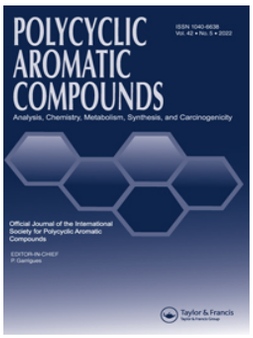

Polycyclic Aromatic Compounds

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Amide-Linked Monocarbonyl Curcumin Analogues: Efficient Synthesis, Antitubercular Activity and Molecular Docking Study

Dnyaneshwar D. Subhedar[a], Mubarak H. Shaikh[a,b], Amol A. Nagargoje[a,c], Satish V. Akolkar[a], Sujit G. Bhansali[d], Dhiman Sarkar[d], and Bapurao B. Shingate[a]

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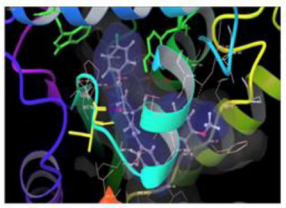
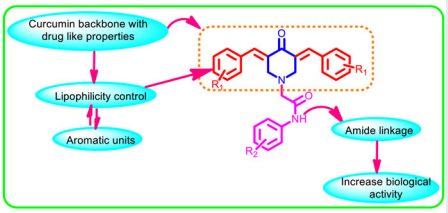
ABSTRACT

An approach toward the synthesis of novel conjugates of 3,5-bis (arylidene)-4-piperidones (DAP) pharmacophore with amide-linkage has been developed *via* one-pot multicomponent reaction of aryl aldehydes, piperidinone and 2-chloro-*N*-phenylacetamide using [Et_3NH][HSO_4] as a catalyst/medium. Both substitutions on arylidene rings and piperidinone nitrogen (substituted 2-chloro-*N*-phenylacetamide) were varied. The synthesized conjugates were evaluated for their *in vitro* antitubercular activity against *M. tuberculosis* $H_{37}Ra$ (*MTB*) and *M. bovis* BCG strains. Among the series, compounds **4f**, **4g, 4i** and **4j** showed remarkable broad spectrum antitubercular activity with low IC_{50} values. Furthermore, computer docking simulations, for the most active conjugates were performed with the active site of mycobacterial enoyl-acyl carrier protein reductase (InhA) support the antitubercular activity. Lower cytotoxicity, high potency and promising activity against *MTB* and *M. Bovis* BCG suggest that amide linked DAP could serve as good leads for further modifications and development.





3D view of binding of compound **4f**

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Bis (arylidene)-4-piperidones; Ionic liquid; Antimycobacterial activity; Cytotoxicity; Molecular docking Study

Introduction

Tuberculosis is the ninth leading cause of death worldwide from a single infectious agent ranking above HIV/AIDS. It is fatal airborne disease caused by *Mycobacterium tuberculosis* (*Mtb*) which affects the lung and also responsible for infection in others sites of body.[1] Global tuberculosis report of 2017 given by World Health Organization (WHO), shows that TB is the leading cause of deaths due to antimicrobial resistance and among people with HIV.[2] Over 6.3 million new TB

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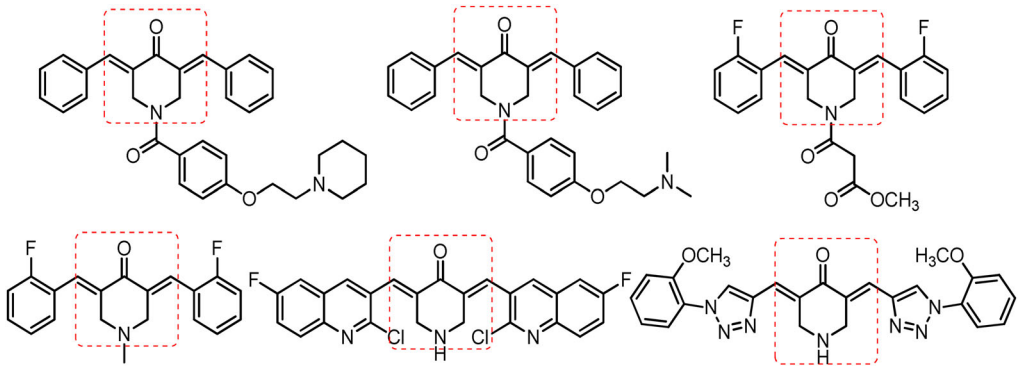


Figure 1. Known MACs as an antimycobacterial agents.

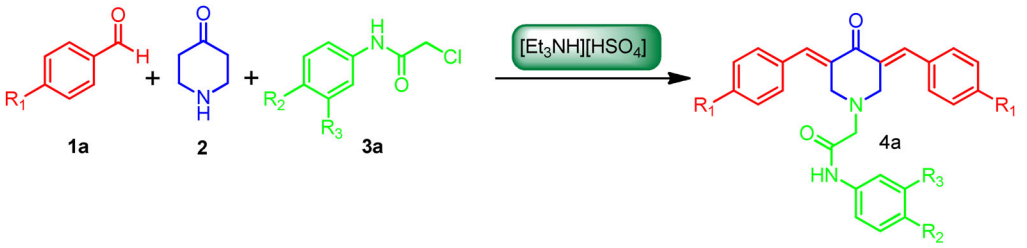
cases were notified to WHO by national authorities. In 2016 WHO estimates that there have been emergence and increasing evidences of extensively drug resistance and multiple drug resistance TB cases which results in global epidemic. Also, 4.9 lacs of new multidrug resistance TB cases were reported as per global tuberculosis report given by WHO. It was also reported that about 6 lacs new TB cases were found to exhibit resistance to most of the front-line drug against TB like rifampicin.[3] Hence, development of new therapeutic agents against TB is the urgent need of time.

In recent years enormous efforts have made by organic chemists across the globe in the development of structurally modified, therapeutically active curcumin analogues/derivatives.[4] Monocarbonyl analogue of curcumin is one of the class of structurally modified and potentially active curcuminoids obtained by removing β-diketone moiety and active methylene group from curcumin.[5,6] Monocarbonyl curcumin analogues exhibit a wide array of biological activities,[7] such as antileishmanial,[8] antiparasitic,[9] anti-inflammatory,[10] antitumor and antioxidant,[11] alzheimer's disease,[12] antibacterial,[13] anticancer[14] and antitubercular activity are well reported[15] (Figure 1).

Owing to the potential importance of curcumin derivatives as a key moieties in life sciences, pharmaceuticals, agriculture, world-wide efforts have made in the last few decades by researchers and various protocols have been developed for their synthesis[16]a using catalysts, such as NaOH/EtOH,[16]b EtOH/KOH,[16]c piperidine/HCl,[16]d AcOH/HCl gas,[16]epiperidine, L-Proline/EtOH[16]f and $MgBr_2.Et_2O/Et_3NH$.[16]g The above methods suffer from one or more limitations such as the use of excess or stoichiometric quantity, corrosive, which are non-recoverable and/or recoverable with tedious separation procedures involving lots of toxic waste generation besides a long reaction time and low yield for the desired product.

Ionic Liquids (ILs) have attracted interest because of alternative green reaction media due to their unique chemical and physical properties such as low vapor pressure, high thermal and chemical stability, good solvating ability, ease of recyclability, and controlled miscibility.[17] Thus, ILs are considered to be a safer alternative to original organic solvents as they are cleaner and safer to use and reuse.[18] The utility of Acidic Bronsted Ionic Liquid (ABIL), particularly $[Et_3NH][HSO_4]$[19] has received considerable attention because, it is an inexpensive, nontoxic catalyst as well as solvent for many organic transformations in excellent yields. Multicomponent reactions (MCRs) are important class of organic transformations and increasing attention due to their, efficiency, atom economy, short reaction times and diversity in organic synthesis.[20] Moreover, use of solvent-free methods in MCRs makes the process safer, cleaner and easier to perform.[21] Thus, the utilization of MCRs coupled with environmentally benign solvent-free condition is highly desirable.

Monocarbonyl analogues of curcumin (MACs) are key constituent and structural backbone of many pharmaceutical and agricultural compounds and the development of more general and cost



Scheme 1. Model reaction.

Table 1. Optimization of solvent and temperature.

Entry	Solvent	Temp	Time (h)	Yield(%)[a]
1	EtOH	rt	6	60
2	MeOH	rt	6	65
3	DMF	rt	6	70
4	THF	rt	6	55
5	CH_3CN	rt	6	50
6	Toluene	rt	6	71
7	Solvent-free	rt	2.5	82

Reaction conditions: **1a** (1 mmol), **2** (1 mmol), **3a** (1 mmol) and 25 mol%. $[Et_3NH][HSO_4]$.
[a]Isolated yield.

effective, one-pot multi-component protocols for their synthesis under more efficient, environment friendly conditions using recyclable, ecofriendly catalysts is still a possibility to explore. Hence keeping in view the potential of MACs as an antitubercular agents and as part of our continuous efforts for the development of bioactive molecules[22] using highly efficient, safer as cost effective protocols,[23,24] we would like to report herein, synthesis and antimycobacterial evaluation of novel MACs by using $[Et_3NH][HSO_4]$ as a medium/catalyst *via* one-pot multicomponent approach.

Results and discussion

Chemistry

In a preliminary experimental investigation of the optimum reaction conditions regarding the solvent, amount of catalyst and temperature. For this, benzaldehyde **1a** (1 mmol), piperidone **2** (1 mmol) and 2-chloro-*N*-phenyl acetamide **3a** (1 mmol) were chosen as standard model substrates for the synthesis of representative compound **4a** *via* Clasien-Schmidt reaction-alkylation as shown in Scheme 1.

The model reaction was carried out with 25 mol% of $[Et_3NH][HSO_4]$ as a catalyst in different solvents (Table 1) at room temperature. In methanol (MeOH) and ethanol (EtOH), the reaction took a longer time (6 h) with a moderate yield of the products (Table 1, entries 1 and 2). In *N*,*N*-Dimethylformamide (DMF), a better yield of the product was obtained (Table 1, entry 3). Further, the reaction carried out in tetrahydrofuran (THF) and acetonitrile (CH_3CN), but again a lower yield of the product (Table 1, entries 4, 5) obtained In Toluene, a good yield of the product was obtained in 6 h (Table1, entry 6). However, when the model reaction was carried out under a solvent-free condition, there is a significant increase in the yield of the product in a shorter period (Table1, entry 7). Thus, solvent-free is the best condition for this Clasien-Schmidt reaction.

To optimize the concentration of catalyst, we further examined the influence of catalyst concentration on the reaction time and percentage yield. So, the model reaction was performed using different concentration of catalyst 5, 10, 15, 20, 25 and 30 mol% of $[Et_3NH][HSO_4]$ at room

Table 2. The effect of catalyst loading on model reaction **4a**.[a]

Entry	Catalyst (mol %)	Yield (%)[b]
1	5	40
2	10	59
3	15	69
4	20	73
5	25	82
6	30	82

[a]Reaction conditions: **1a** (1 mmol), **2** (1 mmol), **3a** (1 mmol) and 25 mol%. $[Et_3NH][HSO_4]$.
[b]Isolated yield.

Table 3. Reusability of $[Et_3NH][HSO_4]$ in the synthesis of **4a**.[a]

Entry	Reaction cycle	Isolated yield (%)[b]
1	1st (fresh run)	82
2	2nd cycle	80
3	3rd cycle	78
4	4th cycle	78
5	5th cycle	74

[a]Reaction conditions: **1a** (1 mmol), **2** (1 mmol), **3a** (1 mmol) and 25 mol%. $[Et_3NH][HSO_4]$,rt.
[b]Isolated yield of products.

temperature under solvent-free conditions and the product **4a** was obtained in 40, 59, 69, 73, 82 and 82% yields, respectively.

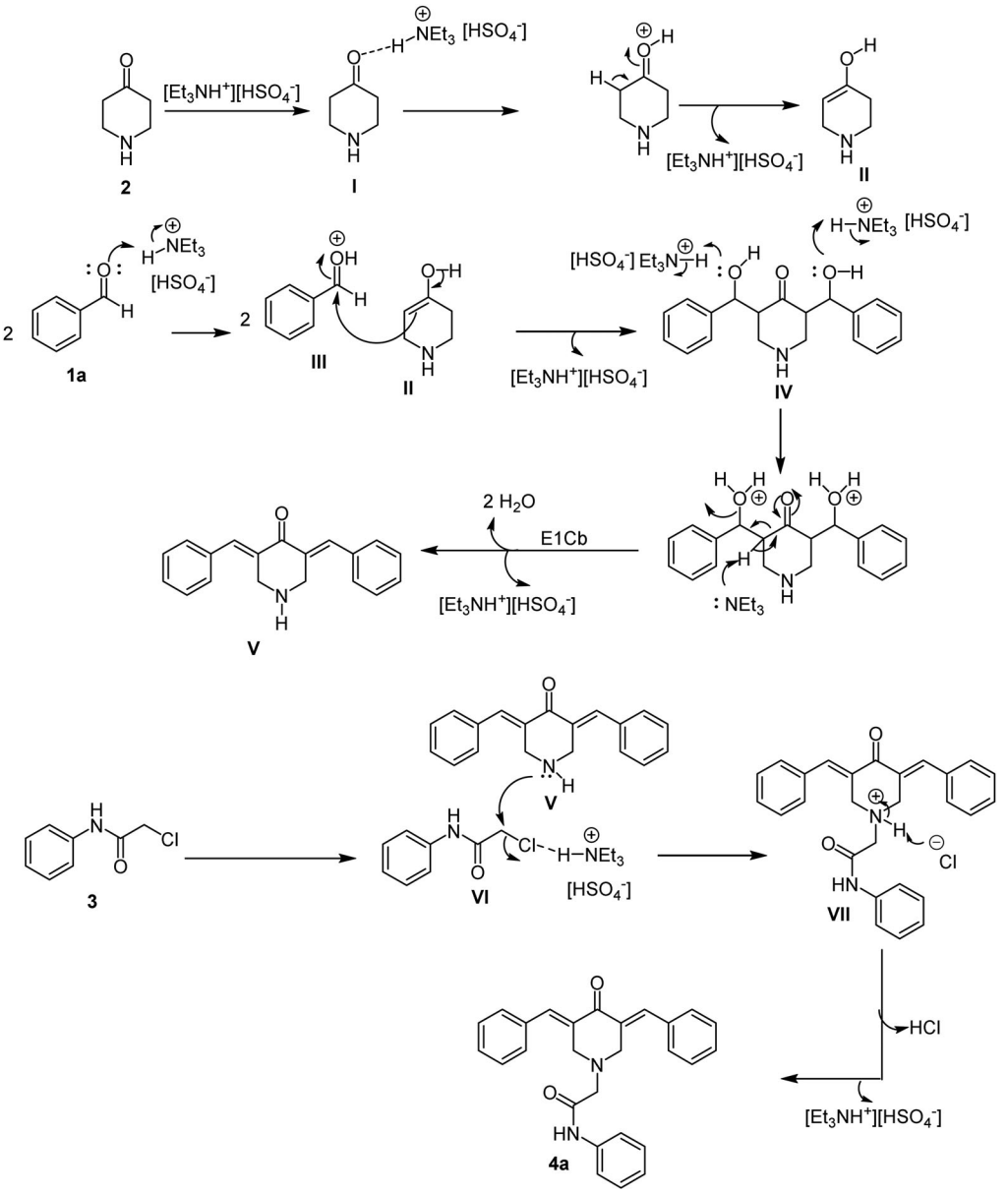
Further, the yield of the product did not improve as the concentration of catalyst increased (Table 2, entry 6). It was therefore concluded that the optimum concentration of catalyst was 25 mol % (Table 2, entry 5). Thus, it is concluded that 25 mol % of $[Et_3NH][HSO_4]$ is sufficient for the best result.

Further, the recyclability of the ionic liquid $[Et_3NH][HSO_4]$was then studied and the results are shown in Table 3. After completion of the reaction, the reaction mixture was poured on ice cold water and the product was extracted using ethyl acetate solvent. The filtrate was subjected for evaporation of water to get viscous liquid, which on cooling afforded ionic liquid. Further, the residual ionic liquid was washed with diethyl ether, dried under vacuum at 60 °C and reused for subsequent reactions. It was then reused at least four consecutive cycles without much appreciable loss in its catalytic activity (Table 3, entries 1–5).

The formation of compound **4a** has been confirmed by IR, ^{1}H NMR, ^{13}C NMR and HRMS spectral study. IR spectrum of **4a** displayed characteristic signals for C = O at 1707 and 1656 cm^{-1}. In the ^{1}H NMR spectrum of compound **4a**, a sharp singlet resonating at around δ 4.47 ppm for four protons, assigned to methylene groups of the piperidine ring. The peak observed at δ 4.12 ppm for the CH_2 group attached to the *N*-phenylacetamide. ^{13}C NMR spectrum was also in agreement with the proposed structure displaying characteristic signals for carbonyl at around δ 187.2 and 169.5 ppm. Similarly, signals resonating at around δ 57.5 and 50.5 have been assigned to methylene carbons of the piperidine and *N*-phenylacetamide ring. The HRMS spectral analysis of the **4a** was also conformity with the proposed structure. Similarly, the structure of other derivatives was confirmed by physical and spectral data (Supplementary information).

A plausible mechanistic pathway is proposed to illustrate the synthesis of monocarbonyl curcumin analogues catalyzed by $[Et_3NH][HSO_4]$ (Scheme 2).

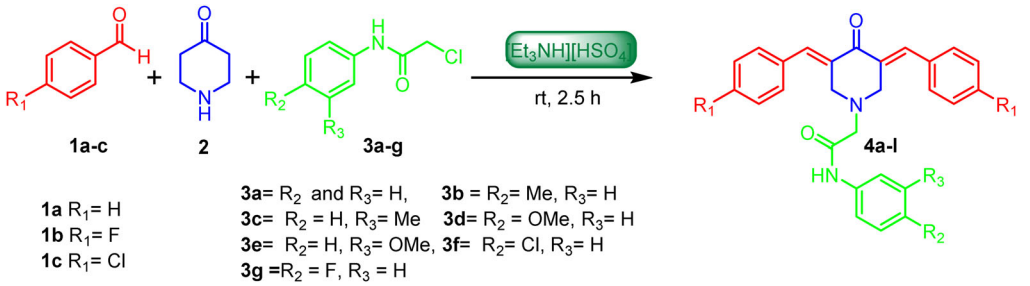
The initial step is believed to be the protonation of the carbonyl carbon of aldehyde **1a** by ionic liquid $[Et_3NH][HSO_4]$ to form intermediate **III** and enolate formation of piperidinone **II** which facilitates the nucleophilic attack of piperidinone to promote the formation of C-C bond to yield intermediate **IV**. The subsequent protonation and elimination of H_2O molecule *via* E_1CB



Scheme 2. Plausible mechanistic catalytic cycle for the synthesis of compound **4a**.

mechanism leads to the formation of intermediate **V**. Ionic liquid increases the electrophilicity of 2-chloro-*N*-phenylacetamide C-Cl carbon shown in structure **VI**. Then nucleophilic substitution of 2-chloro-*N*-phenylacetamide by compound **V** expedite the formation of C-N bond to yield compound **VII**. The final step involves deprotonation of intermediate **VII** leads to regeneration of ionic to yield compound **4a**.

So, having the optimized reaction conditions in hand, the scope and efficiency of this approach were explored for the synthesis of highly functionalized amide linked 3,5-bis (arylidene)-4-piperidones (Scheme 3) and the structures are given in Figure 2. The structures of all the synthesized conjugates were confirmed by their physical data and spectral analysis.



Scheme 3. Synthetic pathway for 3,5-bis (arylidene)-4-piperidones.

Biological activity

Antitubercular activity

In a standard primary screening, all the newly synthesized 3,5-bis (arylidene)-4-piperidones **4a-l** were evaluated for their *in vitro* antitubercular activity against *M. tuberculosis* $H_{37}Ra$ and

M. bovis BCG at concentrations of 30, 10 and 3 μg/mL using an established XTT Reduction Menadione assay (XRMA) method.[25] The drug rifampicin was used as reference.

In general, compounds which showed more than 90% inhibition (SI, Table S1, S2) at 30 μg/mL were confirmed by carrying out dose dependent effect using a range from 50 to 0.39 μg/mL to determine IC_{50} and MIC with serial dilution in DMSO (Table 4).

Among all the prepared xanthene conjugates, compounds **4f, 4h, 4i, 4j, 4k** and **4l** are found prominent antimycobacterial activities against *MTB* and *M. bovis BCG* strain with MIC values 1.89–26.37 and 2.69–29.14 µg/mL, respectively. However, rest of the xanthene conjugates **4a, 4b, 4c, 4d, 4e** and **4g** are inactive against *MTB* and *M. bovis BCG* strain with MIC = >30 µg/mL.

Cytotoxic activity

The most active conjugates **4f, 4h, 4i, 4j, 4k** and **4l** were further evaluated against human cancer cell lines (MCF-7, A549 and HCT 116) for toxicity and results are given in Table 5. The GI_{50}/GI_{90} (>250 µg/mL) values of all the compounds (except **4k**) indicate that the compounds are potent and specific inhibitors against *MTB*. The primary cytotoxic study information is given in supporting information (SI S3, S4 and S5).

Selectivity index (SI)

The selectivity index reflects the concentration of the compound at which it is active against *mycobacteria* but not toxic toward host cells. A higher selectivity index of compounds indicates that the can be used as a therapeutic agent. The compound **4f** (SI > 12) showed very high SI, which is actually good inhibitor of *MTB* and *M. bovis* BCG and the results are shown in Table 6.

Although the selectivity index of rifampicin is very high, it is important to consider the significance of this study with respect to the developing resistance among microorganisms against available antibiotics. According to a study of Hartkoorn et al.[26] on the drug susceptibility of TB, antimycobacterial activity was considered to be specific when the selectivity index was >10. In the current study compound **4f** exhibited highest selectivity index of >10, indicating their potential as an antitubercular agent and should be investigated further.

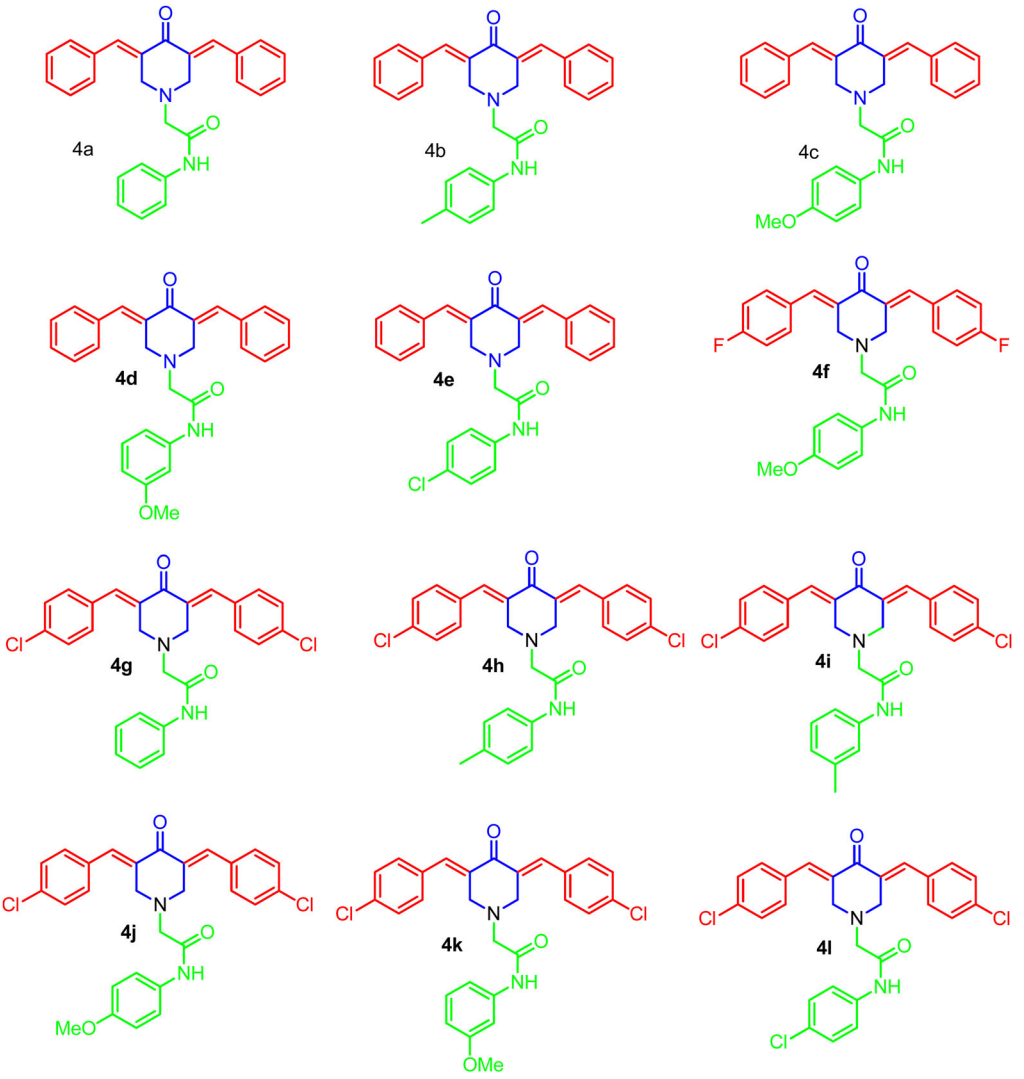


Figure 2. Structures of synthesized MACs.

Table 4. Antitubercular screening results of compounds **4a-l** (μg/mL)[a].

	MTB H37Ra		*M. bovis* BCG	
Compound	MIC	IC_{50}	MIC	IC_{50}
4a	>30	>30	>30	>30
4b	>30	>30	>30	>30
4c	>30	>30	>30	>30
4d	>30	>30	>30	>30
4e	>30	>30	>30	>30
4f	20.57	1.89	22.33	2.69
4g	>30	6.85	>30	7.33
4h	>30	>30	>30	>30
4i	>30	18.33	>30	6.94
4j	28.46	2.37	29.42	2.85
4k	>30	26.37	>30	29.14
4l	>30	27.64	>30	23.4
[b]Rifampicin	0.045	0.0017	0.017	0.0015

[a]MIC in μg/mL. Antitubercular activity of all agents was firm by serial dose dependent dilutions method.
[b]Standard antitubercular drug rifampicin as a positive control. Data were expressed as the means of triplication. SD (±): Standard Deviation.

Table 5. Cytotoxicity activity.

	MCF-7		A549		HCT-116	
Compound	GI_{50}	GI_{90}	GI_{50}	GI_{90}	GI_{50}	GI_{90}
4f	>250	>250	>250	>250	>250	>250
4h	>250	>250	>250	>250	>250	>250
4i	>250	>250	>250	>250	>250	>250
4j	>250	>250	>250	>250	>250	>250
4k	188.58	>250	225.51	>250	219.11	>250
4l	>250	>250	>250	>250	>250	>250
Rifampicin	>100	>100	>100	>100	>100	>100
Paclitaxel	0.0048	0.075	0.0035	0.0706	0.1279	5.715

The GI_{50} (cytotoxicity) values were calculated as the concentration of compounds resulting in 50% reduction of absorbance compared to untreated cells.

Table 6. SI of selected 3,5-*bis* (arylidene)-4-piperidones.

	MCF-7		A549		HCT 116	
Compound	*MTB*	BCG	*MTB*	BCG	*MTB*	BCG
4f	>12	>11	>12	>11	>12	>11
4h	>8	>8	>8	>8	>8	>8
4i	>8	>8	>8	>8	>8	>8
4j	>6	>6	>8	>8	>7	>7
4k	>9	>8	>9	>8	>9	>8
4l	>8	>8	>8	>8	>8	>8
Rifampicin	>133	>123	>133	>123	>133	>123

Table 7. Antibacterial activity of curcumin derivatives (MIC in μg/mL).

Compound	Gram-negative		Gram-positive	
	E. coli	*P. fluorescens*	*S. aureus*	*B. subtilis*
4f	>100	>100	>100	>100
4h	>100	>100	>100	>100
4i	18.54	20.99	20.61	22.48
4j	>100	>100	>100	>100
4k	>100	>100	>100	>100
4l	28.32	22.87	26.57	26.65
Ampicillin	1.46	4.36	1	10.32
Kanamycin	1.62	0.49	>30	1.35

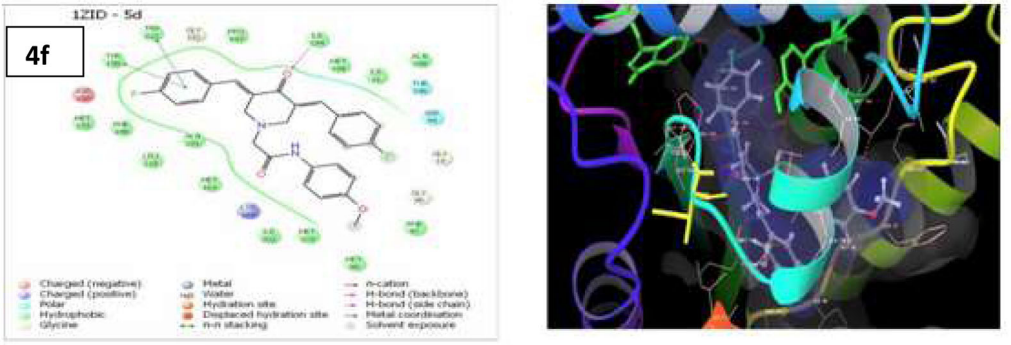
Antibacterial activity

For investigating the specificity of conjugates **4f, 4h, 4i, 4j, 4k** and **4l** were further screened for their anti-bacterial activity against four bacterial strains. All the compounds (except **4i** and **4l**) showed higher specificity toward *MTB*, because they display no anti-bacterial activity upto 100 μg/mL (Tables 6 and 7).

Computational study

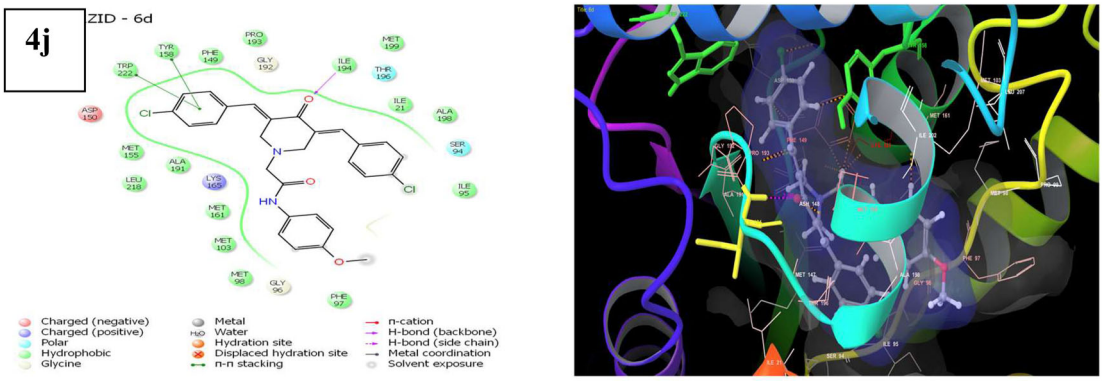
Molecular docking

Studies of whole genome sequence of *MTB* H37Ra showed the presence of genes which encodes the components of the FAS-II system along with enzyme enoyl-acyl carrier protein reductase (InhA) aids in elongation and synthesis of mycolic acids.[27] We have carried out the docking study of benzylidene-4-oxopiperidin-1-yl-*N*-phenylacetamide derivatives against mycobacterial enoyl-acyl carrier protein reductase (InhA) which could give the important leads for treatment of TB.



2D view of binding of compound **5d** 3D view of binding of compound **5d**

Figure 3. 2D and 3D view of binding of compound **4f** with the active site of InhA.



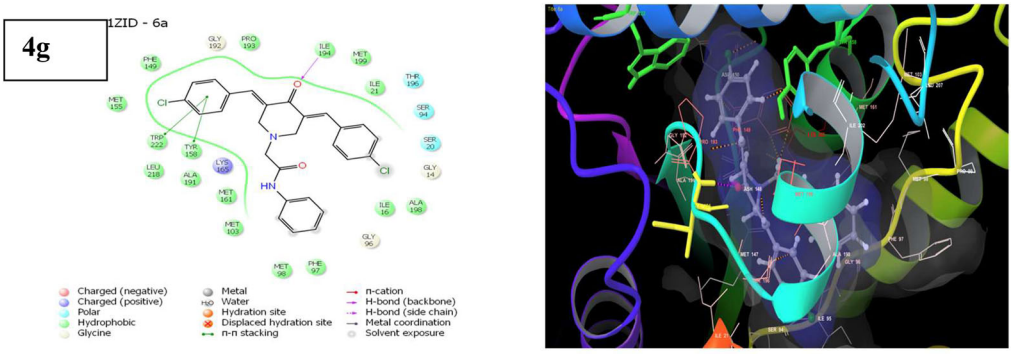
2D view of binding of compound **6d** 3D view of binding of compound **6d**

Figure 4. 2D and 3D view of binding of compound **4j** with the active site of InhA.

All the conjugates were successfully docked into the active site of mycobacterial InhA. The docking score of most active compounds **4f**, **4g** and **4j** were found to be −8.102, −7.960 and −7.816, respectively. The interactions of these molecules with the active site of mycobacterial InhA is shown in Figures 3–5, respectively. The lowest energy docking pose for **4f**, **4g** and **4j** revealed the presence of hydrogen bonding interactions between 4-oxo group of piperidine ring and Ile194 with a distance of 1.966 Å, 2.086 Å and 1.900 Å as observed in Figures 3–5, respectively. These firm hydrogen bonding interactions helps in 3D orientation of these ligands within the active site which increases the steric and electrostatic interactions of ligands with the amino acid residues present within the active site of mycobacterial InhA.

The per residue interaction analysis showed that several strong van der Waals and electrostatic interactions which played an important role for binding of benzylidene-4-oxopiperidin-1-yl-*N*-phenylacetamide derivatives with the active site of mycobacterial InhA. (Data represented in Table S6 in SI.) In addition, one of the 4-fluoro phenyl ring of compound **4f** and 4-chloro phenyl ring of compound **4g** and **4j** showed the two π-π stacking interactions with amino acid Tyr158 and Trp222 which were enclosed in the hydrophobic pocket of the active site of mycobacterial InhA formed by the amino acids Pro193, Trp222, Met155 and Phe149 as observed in Figures 3–5.

Further, the other 4-fluoro phenyl ring of compound **4f** and 4-chloro phenyl ring of compound **4g** and **4j** fits into the hydrophobic pocket formed by the amino acid residues Ile21,



2D view of binding of compound **6a** 3D view of binding of compound **6a**

Figure 5. 2D and 3D view of binding of compound **4g** with the active site of InhA.

Ala198, Ile95 and Ile16 present within the active site of mycobacterial InhA. Also, the phenyl ring attached to acetamide group is embedded in the hydrophobic pocket formed by theMet103, Mett98 and Phe97 amino acid residues present within the active site of mycobacterial InhA. Therefore, from the docking studies, it is clear that these compounds have significant binding with the active site of mycobacterial InhA. The hydrophobic substituents on aromatic rings attached to piperidin-4-one ring are necessary for firm binding through π-π stacking interactions with the active site of mycobacterial InhA. The –C = O group attached to the piperidine is essential for firm binding with the active site through strong hydrogen bonding interactions with the Ile194 amino acid residue present within the active site of mycobacterial InhA. Also, the phenyl ring attached to the acetamide group is essential for binding since it perfectly fits into hydrophobic pocket formed by the amino acid residues of active site which helps in the firm binding of these compounds with the active site of mycobacterial InhA. Hence, the mode of action of antitubercular activity for these compounds might be through the inhibition of active site of mycobacterial InhA.

Conclusions

In conclusion, a novel, highly efficient, one-pot multi-component protocol has been developed for the synthesis of 3,5-bis (arylidene)-4-piperidones in excellent yields using $[Et_3NH][HSO_4]$ as a catalyst at room temperature in a short reaction time. These analogues were evaluated for the first time for antitubercular activity against the *MTB* H37Ra and *M. Bovis* BCG strains. However, the compounds **4f**, **4g** and **4j** exhibited IC_{50} values of 1.89, 6.85 and 2.37 µg/ml against *MTB* H37Ra, respectively. Compounds **4f**, **4g, 4i** and **4j** showing excellent antitubercular activity against *M. Bovis* BCG strain with IC_{50} values of 2.69, 7.33, 6.94 and 2.85 µg/mL, respectively. From the docking studies, the most active conjugates **4f**, **4g** and **4j** showed hydrogen binding with the amino acid residue Ile194 and two π-π stacking hydrophobic interactions with the amino acid residue Tyr158 and Trp222 present in the active site of *MTB* InhA. Furthermore, evaluation of the data on the cytotoxicity, antimicrobial activity and docking studies shows that the conjugates **4f**, **4g, 4i** and **4j** are highly selective toward the *MTB* H37Raand *M. Bovis* BCG strain.

Experimental section

Materials and methods

All chemicals and reagents were procured from Sigma Aldrich, S.D. Fine chemical and commercial suppliers and used without further purification. The completion of the reactions was

monitored by thin-layer chromatography (TLC) on aluminum plates coated with silica gel 60 F_{254}, 0.25 mm thickness (Merck). The detection of the components was made by exposure to iodine vapors or UV light. Melting points were determined by open capillary methods and are uncorrected. The products were characterized using ^{1}H NMR, ^{13}C NMR spectra and HRMS. The NMR spectra of the product were obtained using a Bruker AC-400MHz spectrometer with TMS as the internal standard. High-resolution mass spectra (HRMS) were recorded on Agilent 6520 (QTOF) mass spectrometer.

Experimental procedure for the synthesis of $[Et_3NH][HSO_4]$

The synthesis of ionic liquid was carried out in a 100 mL round-bottom flask, which was immersed in a recirculating heated water-bath and fitted with a reflux condenser. Sulfuric acid (98%) (1.96 g, 0.02 mol) was added drop wise from triethylamine (2.02 g, 0.02 mol) stirring at 60 °C for 1 h. After the addition, the reaction mixture was stirred for an additional period of 1 h at 70 °C to ensure the reaction had proceeded to completion. The traces of water were removed by heating the residue at 80 °C in high vacuum until the weight of the residue remains constant.

Triethylammonium hydrogen sulfate $[Et_3NH][HSO_4]$

^{1}H NMR (400 MHz, DMSO-d_6): δ ppm = 1.16–1.23 (t, 9H, 3 × CH_3), 3.04–3.15 (q, 6H, 3 × CH_2) and 8.85 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm = 11.03 (CH_3) and 52.6 (CH_2); FT-IR cm^{-1} 3024 (for N-H stretch), 2815.74 (for C-H stretch), 1231.96 (for C-N stretch).

General procedure for one-pot synthesis of 2-((3E,5E)-3,5-dibenzylidene-4-oxopiperidin-1-yl)-N-phenylacetamide (4a-l)

A mixture of the substituted aldehyde (**1a-c**) (1 mmol), piperidinone (**2**) (1 mmol) and various 2-chloro-*N*-phenyl acetamides (**3a-g**) (1 mmol) in 25 mol% $[Et_3NH][HSO_4]$ catalyst was stirred at room temperature for 2.5 h. The progress of the reaction was monitored by TLC. After the completion of reaction confirmed by the TLC using *n*-hexane: ethyl acetate (8:2) as solvent system. After completion of the reaction, the reaction mixture was poured on ice cold water and the product was extracted using ethyl acetate solvent. The organic layer evaporated under vacuum on rotavapor to get crude solid which further crystallized from ethanol to obtain a pure solid product (**4a-l**). The aqueous filtrate was subjected for evaporation of water to get viscous liquid, which on cooling afforded ionic liquid. Further, the residual ionic liquid was washed with diethyl ether, dried under vacuum at 60 °C and reused for subsequent reactions. It was then reused at least four consecutive cycles without much appreciable loss in its catalytic activity.

2-((3E,5E)-3,5-Dibenzylidene-4-oxopiperidin-1-yl)-N-phenylacetamide (**4a**): The compound **4a** was obtained from **1a**, **2** and **3a** as yellow solid; Yield: 82%; Mp: 182–184 °C; IR (cm^{-1}): 3030, 2985, 1707, 1656, 1591, 1555 and 1493; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 9.76 (s, 1H, NH), 7.83 (s, 2H, -C = H), 7.67–7.65 (m, 4H, Ar-H), 7.59–7.30 (m, 10H, Ar-H), 4.47 (s, 4H, -CH_2) and 4.12 (s, 2H, -CH_2); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 187.2, 169.5, 145.8, 137.0, 136.6, 132.3, 131.5, 131.0, 130.9, 127.5, 126.7, 125.0, 123.3, 121.9, 119.7, 57.5 and 50.5; HRMS (ESI-qTOF): Calcd for $C_{27}H_{25}N_2O_2$ $[M + H]^+$, 409.2838, found: 409.2842.

2-((3E,5E)-3,5-Dibenzylidene-4-oxopiperidin-1-yl)-N-(p-tolyl)acetamide (**4b**): The compound **4b** was obtained from **1a**, **2** and **3b** as pale yellow solid; Yield: 75%; Mp: 205–207 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.75 (s, 2H, -C = H), 7.59–7.48 (m, 10H, Ar-H), 7.46–7.29 (m, 4H, Ar-H), 4.48 (s, 4H, -CH_2), 4.11 (s, 2H, -CH_2) and 2.42 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 183.8, 162.2, 142.2, 134.9, 131.7, 129.9, 129.8, 128.3, 124.2, 123.1, 115.2, 115.0, 114.6, 55.2, 51.3 and 21.1.

2-((3E,5E)-3,5-Dibenzylidene-4-oxopiperidin-1-yl)-N-(4-methoxyphenyl) acetamide (**4c**): The compound **4c** was obtained from **1a**, **2** and **3c** as yellow solid; Yield: 76%; Mp: 181–183 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.69 (s, 2H, -C = H), 7.54–7.36 (m, 12H, Ar-H), 7.04–703 (m, 2H, Ar-H), 4.57 (s, 4H, -CH_2), 4.37 (s, 2H, -CH_2) and 3.84 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 184.9, 155.7, 136.5, 135.2, 132.2, 128.6, 125.6, 125.5, 123.5, 121.8, 121.3, 119.7, 119.1, 111.1, 57.3, 54.5 and 49.9; HRMS (ESI-qTOF): Calcd for $C_{28}H_{27}N_2O_3$ $[M + H]^+$, 439.1555, found: 439.1586.

2-((3E,5E)-3,5-Dibenzylidene-4-oxopiperidin-1-yl)-N-(3-methoxyphenyl) acetamide (**4d**): The compound **4d** was obtained from **1a**, **2** and **3d** as yellow solid; Yield: 71%;Mp: 198–200 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.84 (s, 2H, -C = H), 7.56–7.08 (m, 11H, Ar-H), 7.02–6.73 (m, 3H, Ar-H), 4.79 (s, 4H, -CH_2), 4.46 (s, 2H, -CH_2) and 3.89 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 185.1, 160.0, 140.3, 137.8, 135.3, 131.5, 129.4, 129.1, 127.8, 127.5, 127.1, 126.2, 124.3, 123.4, 122.6, 119.9, 56.4, 53.7 and 49.2.

N-(4-Chlorophenyl)-2-((3E,5E)-3,5-dibenzylidene-4-oxopiperidin-1-yl) acetamide (**4e**): The compound **4e** was obtained from **1a**, **2** and **3f** as pale yellow solid; Yield: 76%; Mp: 207–209 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.77 (s, 2H, -C = H), 7.61–7.46 (m, 12H, Ar-H), 7.38–7.36 (m, 2H, Ar-H), 4.53 (s, 4H, -CH_2) and 4.16 (s, 2H, -CH_2); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 184.3, 168.1, 141.1, 136.7, 132.5, 131.6, 130.9, 137.3, 126.7, 126.3, 122.6, 122.3, 122.0, 121.8, 119.5, 55.3 and 49.6; HRMS (ESI-qTOF): Calcd for $C_{27}H_{24}ClN_2O_2$ $[M + H]^+$, 443.2032, found: 443.2005

2-((3E,5E)-3,5-Bis(4-fluorobenzylidene)-4-oxopiperidin-1-yl)-N-(4-methoxyphenyl)acetamide (**4f**): The compound **4f** was obtained from **1b**, **2** and **3f** as yellow solid; Yield: 78%; Mp: 232–234 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.83 (s, 2H, -C = H), 7.60–7.58 (m, 4H, Ar-H), 7.39–7.04 (m, 8H, Ar-H), 4.59 (s, 4H, -CH_2), 4.03 (s, 2H, -CH_2) and 3.91 (s, 3H, OCH_3); ^{13}C NMR (101 MHz, DMSO) δ 183.9, 163.4, 162.1, 160.4, 136.1, 132.4, 130.9, 129.5, 127.5, 125.5, 122.1, 115.83, 57.3, 52.78, 46.58; HRMS (ESI-qTOF): Calcd for $C_{28}H_{25}F_2N_2O_3$ $[M + H]^+$, 475.2342, found: 475.2364

2-((3E,5E)-3,5-Bis(4-chlorobenzylidene)-4-oxopiperidin-1-yl)-N-phenylacetamide (**4g**): The compound **4g** was obtained from **1c**, **2** and **3g** as pale yellow solid; Yield: 80%; Mp: 186–188 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.79 (s, 2H, -C = H), 7.53–7.46 (m, 4H, Ar-H), 7.44–7.16 (m, 9H, Ar-H), 4.40 (s, 4H, -CH_2) and 4.08 (s, 2H, -CH_2); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 184.1, 166.7, 160.8, 148.8, 148.2, 142.2, 138.7, 129.9, 129.8, 128.6, 128.4, 124.2, 123.1, 115.2, 115.0, 114.0, 57.4 and 51.5; ESI-MS: Calcd for $C_{27}H_{23}Cl_2N_2O_2$ $[M + H]^+$, 478.1, found: 478.0.

2-((3E,5E)-3,5-Bis(4-chlorobenzylidene)-4-oxopiperidin-1-yl)-N-(p-tolyl)acetamide (**4h**): The compound **4h** was obtained from **1c**, **2** and **3b** as pale yellow solid; Yield: 82%; Mp: 175–177 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.77 (s, 2H, -C = H), 7.49–7.43 (m, 8H, Ar-H), 7.30–7.25 (m, 4H, Ar-H), 4.50 (s, 4H, -CH_2), 3.98 (s, 2H, -CH_2) and 2.37 (s, 3H, CH_3); 184.4, 143.1, 139.5, 135.1, 134.5, 133.5, 130.5, 129.7, 128.4, 125.3, 124.4, 115.3, 57.3, 49.6 and 21.5; ESI-MS: Calcd for $C_{28}H_{25}Cl_2N_2O_2$ $[M + H]^+$, 492.2, found: 492.0.

2-((3E,5E)-3,5-Bis(4-chlorobenzylidene)-4-oxopiperidin-1-yl)-N-(m-tolyl)acetamide (**4i**): The compound **4i** was obtained from **1c**, **2** and **3c** as pale yellow solid; Yield: 75%; Mp: 201–203 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.81 (s, 2H, -C = H), 7.47–7.43 (m, 8H, Ar-H), 7.30–7.02 (m, 4H, Ar-H), 4.48 (s, 4H, -CH_2), 3.90 (s, 2H, -CH_2) and 2.40 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 185.8, 167.8, 144.8, 143.6, 139.5, 134.1, 129.3, 120.3, 119.2, 57.9, 55.8 and 21.2; ESI-MS: Calcd for $C_{28}H_{25}Cl_2N_2O_2$ $[M + H]^+$, 492.4, found: 492.0.

2-((3E,5E)-3,5-Bis(4-chlorobenzylidene)-4-oxopiperidin-1-yl)-N-(4-methoxyphenyl)acetamide (**4j**): The compound **4j** was obtained from **1c**, **2** and **3d** as yellow solid; Yield: 74%; Mp: 217–219 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.78 (s, 2H, -C = H), 7.49–7.43 (m, 10H, Ar-H), 7.00–6.99 (m, 2H, Ar-H), 4.48 (s, 4H, -CH_2), 3.96 (s, 2H, -CH_2) and 3.86 (s, 3H, OCH_3); ^{13}C

NMR (100 MHz, DMSO-d_6, δppm): 186.4, 161.3, 137.6, 136.7, 132.7, 129.0, 126.2, 126.1, 124.9, 124.8, 124.0, 122.4, 121.7, 120.1, 56.8, 52.7 and 47.4; HRMS (ESI-qTOF): Calcd for $C_{28}H_{25}Cl_2N_2O_3$ $[M+H]^+$, 508.2652, found: 508.2629

2-((3E,5E)-3,5-Bis(4-chlorobenzylidene)-4-oxopiperidin-1-yl)-N-(3-methoxyphenyl)acetamide (**4k**): The compound **4k** was obtained from **1c**, **2** and **3e** as yellow solid; Yield: 76%; Mp: 221–223 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 7.95 (s, 2H, -C = H), 7.65–7.53 (m, 9H, Ar-H), 7.11–6.85 (m, 3H, Ar-H), 4.53 (s, 4H, -CH_2), 3.93 (s, 2H, -CH_2) and 3.88 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 185.1, 161.7, 136.5, 134.6, 129.7, 129.3, 126.3, 126.0, 125.6, 125.3, 122.9, 122.5, 122.0, 121.2, 120.4, 120.2, 119.8, 119.3, 56.1, 52.4 and 49.0.

2-((3E,5E)-3,5-Bis(4-chlorobenzylidene)-4-oxopiperidin-1-yl)-N-(4-chlorophenyl)acetamide (**4l**): The compound **4l** was obtained from **1c**, **2** and **3f** as yellow solid; Yield: 78%; Mp: 190–192 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δppm): 8.05 (s, 2H, -C = H), 7.84–7.80 (m, 9H, Ar-H), 7.76–7.64 (m, 3H, Ar-H), 4.52 (s, 4H, -CH_2) and 4.26 (s, 2H, -CH_2); ^{13}C NMR (100 MHz, DMSO-d_6, δppm): 187.0, 168.9, 146.0, 144.8, 137.7, 136.7, 135.3, 130.4, 129.8, 132.4, 59.1 and 57.0; HRMS (ESI-qTOF): Calcd for $C_{27}H_{22}Cl_3N_2O_2$ $[M+H]^+$, 512.4762, found: 512.4727.

Biological assay

Antitubercular activity

All the chemicals such as sodium salt XTT, DMSO, sulfanilic acid, sodium nitrate, NEED and rifampicin were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA. Compounds were dissolved in DMSO and used as stock solution for further antimycobacterial testing. Microbial strains such as Mycobacterium bovis BCG (ATCC 35734) and Mycobacterium tuberculosis H_{37}Ra (ATCC 25177) were obtained from AstraZeneca, India. The stock culture was maintained at −80 °C and sub cultured once in a liquid medium before inoculation into an experimental culture. Cultures were grown in Dubos media (enrichment media). Mycobacterium pheli medium (minimal essential medium) was used for antimycobacterial assay. It contains 0.5 g KH_2PO_4, 0.25 g trisodium citrate, 60 mg $MgSO_4$, 0.5 g aspargine and 2 mL glycerol in distilled water (100 mL) followed by pH adjustment to 6.6. All the newly synthesized compounds were screened *in vitro* against two Mycobacterium species such as Mycobacterium tuberculosis H_{37}Ra and Mycobacterium bovis BCG. Both species of Mycobacterium were grown in Mycobacterium pheli medium. Screening of Mycobacterium tuberculosis H_{37}Ra was done by using XTT reduction menadione assay (XRMA) and Mycobacterium Bovis BCG screening was done by using NR (Nitrate reductase) assay, both of them were developed earlier in our lab.[25] Briefly, 2.5 μl of these inhibitor solutions were added in a total volume of 250 μl of Mycobacterium pheli medium consisting of bacilli. The incubation was terminated on the 8th day for Active and 12 days for Dormant MTB culture. The XRMA and NR was then carried out to estimate viable cells present in different wells of the assay plate. The optical density was read on a micro plate reader (Spectramax plus384 plate reader, Molecular Devices Inc) at 470 nm filter for XTT and at 540 nm filter for NR against a blank prepared from cell-free wells. Absorbance given by cells treated with the vehicle alone was taken as 100% cell growth. Primary screening was done at 30, 10 and 3 μg/ml. Compounds showing 90% inhibition of bacilli, at or lower than 30 μg/mL were selected for further dose response curve. All experiments were performed in triplicates and the quantitative value was expressed as the average ± standard deviation. MIC and IC_{50} values of selected compound were calculated from their dose response curves by using Origin 6 software. % Inhibition was calculated by using following formula: % Inhibition = [(absorbance of compound − absorbance of Test)/(absorbance of Control − absorbance of Blank)] × 100, where control is the medium with bacilli along with vehicle and blank is cell free medium.

Cytotoxicity activity

Three human cancer cell lines, HeLa (human cervical cancer cell line), A549 (human lung adenocarcinoma cell line) and PANC-1 (human pancreas carcinoma cell line) were used to check the cytotoxicity of compounds. The cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in T 25 flasks with 10% (v/v) fetal bovine serum (FBS) containing Dulbecco's Modified Eagle Medium (DMEM). Cell line containing T 25 flasks were maintained at 37 °C under 5% CO_2 and 95% air in a humidified atmosphere. Medium were replaced twice a week. All the compounds were tested for their cytotoxicity against HeLa, A549 and PANC-1 cell line by using modified MTT assay.[28] Briefly, cells were seeded as, 1.5×10^4 cells/ml for HeLa, 1×10^4-cells/well for A549 and PANC-1 in a 96 well plate. The plates were incubated for 24 h into CO_2 incubator (37 °C under 5% CO_2 and 95% air in a humidified atmosphere) to adhere the cells. After incubation, compound was added in such a way that final concentration becomes 30, 10, and 3 µg/ml in the test well. Concentrations ranges of compound were selected as 30, 10 and 3 µg/ml of each. Again, plates were incubated for additional 72 h for HeLa and 48 h for A549 and PANC-1 to see the effect of compound on cells. After that, cell medium was replaced with 100 µl of Glucose-MTT (0.5 mg/ml)-PBS medium and kept the plate for 2–4 h to form the reduced MTT or Formazan crystals. This reduced MTT or Formazan crystals were solubilized by addition of acidified isopropanol. The optical density was read on a micro plate reader (Spectramax plus 384 plate reader, Molecular Devices Inc) at 470 nm filter against a blank prepared from cell-free wells. Absorbance given by cells treated with the vehicle alone was taken as 100% cell growth. All the experiments were performed in triplicates and the quantitative value was expressed as the average ± standard deviation. GI_{50} and MIC values were calculated by plotting the graphs, by using Origin Pro software. The viability and growth in the presence of test material is calculated by using the following formula: % cytotoxicity = [(average absorbance of control-absorbance of compound)/(absorbance of control-absorbance of blank)] × 100, where control is the culture medium with cells and DMSO and blank is the culture medium without cells.

Selectivity index

The selectivity index (SI) was calculated by dividing the 50% growth inhibition concentration (GI_{50}) for cell lines (HeLa, A549 and PANC-1) by the MIC for *in vitro* activity against active/dormant MTB and BCG.[29]

Anti-bacterial activity

All bacterial cultures were first grown in LB media at 37 °C at 180 RPM. Once the culture reaches 1 O.D, it is used for antibacterial assay. Bacterial strains *E. coli* (NCIM 2688), *P. fluorescens* (NCIM 2036) as Gram-negative and *B. subtilis* (NCIM 2079), *S. aureus* (NCIM 2010) as Gram-positive were obtained from NCIM (NCL, Pune) and were grown in Luria Burtony medium from Himedia, India. The assay was performed in 96 well plates after 8 and 12 h. for Gram-negative and Gram-positive bacteria, respectively. 0.1% of 1 OD culture at 620 nm was used for screening.[30] 0.1% inoculated culture was added in to each well of 96 well plate containing the compounds to be tested. Optical density for each plate was measured at 620 nm after 8 h for Gram negative bacteria and after 12 h for Gram-positive bacteria.

Computational study

Molecular docking study

To understand mode of action for antitubercular activity of the synthesized benzylidene-4-oxopiperidin-1-yl-*N*-phenylacetamide derivatives, molecular modeling and docking studies were

performed. Glide 5.8[31] was used to perform docking studies using crystal structure of mycobacterium tuberculosis enoyl-acyl carrier protein reductase (InhA) (PDB id:1ZID).

The structures of the compounds were drawn using 2D-sketcher present in Maestro 9.3[32] and were converted from 2D into 3D representations and saved in maestro format. For further computational studies, compounds were prepared using LigPrep 2.5[33] which gives the low-energy conformers, 3D structures with correct chiralities for each successfully processed input structure. The protein was purified using the protein preparation wizard present in Maestro 9.3. All the water molecules were deleted. Bond order was assigned and H-atoms were added to the protein, including the protons necessary to define the correct ionization and tautomeric states of the amino acid residues. Missing residues of the side chain were added using Prime 3.1.[34] Protein refinement was done in two steps. Initially, orientation of polar hydrogens, flip terminal amides and histidines was optimized and the protonation states were adjusted. Further, steric clashes potentially existing in the protein were relaxed using the OPLS-2005 force field present in the impact refinement module. Minimization was terminated when the energy converged or the root mean square deviation reached a maximum cut off of 0.30 Å.[35] To predict the active site of the receptor, a grid was generated using grid generation panel of glide with the default settings. Grid is generated to define the binding site of co crystallized ligand in the receptor. The ligand was selected to define the position of active site and size of the enclosing box was set to 20 Å to include the significant part of 1ZID.

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Disclosure statement

There are no conflicts of interest.

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