enzyme. (Pink bond represents the hydrogen bonding between ligand and receptor and Blue bond indicates the Pi-Pi stacking interaction, Purple colored structure represents the molecule) >

< Figure 4. The docking pose of compound **5a** into the active pocket of DdlB enzyme.>

< Figure 5. The docking pose of compound **5j** into the active pocket of DdlB enzyme. >

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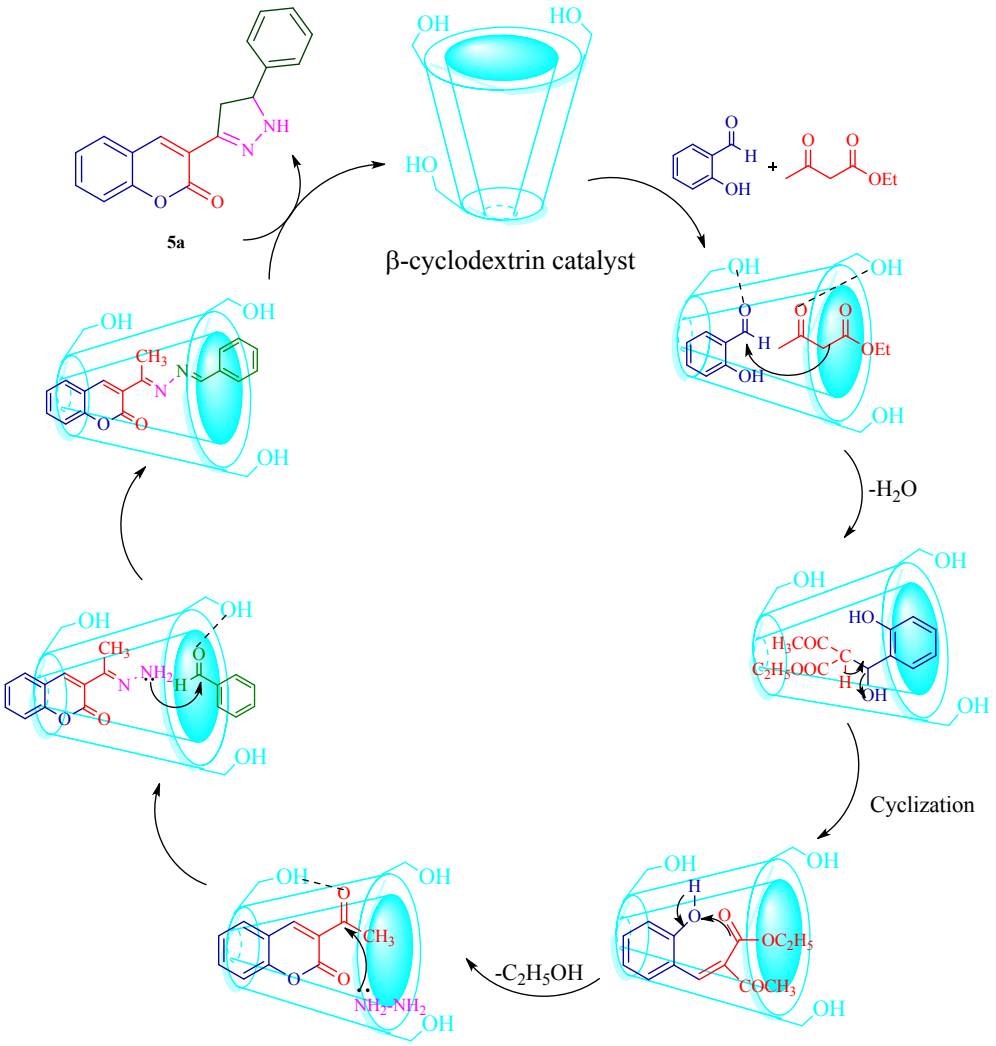
Reaction mechanism



Scheme 3. Plausible reaction mechanism of coumarins linked to pyrazoline derivative

4. Conclusion

We have explored a facile construction of biologically active coumarins linked to pyrazoline by the multicomponent approach. A new, simple, efficient and environmentally benign method involving the usage of β-cyclodextrin as a supramolecular catalyst in water for the synthesis of coumarins-pyrazoline was developed. The synthesized novel molecules are inhibitors of DdlB, with IC50 values in the micromolar range. The result shows that coumarin attached to pyrazoline moiety is essential for inhibitory activity against DdlB. The designed compounds represent an important starting point for further optimization and modification, to improve these inhibitory activities against DdlB. These types of inhibitors have the potential to

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be developed into drugs that would reverse bacterial resistance to D-Cycloserine. The synthesized compound **5f** was found to be the most potent D-alanine-D-alanine ligase enzyme inhibitor. The compound **5g** was found to be the second most potent inhibitor of the DdlB enzyme with IC_{50} value 111 μM. Furthermore, the potency of these compounds against DdlB suggests that it will be possible to develop broad-spectrum antimicrobials that target both Gram-negative and Gram-positive infections.

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Graphical Abstract

A new efficient domino approach for the synthesis of coumarin-pyrazolines as antimicrobial agents targeting bacterial D-alanine-D-alanine ligase

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