Synthesis, Docking, and Pharmacological Evaluation of Derivatives of α-Aminoketones Appended to Sydnones as Potent Antitubercular and Antifungal Scaffolds

Atukuri Dorababu,[a] Ravindra R. Kamble,[a]* Saba Kauser J. Shaikh,[a] Shilpa M. Somagond,[a] Praveen K. Bayannavar,[a] and Shrinivas D. Joshi[b]

[a]Department of Chemistry, Karnatak University Dharwad, Pavate Nagar, Dharwad, Karnataka 580003, India
[b]Department of Pharmaceutical Chemistry, SET's College of Pharmacy, Novel Drug Design and Discovery Laboratory, Sangolli Rayanna Nagar, Dharwad, Karnataka 580002, India
*E-mail: kamchem9@gmail.com

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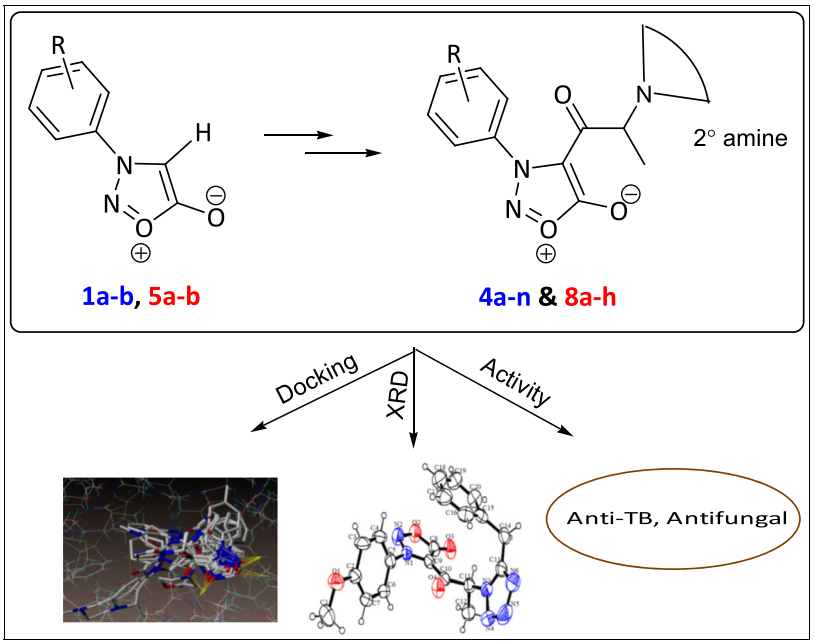


3-Arylsydnones are reported to possess striking pharmaceutical potency. α-Aminoketone, a biologically active structural unit, is built at the fourth (electrophilic) position of sydnone and further derivatized with secondary amine and tetrazoles. The α-aminoketone derivatives of sydnones coupled with secondary amines **4a**–**n** were docked on enoyl acyl carrier protein (ACP) reductase from *Mycobacterium tuberculosis*, which revealed that compounds **4b**, **4f**, and **4i** showed efficient C score values with different binding modes and hydrogen bonding. Further, these compounds were screened for antimycobacterial activity; among them, compound **4f** displayed sensitivity at 6.25 μg/mL compared with the standard drug (Streptomycin) against *M. tuberculosis* ($H_{37}R_V$ strain). In addition to this, α-aminoketone derivatives of sydnones coupled with tetrazoles **8a**–**h** were evaluated for antifungal activity. In the antifungal activity, compound **8b** has exhibited potent activity at 6.25 μg/mL against *Candida albicans* and compound **8g** at 0.4 μg/mL against *Aspergillus fumigatus*. The antifungal activities are comparatively better than standard antifungal agent Fluconazole at these drug concentrations. Alongside characterization of the final compounds by Fourier transform infrared, mass, ^{1}H NMR, and ^{13}C NMR spectral analyses, compounds **8b** and **8g** were confirmed by X-ray crystallographic studies.

INTRODUCTION

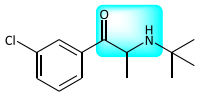
One of the class of mesoionic compounds, sydnones are non-benzeniod aromatic heterocyclic compounds. They have shown good antibacterial and antifungal activity [1] and are widely studied for some important biological activities like antiviral, antitumor, antimicrobial, anti-inflammatory, anticancer, analgesic, anthelmintic, and antihypertensive activities [2].

The heterocyclic compounds containing aminoketone moiety have exhibited a striking pharmaceutical activity, which is evident from the vast literature of aminoketone derivatives. They have possessed a wide variety of antimicrobial activities, and these compounds are also

highly active in some physiological activities. Chiral α-amino ketones represent an important class of compounds as evidenced by their biological activity (**i**, Fig. 1) [3]. They are important structural elements in chiral drugs such as α-adrenergic or β-adrenergic blockers and agonists in the treatment of cardiovascular disease (cardiac failure), asthma, and glaucoma [4]. Recently, reported aryl alkanol piperazine derivatives were discovered with the reuptake inhibition activities of 5-hydroxytryptamine and noradrenaline, which may have potential utility in treating the depression [5]. Aryl α-amino ketones are used as antidepressants in the clinical treatment [6,7]. Drugs such as Bupropion (**i**) block reuptake at both noradrenaline and dopaminergic neurons are also known as dual reuptake inhibitors providing good efficacy and tolerability with respect to antide pression activity [8–10]. Peptidyl ketone derivatives of α-amino ketones have significant value for the development of molecular therapeutics and diagnostic tools in chemical biology [11,12].

4-Bromo-*N*-phenylsydnone derivatives when reacted with secondary amines gave some potent pharmaceutical moieties having α-aminoketone moiety, which have shown excellent antitubercular activity [13]. Ye *et al*. [14] synthesized a series of 3-[2-(2-methoxyphenyl)2-oxoethyl] quinazolinones as anticoccidial agents by modifying the quinazolinone ring of *Febrifugine* against *Eimeria tenella* in the chicken. Kumar *et al*. [15] reported synthesis of α-aminoketone derivatives and reported that the compound has exhibited good anti-inflammatory activity.

A group of aminoketone derivatives of 2,4-disubstituted thiazoles were tested *in vivo* for their anti-inflammatory activity and have shown excellent activity. The activity was studied by means of the carrageenin-induced mouse paw edema [16]. α-Amino and β-amino ketone analogs of amino acids were synthesized as potential antagonists of amino acids in microbial metabolism [17]. Aminoketones appended to nabumetone moiety prepared *via* Mannich reaction have exhibited significant antidiabetic activity [18]. α-Aminoketone hydrochlorides synthesized by Gevorgyan *et al*. possessed moderate antibacterial and pronounced peripheral *n*-cholinolytic activity [19]. Aminoketones have also been reported as good analgesic agents [20]. α-Aminoketones built on pyrimidine have exhibited potent antimicrobial activity, in particular



i

Figure 1. Chiral drug containing α-aminoketone moiety. [Color figure can be viewed at wileyonlinelibrary.com]

promising antifungal activity against *Candida albicans* [21]. α-Aminotetralones possessing α-aminoketone structural unit have been synthesized by Dunsmore *et al*. [22] and were found to have striking antibacterial activity where in the lead compounds have inhibited MurA and MurZ enzymes of bacterium *Staphylococcus aureus*. They have also proved that an amine motif is essential for its antibacterial activity. 6-Amino-2,4-lutidine carbo xamides having α-aminoketone structural motif were designed using 2-aminopyridine and reported to possess good systemic and topic inflammatory inhibition activities [23]. Antianalgesics were prepared using indole as structural unit and thereafter appending α-amidoamine moiety on it [24].

5-Aryltetrazole derivatives were synthesized and evaluated for their antifungal potency [25]. Potent antifungal tetrazoles were designed and synthesized, and their structure–activity relationship was studied [26]. Dai *et al*. [27] have synthesized the tetrazole derivatives possessing both antibacterial and antifungal potencies. Novel tetrazole ring appending to acyl-hydrazones was reported and screened for their striking antifungal activity [28]. Staniszewska *et al*. [29] have prepared 1,5 and 2,5-disubstituted tetrazoles that exhibited promising anti fungal activity towards *C. albicans* fungus. A set of hybrid compounds of ciprofloxacin and 5-aryl tetrazoles was engineered *via* $COCH_2$ linkage [30]. The lead compounds were reported to have antifungal potency against different fungal strains. 5-Thio-substituted tetrazole derivatives were synthesized, and their antibacterial and antifungal activities are evaluated [31].

Spirocyclic tertiary amines designed and synthesized by Badiola *et al*. [32] have exhibited prominent antitubercular activity. Pentacyclo-undecane derivatized tetra-amines having tertiary amine units in the cyclic form were prepared and found to be potent antitubercular agents [33]. Bisquinoline analogs of TMC207 and confor mational-constrained molecules possessing tertiary amine unit were reported, and they possessed remarkable antitubercular potency [34]. A set of 9-benzylpurines possessing tertiary amine unit was synthesized among which 2-chloro-4(2-furanyl)-9-benzylpurine was reported to inhibit *Mycobacterium tuberculosis* $H_{37}R_V$ with lowest minimum inhibitory concentration (MIC) value [35]. 6-Chloro-3-phenyl-4-thioxo-2*H*-1,3-benzoxazine-2(3*H*)-ones and 6-chloro-3-phenyl-2*H*-1,3-benzoxazine-2,4(3*H*)-dithiones in which tertiary amine structure unit in the form of cyclic ring were synthesized and found to be good antimycobacterial agents [36]. Hence, the amino ketone group is pharmacologically active moiety with respect to anti-inflammatory and antifungal activities. Alongside, antifungal nature of tetrazole unit and antitubercular potency of tertiary amine moiety have prompted us to prepare the tetrazole and tertiary amine

derivatized α-aminoketones of sydnones and evaluate their biological activity.

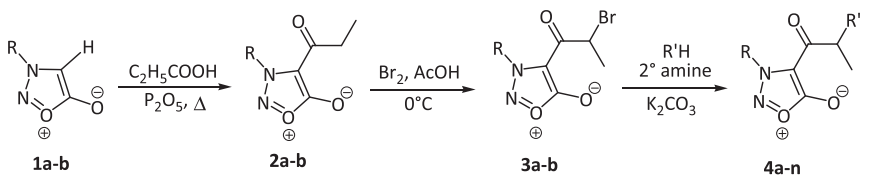
RESULTS AND DISCUSSION

Chemistry. Propionyl group is introduced at the fourth position of 3-arylsydnones **1a**–**b** *via* electrophilic substitution affording 1-(3-arylsydnon-4-yl)propan-1-ones, **2a**–**b**, which were monobrominated at α-position of propionyl group to yield 2-bromo-1-(3-arylsydnon-4-yl) propan-1-ones, **3a**–**b**, and subsequent nucleophilic substitution of **3a**–**b** using various secondary amines and tetrazoles gave α-aminoketones, 2-(sec-amino)-1-(3-aryl sydnon-4-yl)propan-1-ones **4a**–**n** (Scheme 1). Likewise, compounds **5a**–**b** are propionylated at 4-position to afford 1-(3-arylsydnon-4-yl)propan-1-ones, **6a**–**b**, which on bromination yielded 2-bromo-1-(3-arylsydnon-4-yl)pro pan-1-ones, **7a**–**b**. Finally, intermediates **7a**–**b** are reacted with benzyl tetrazoles and thiobenzyl tetrazoles to obtain 2-(tetrazolo)-1-(3-arylsydnon-4-yl)propan-1-ones, **8a**–**h** (Scheme 2).

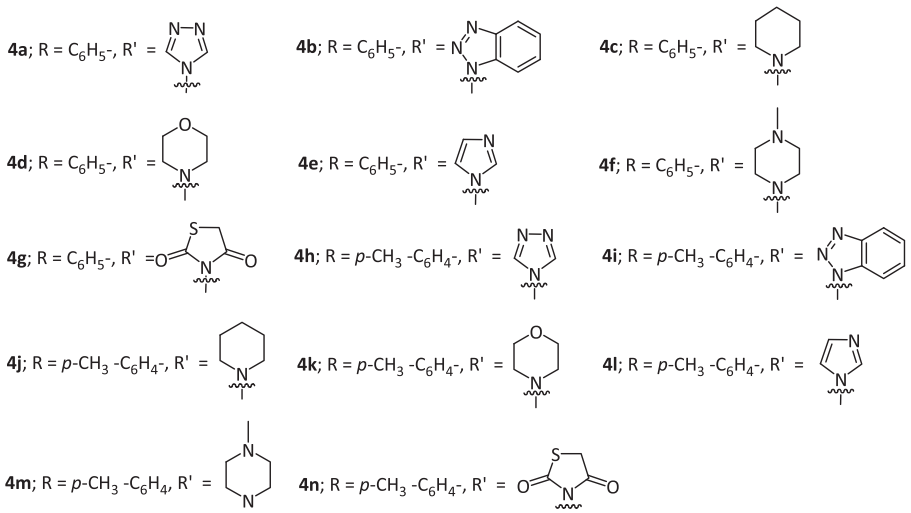
Benzyl tetrazole and benzyl thiotetrazole possess two nucleophilic sites at the third and fifth positions because of tautomerism of NH proton. Hence, these give two isomers regioselectively in which 1,5-isomer as minor product and 1,3-isomer as major product. 1,3-Isomer is major product because it is sterically less hindered when compared with 1,5-isomer. The isomers were separated using column chromatography (hexane : ethyl acetate) and analyzed. The two different isomers were identified on the basis of chemical shift of methine proton flanked by carbonyl group at the fourth position of sydnone and nitrogen of tetrazole ring. The methine proton in 1,3-isomer appeared to be down field when compared with that of 1,5-isomer as in 1,3-isomer nucleophilic nitrogen of tetrazole is flanked by nitrogen atoms of tetrazole ring on either sides, whereas in 1,5-isomer, nucleophilic nitrogen of tetrazole is flanked by nitrogen atom and carbon atom of tetrazole ring. This was also confirmed by X-ray diffraction analysis of 1,5-isomer 2-(5-benzyl-1*H*-tetrazol-1-yl)-1-(3-(4-methoxyphenyl)sydnon-4-yl)propan-1-one, **8b** (CCDC No. 1884833) and 1,3-isomer of compound **8g** (CCDC No. 1884831) (Figs 2 and 3 and Scheme 2). Details of X-ray diffraction analysis can be found in the Supporting Information.

Spectral analysis. In spectral analysis of final compounds, a band in the range 1762–1777 cm^{-1} was due to sydnone carbonyl stretching. Stretching in the range 1673–1689 cm^{-1} was attributed to propionyl carbonyl group. ^1H NMR spectra exhibited a doublet and a quartet for methyl and methine protons in the

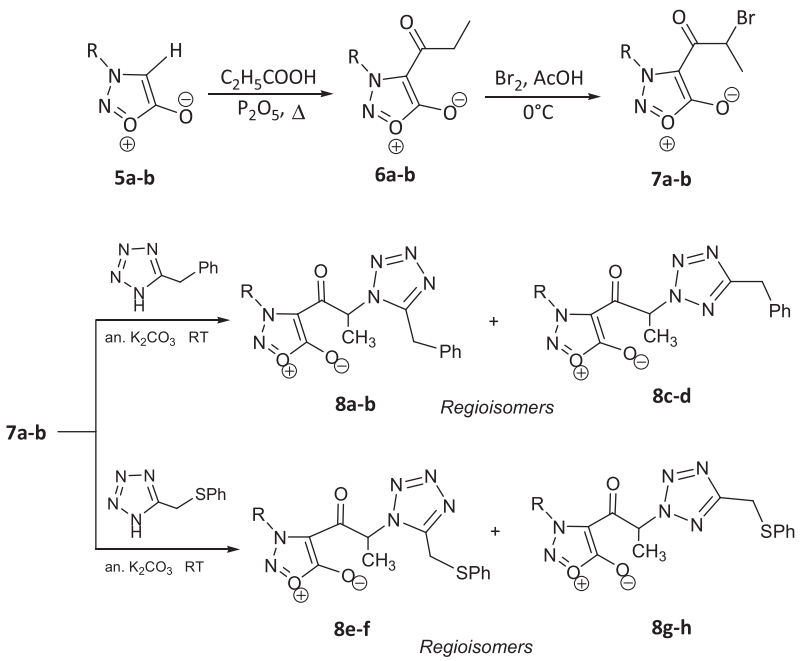
Scheme 1. Synthesis of α-aminoketone derivatives of sydnones.



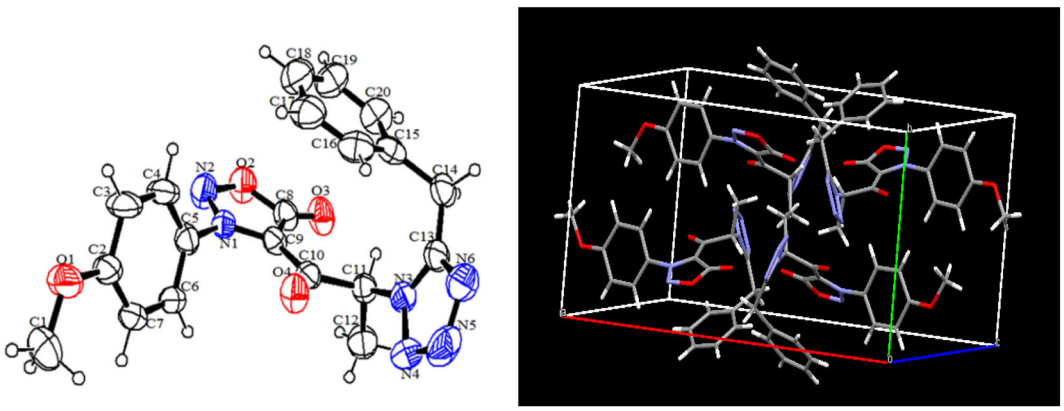
1a; R = phenyl, **1b**; R = *p*-tolyl, **2a**; R = phenyl, **2b**; R = *p*-tolyl, **3a**; R = phenyl, **3b**; R = *p*-tolyl



Scheme 2. Synthesis of α-aminoketone derivatives of sydnones coupled with tetrazoles.



5a; R = phenyl, **5b**; R = 4-OCH_3-C_6H_4-, **6a**; R = phenyl, **6b**; R = 4-OCH_3-C_6H_4-, 7**a**; R = Ph, 7**b**; = 4-OCH_3-C_6H_4-, **8a**; R = Ph (1,5-isomer), **8b**; R = 4-OCH_3-C_6H_4- (1,5-isomer), **8c**; R = Ph (1,3-isomer), **8d**; R = 4-OCH_3-C_6H_4- (1,3-isomer), **8e**; R = Ph (1,5-isomer), **8f**; R = 4-OCH_3-C_6H_4- (1,5-isomer), **8g**; R = Ph (1,3-isomer), **8h**; R = 4-OCH_3-C_6H_4- (1,3-isomer).



(a) **(b)**

Figure 2. (a) ORTEP projection of compound **8b** at 50% probability level. (b) Packing diagram of compound **8b**. [Color figure can be viewed at wileyonlinelibrary.com]

range 1.23–1.99 ppm and 5.13–6.45 ppm, respectively. A peak in the range 2.21–2.35 ppm was due to *p*-tolyl methyl protons in compounds **4h**–**n**. In case of compounds **4f** and **4m**, piperidine and *N*-methylpiperidine protons have resonated in the range 1.24–2.32 ppm and 2.73–2.82 ppm, respectively. A singlet in the range 3.35–3.37 ppm was due to thiazolidinone protons in compounds **4g** and **4n**. Resonance of imidazole and triazole protons was in the range 6.87–7.54 ppm and 8.05–8.44 ppm, respectively. Multiplet in the range 2.82–3.96 ppm was due to the resonance of morpholine protons in compounds **4d** and **4k**. Aromatic protons have resonated in the range 6.83–8.11 ppm.

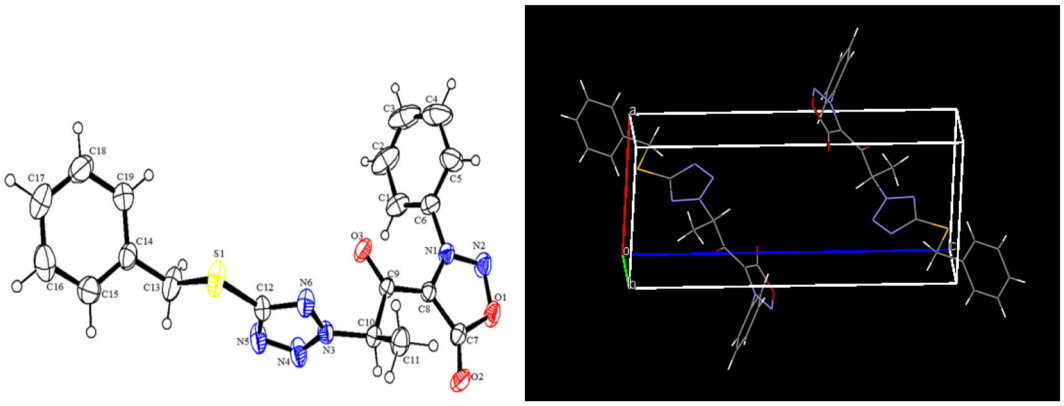


Figure 3. (a) ORTEP projection of compound **8g** at 50% probability level. (b) Packing diagram of compound **8g**. [Color figure can be viewed at wileyonlinelibrary.com]

In the infrared spectra of **8a–h**, stretching of sydnone carbonyl propionyl carbonyl groups were in the range 1769–1782 and 1671–1686 cm^{-1}, whereas in ^{1}H NMR spectra, methyl protons have resonated in the range 1.73–1.90 ppm as doublet. A singlet and a quartet in the range 4.18–4.52 and 5.87–6.39 ppm were due to the resonance of methylene and methine protons, respectively. Resonance of methoxy protons is in the range 3.76–3.84 ppm. Aromatic protons have resonated in the range 7.09–7.74 ppm. In the ^{13}C NMR spectra, number of signals was equal to the number of magnetically nonequivalent carbon atoms. Also, all the final compounds have shown molecular ion peaks corresponding to their molecular mass.

PHARMACOLOGY

Docking simulation. To investigate the mechanism of antitubercular activity and detailed intermolecular interactions between the synthesized compounds, molecular docking studies were performed on the crystal structure of *M. tuberculosis* enoyl reductase (InhA) complexed with 1-cyclohexyl-*N*-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK, 1.62 Å X-ray resolution) using the Surflex-Dock program of Sybyl-X 2.0 software. On the basis of greater level of resistance associated with Isonicotinylhydrazide (INH) isolates against InhA, docking studies were performed on InhA complex with 1-cyclohexyl-*N*-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide, which indicated the presence of drug–receptor interactions. All the 14 inhibitors were docked into the active site of Enoyl-acyl carrier protein reductase (ENR) as shown in Figure 4. The predicted binding energies of the compounds are listed in Table 1. The docking study revealed that all the compounds have showed very good docking score against *M. tuberculosis*. As depicted in Figure 5, compound **4f** makes one hydrogen bonding interaction at the active site of the enzyme (PDB ID: 4TZK), and carbonyl group makes interaction with hydrogen of TYR158 (C═O—H-TYR158, 2.74 Å). As depicted in

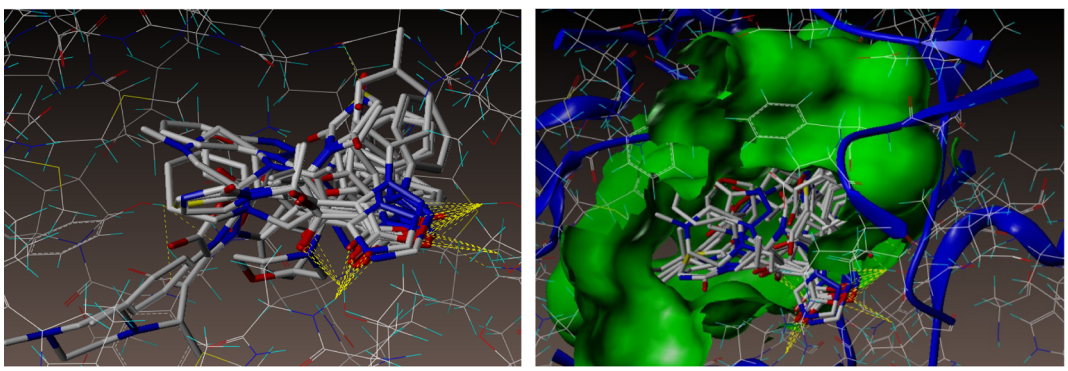


Figure 4. Docked view of all the compounds at the active site of the enzyme PDB ID 4TZK. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1

Surflex Docking score (kcal/mol) of the derivatives.

Compounds	Total[a]	Crash[b]	Polar[c]	D score[d]	PMF score[e]	G score[f]	Chem score[g]
4a	4.71	−0.58	4.03	−30.673	−2.773	−114.514	−17.365
4b	**6.28**	**−0.73**	**2.69**	**−54.296**	**17.741**	**−158.462**	**−24.809**
4c	4.40	−0.920	2.31	−45.381	14.662	−126.644	−20.823
4d	5.19	−0.62	2.96	−42.620	10.472	−132.427	−20.620
4e	4.89	−0.37	3.11	−31.795	7.483	−101.532	−20.175
4f	**7.09**	**−1.30**	**0.00**	**−111.964**	**−23.696**	**−242.241**	**−29.028**
4g	4.54	−0.44	3.08	−40.544	12.190	−108.211	−20.405
4h	5.55	−0.81	2.74	−48.632	17.895	−139.766	−16.688
4i	**5.90**	**−0.72**	**3.40**	**−48.973**	**15.784**	**−151.220**	**−25.324**
4j	5.44	−0.75	0.96	−84.891	−20.690	−153.288	−25.030
4k	5.56	−0.62	2.78	−55.692	9.058	−130.807	−22.391
4l	4.96	−0.69	2.71	−39.895	−5.982	−132.139	−22.033
4m	5.59	−0.72	2.60	−50.18	5.458	−147.576	−26.966
4n	5.25	−0.78	3.22	−51.960	4.431	−149.577	−22.618
4TZK	8.73	−1.39	1.18	−168.11	−49.19	−285.29	−37.47

Bold emphasizes the relevance of highest C-score values of the compounds under study obtained from Docking simulation.
[a]C score (consensus score) integrates a number of popular scoring functions for ranking the affinity of ligands bound to the active site of a receptor and reports the output of total score.
[b]Crash score reveals the inappropriate penetration into the binding site. Crash scores close to 0 are favorable. Negative numbers indicate penetration.
[c]Polar indicates the contribution of the polar interactions to the total score. The polar score may be useful for excluding docking results that make no hydrogen bonds.
[d]D score for charge and van der Waals interactions between the protein and the ligand.
[e]PMF (potential of mean force) score indicates the Helmholtz free energies of interactions for protein–ligand atom pairs.
[f]G score shows hydrogen bonding, complex (ligand–protein), and internal (ligand–ligand) energies.
[g]Chem score points for H-bonding, lipophilic contact, and rotational entropy, along with an intercept term.

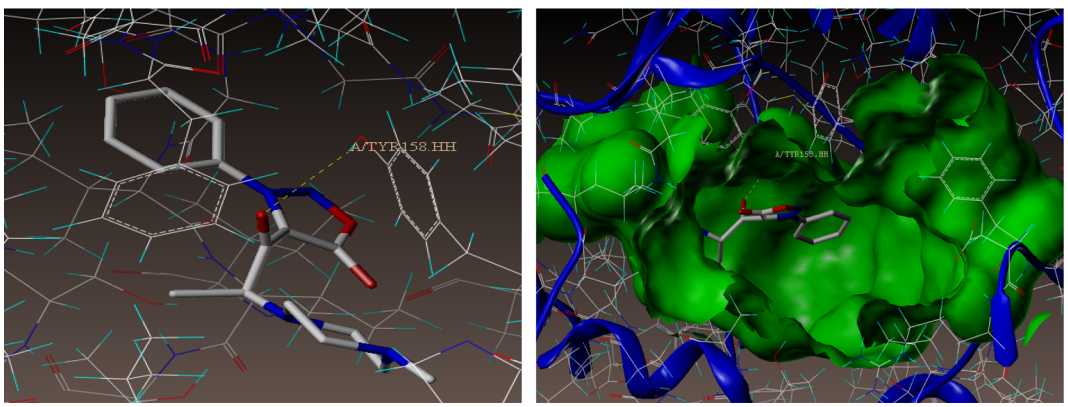
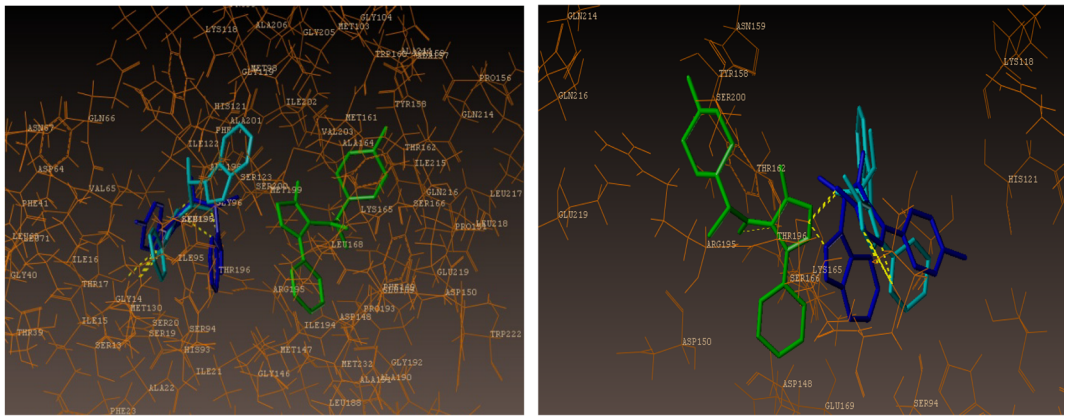


Figure 5. Docked view of compound **4f** at the active site of the enzyme PDB ID 4TZK. [Color figure can be viewed at wileyonlinelibrary.com]

Figure S7, compound **4b** makes five hydrogen bonding interactions at the active site of the enzyme (PDB ID: 4TZK); among those four interactions were of nitrogen atom present at the second position of oxadiazole ring with hydrogen of SER20 (N—H-SER20, 1.83 Å), oxygen atom present at the first position of oxadiazole ring makes two hydrogen bonding interactions with hydrogen of SER20 and THR17 (O—H-SER20, 2.73 Å; O—H-THR17, 2.73 Å), oxygen present at the fifth position of the oxadiazole ring with hydrogen of ALA198 (C–O—H-ALA198, 1.83 Å), and remaining another hydrogen bonding interaction raised from the carbonyl group with hydrogen of THR196 (C═O—H-THR196, 2.36 Å).

As depicted in Figure S8, compound **4i** makes five hydrogen bonding interactions at the active site of the enzyme (PDB ID: 4TZK): nitrogen atom present at the second position of oxadiazole ring with hydrogen of SER20 (N—H-SER20, 2.53 Å), oxygen atom present at the first position of oxadiazole ring makes one hydrogen bonding interaction with hydrogen of SER20 (O—H-SER20, 1.98 Å), oxygen present at the fifth position of the oxadiazole ring makes two hydrogen bonding interactions with hydrogen of THR196 and ALA198 (C–



(a) **(b)**

Figure 6. (a) Hydrophobic amino acids surrounded to compounds **4f** (green color), **4b** (cyan color), and **4i** (blue color). (b) Hydrophilic amino acids surrounded to compounds **4f**, **4b**, and **4i**. [Color figure can be viewed at wileyonlinelibrary.com]

O—H-THR196, 2.45 Å; C–O—H-ALA198, 1.96 Å), and remaining another hydrogen bonding interaction raised from the nitrogen atom of present at the second position of triazole ring with hydrogen of THR196 (N—H-THR196, 2.10 Å). Figure 6 represents the hydrophobic and hydrophilic amino acids surrounded to the studied compounds **4f**, **4b**, and **4i**. All the compounds showed consensus score in the range 7.09–4.40, indicating the summary of all forces of interaction between ligands and the InhA. Charge and van der Waals interactions between protein and ligands varied from −0.30 to −1.30. The Helmholtz free energies of interactions for protein ligands atom pairs range between −23.69 and 17.89. However, its H-bonding, complex (ligand–protein), and internal (ligand–ligand) energies range from −101.53 to −242.24, while those values −16.68 to −29.02 indicate the ligands due to H-bonding, lipophilic contact, and rotational entropy, as well as intercept terms. These scores indicate that molecules preferentially bind to InhA in comparison with the reference 4TZK ligand (Table 1).

BIOLOGY

Antitubercular activity using Alamar Blue dye. Compounds **4a–n** were tested for antitubercular activity against strain *M. tuberculosis* ($H_{37}R_V$ strain) at different drug concentrations using standard antitubercular drugs. When compared with standard drugs, compound **4f** has shown sensitivity (activity) as good as standard drugs Streptomycin at 6.25 μg/mL. The remaining compounds have sensitivity at higher drug concentrations as shown in Table 2.

Antifungal activity. Compounds **8a–h** were tested for antifungal activity against *C. albicans* at different concentrations. Compound **8b** has shown activity better than standard drug Fluconazole at MIC 6.25 μg/mL against *C. albicans*. Compound **8f** has exhibited activity as good as standard drug Fluconazole at MIC 12.5 μg/

Table 2

Results of antitubercular activity of compounds **4a–n**.

Test compounds	MIC (μg/mL)
4a	50
4b	50
4c	50
4d	50
4e	25
4f	6.25
4g	25
4h	50
4i	50
4j	50
4k	50
4l	50
4m	12.5
4n	50

MIC, minimum inhibitory concentration.
Standard values for the antitubercular test: Pyrazinamide, 3.125 μg/mL; Streptomycin, 6.25 μg/mL; Ciprofloxacin, 3.125 μg/mL.

Table 3

Minimum inhibitory concentration (MIC) results for antifungal activity.

Test compounds	MIC (μg/mL)	
	C. albicans	*A. fumigatus*
8a	100	100
8b	6.25	25
8c	100	100
8d	100	3.12
8e	100	25
8f	12.5	6.25
8g	100	0.40
8h	100	100

Fluconazole: *C. albicans*, 16 μg/mL; *A. fumigates*, 8 μg/mL.

mL. The remaining derivatives have shown weak activity. Compounds **8a**–**h** were tested for antifungal activity against *Aspergillus fumigatus* at different concentrations. Compound **8g** exhibited highly potent activity at MIC 0.4 μg/mL, which is better than standard drug Fluconazole. Compounds **8d** and **8f** have exhibited good activity at MIC 3.12 and 6.25 μg/mL, respectively. The remaining compounds have shown sensitivity at higher drug concentrations as shown in Table 3.

CONCLUSIONS

Enoyl reductase is involved in the biosynthesis of mycolic acids, a major component of mycobacterial cell walls. Hence, final compounds were docked on the crystal structure of *enoyl reductase* (InhA) complexed with PDB ID 4TZK. The docking study revealed that all the compounds have showed very good docking score against *M. tuberculosis*. Among the final compounds, compound **4f** has exhibited best C score. It indicates that the compound has penetrated well enough into the active site with less polar interactions. Considerably high van der Waals interactions between enzyme and the compound can be observed. Further high Helmholtz free energies of interaction, hydrogen bonding, and a good lipophilic contact between the compound and the enzyme can reveal from the C score.

Most of the final compounds have exhibited good antitubercular activity and some at higher drug concentration. Compound **4m** has shown sensitivity at 12.5 μg/mL and compound **4f** at 6.25 μg/mL. It may be due to the presence of secondary amine piperazine and that might have enhanced the antitubercular activity in those aminoketones.

In case of antifungal activity against *C. albicans*, only some compounds have exhibited activity. Among those compounds **8b** and **8f** have better activity at 6.25 and 12.5 μg/mL, respectively. From the aforementioned observations, it can be concluded that 1,5-isomeric position and methoxy group on the benzene ring result in the good activity. However, compound **8b** has shown comparatively better activity than compound **8f**. This indicates that presence of sulfur atom in compound **8f** has decreased its activity. In case of activity against *A. fumigatus*, compound **8g** has exhibited excellent activity, and compounds **8d** and **8f** have shown moderate activity. This indicates that 1,3-isomeric position with inclusion of sulfur atom and plane benzene ring attached to sydnone ring has resulted in excellent activity. Change of isomeric position from 1,3 to 1,5 or introduction of methoxy group onto the benzene ring has gradually decreased the activity.

EXPERIMENTAL

Melting points were determined in open capillaries. Fourier transform infrared spectra (KBr) were recorded on Nicolet Impact-410 FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300 MHz FT-NMR spectrometer with tetramethylsilane as an internal standard. Mass spectra were recorded using Finnegan MAT (Model MAT 8200) spectrometer, and elemental analyses were carried out using Heraeus CHN rapid analyzer. Purity of the compounds was checked by thin-layer chromatography (TLC) on silica gel plate using *n*-hexane and ethyl acetate as eluent.

General procedure for the synthesis of 2a. Compound **1a** (6.17 mmol) was reacted phosphorus pentoxide (18.51 mmol) and propionic acid (6.17 mmol) in xylene at reflux conditions. After completion of the reaction (8–10 h), xylene was separated from the reaction mixture, concentrated, and then cooled to obtain brown compound of **2a**. It was recrystallized from xylene, and the yield was calculated to be in the range 66–81% (0.88–1.07 g). Similarly, compound **2b** was prepared that required 8–11 h of reaction completion time, and the yield was found to be 62–74% (0.88–1.05 g). Compounds **6a** and **6b** were also prepared from the method used for preparation of **2a** and **2b**. Crude compounds were recrystallized from xylene, and the yield recorded was found to be 68–70% (0.91–1.07 g).

General procedure for the preparation of 3a. Compound **2a** (4.03 mmol) was dissolved in chloroform (20 mL). To this mixture, bromine (4.03 mmol) was added dropwise with constant stirring at room temperature. After completion of the reaction (3–5 h), the reaction mixture was concentrated to obtain brown compound **3a**, which was recrystallized from ethanol, and the yield was reported to be 70–76% (0.95–1.03 g). Similarly, compound **3b** was prepared where in reaction completion time was 4–6 h and the yield produced was 71–80% (0.88–0.99 g).

Compounds **7a** and **7b** were prepared using method from which compounds **3a** and **3b** were prepared. The intermediates were purified from ethanol, and the yield was found to be 68–71% (1.10–1.22 g).

General procedure for the preparation of 1-(3-arylsydnon-4-yl)-2-(1*H*-1,2,4-triazol-1-yl)propan-1-one 4a. Compound **3a** (1 mmol) was dissolved in acetone (15 mL) and stirred in a 50-mL round bottom flask at 0°C. To the aforementioned reaction mixture, anhydrous K_2CO_3 (1 mmol) and secondary amine (1 mmol) were added. Stirring was continued till the completion of reaction monitored by TLC. Reaction mixture was filtered, and solvent was evaporated to obtain crude product. Crude product was further purified by column chromatography

(10% ethyl acetate : hexane as eluent system). Similarly, compounds **4b**–**n** were prepared.

1-(3-Phenylsydnon-4-yl)-2-(1H-1,2,4-triazol-1-yl)propan-1-one 4a. Obtained as white solid (600 mg) in 71% yield; mp 68–70°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.44 (s, 1H, triazole CH), 8.05 (s, 1H, triazole CH), 7.01–7.46 (m, 5H, Ar–H), 5.32 (q, 1H, methine CH), 1.34 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.63, 164.72, 154.5, 152.97, 137.64, 128.73, 125.14, 117.37, 105.97, 62.67, 19.32. MS m/z (%): 285 (M^+). *Anal*. Calcd. for $C_{13}H_{11}N_5O_3$: C, 54.74; H, 3.89; N, 24.55%. Found: C, 54.80; H, 3.94; N, 24.63%.

2-(1H-Benzo[d][1,2,3]triazol-1-yl)-1-(3-phenylsydnon-4-yl) propan-1-one 4b. Obtained as yellowish white solid (1 g) in 78% yield; mp 86–88°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.37–8.04 (m, 9H, ArH), 6.45 (q, 1H, methine CH), 1.99 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.63, 164.72, 145.34, 142.39, 138.57, 137.64, 128.73, 128.42, 127.91, 125.14, 122.68, 117.37, 105.44, 61.87, 19.85. LCMS m/z (%): 335 (M^+). *Anal*. Calcd. for $C_{17}H_{13}N_5O_3$: C, 60.89; H, 3.91; N, 20.89%. Found: C, 60.96; H, 3.99; N, 20.96%.

1-(3-Phenylsydnon-4-yl)-2-(piperidin-1-yl)propan-1-one 4c. Obtained as brown solid (700 mg) in 58% yield; mp 120–122°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.07–7.47 (m, 5H, ArH), 5.52 (q, 1H, methine CH), 1.41 (d, 3H, CH_3), 1.47–2.32 (m, 10H, piperidine CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.32, 164.63, 138.44, 128.71, 125.18, 118.33, 105.36, 62.61, 51.12, 27.54, 20.52, 19.32. MS m/z (%): 301 (M^+). *Anal*. Calcd. for $C_{16}H_{19}N_3O_3$: C, 63.77; H, 6.36; N, 13.94%. Found: C, 63.85; H, 6.43; N, 14.01%.

2-Morpholino-1-(3-phenylsydnon-4-yl)propan-1-one 4d. Obtained as yellow solid (550 mg) in 60% yield; mp 124–126°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.02–7.49 (m, 5H, ArH), 5.59 (q, 1H, methine CH), 3.96 (t, 4H, morpholine OCH_2), 2.84 (t, 4H, morpholine N–CH_2), 1.81 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.34, 164.63, 138.51, 127.98, 125.78, 118.54, 105.76, 61.61, 61.34, 57.54, 20.12. MS m/z (%): 303 (M^+). *Anal*. Calcd. for $C_{15}H_{17}N_3O_4$: C, 59.40; H, 5.65; N, 13.85%. Found: C, 59.48; H, 5.74; N, 13.92%.

2-(1H-Imidazol-1-yl)-1-(3-phenylsydnon-4-yl)propan-1-one 4e. Obtained as white solid (900 mg) in 67% yield; mp 121–123°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.54 (s, 1H, imidazole CH), 7.10 (d, 1H, imidazole CH), 6.87 (d, 1H, imidazole CH), 6.83–7.45 (m, 5H, ArH), 5.22 (q, 1H, methine CH), 1.43 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.45, 162.53, 138.43, 127.61, 121.96, 137.54, 128.15, 126.32, 118.74, 105.85, 60.43, 19.82. MS m/z (%): 284 (M^+). *Anal*. Calcd. for $C_{14}H_{12}N_4O_3$: C, 59.15; H, 4.25; N, 19.71%. Found: C, 59.21; H, 4.32; N, 19.78%.

2-(4-Methylpiperazin-1-yl)-1-(3-phenylsydnon-4-yl)propan-1-one 4f. Obtained as yellow solid (550 mg) in 66% yield; mp 101–103°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.95–7.54 (m, 5H, ArH), 5.50 (q, 1H, methine CH), 2.73 (t, 4H, piperazine CH_2), 2.71 (t, 4H, piperazine CH_2), 2.31 (s, 3H, *N*–CH_3), 1.73 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.54, 164.83, 138.31, 127.68, 125.58, 118.59, 105.58, 61.51, 60.22, 58.14, 42.11, 19.24. MS m/z (%): 316 (M^+). *Anal*. Calcd. for $C_{16}H_{20}N_4O_3$: C, 60.75; H, 6.37; N, 17.71%. Found: C, 60.84; H, 6.45; N, 17.79%.

3-(1-Oxo-1-(3-phenylsydnon-4-yl)propan-2-yl)thiazolidin-2,4-dione 4g. Obtained as brown solid (800 mg) in 73% yield; mp 134–136°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.67–7.75 (m, 5H, ArH), 5.13 (q, 1H, methine CH), 3.35 (s, 2H, methylene CH_2), 1.36 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.98, 165.35, 151.34, 142.88, 139.64, 128.78, 125.18, 116.36, 107.04, 63.97, 60.18, 20.76. MS m/z (%): 333.0 (M^+). *Anal*. Calcd. for $C_{14}H_{11}N_3O_5S$: C, 50.45; H, 3.33; N, 12.61%. Found: C, 50.51; H, 3.44; N, 12.68%.

1-(3-p-Tolylsydnon-4-yl)-2-(1H-1,2,4-triazol-1-yl)propan-1-one 4h. Obtained as brown solid (1 g) in 80% yield; mp 140–142°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.54 (s, 1H, triazole CH), 8.10 (s, 1H, triazole CH), 7.11–7.56 (m, 4H, ArH), 5.14 (q, 1H, methine CH), 2.35 (s, 3H, *p*-tolyl-CH_3), 1.36 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.33, 164.32, 154.67, 151.04, 138.60, 128.38, 125.12, 117.30, 106.45, 61.54, 23.16, 20.34. MS m/z (%): 299.1 (M^+). *Anal*. Calcd. for $C_{14}H_{13}N_5O_3$: C, 56.18; H, 4.38; N, 23.40%. Found: C, 56.25; H, 4.45; N, 23.50%.

2-(1H-Benzo[d][1,2,3]triazol-1-yl)-1-(3-p-tolylsydnon-4-yl)propan-1-one 4i. Obtained as white solid (650 mg) in 80% yield; mp 132–134°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.32–8.11 (m, 8H, ArH), 6.42 (q, 1H, methine CH), 2.21 (s, 3H, *p*-tolyl CH_3), 1.94 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.65, 164.42, 145.75, 142.39, 138.52, 136.84, 128.74, 128.12, 127.90, 124.04, 122.38, 117.27, 104.64, 61.69, 21.11, 19.58. MS m/z (%): 349 (M^+). *Anal*. Calcd. for $C_{18}H_{15}N_5O_3$: C, 61.89; H, 4.33; N, 20.05%. Found: C, 61.96; H, 4.44; N, 20.13%.

1-(3-p-Tolylsydnon-4-yl)-2-(piperidin-1-yl)propan-1-one 4j. Obtained as brown solid (450 mg) in 85% yield; mp 121–123°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.99–7.54 (m, 4H, ArH), 5.37 (q, 1H, methine CH), 2.25 (s, 3H, *p*-tolyl CH_3), 1.48 (d, 3H, CH_3), 1.24–2.11 (m, 10H, piperidine CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.36, 164.73, 138.41, 128.51, 125.39, 118.63, 104.39, 62.41, 53.19, 27.54, 23.38, 21.32, 19.72. MS m/z (%): 315 (M^+). *Anal*. Calcd. for $C_{17}H_{21}N_3O_3$: C, 64.74; H, 6.71; N, 13.32%. Found: C, 69.82; H, 6.77; N, 13.47%.

2-Morpholino-1-(3-p-tolylsydnon-4-yl)propan-1-one 4k. Obtained as pale brown solid (600 mg) in 85% yield; mp 139–114°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.86–7.51 (m, 4H, ArH), 5.53 (q, 1H, methine CH), 3.93 (t, 4H, morpholine OCH_2), 2.82 (t, 4H, morpholine N–CH_2),

2.21 (s, 3H, *p*-tolyl CH_3), 1.73 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.54, 164.83, 138.31, 127.68, 125.18, 118.54, 105.36, 61.81, 60.42, 58.44, 24.11, 19.24. MS m/z (%): 317 (M^+). *Anal*. Calcd. for $C_{16}H_{19}N_3O_4$ (317.14): C, 60.56; H, 6.03; N, 13.24%. Found: C, 60.64; H, 6.12; N, 13.30%.

2-(1H-Imidazol-1-yl)-1-(3-p-tolylsydnon-4-yl)propan-1-one 4l. Obtained as white solid (900 mg) in 81% yield; mp 104–106°C. ^1H NMR (400 MHz, DMSO-d_6): δ 7.43 (s, 1H, imidazole CH), 7.13 (d, 1H, imidazole CH), 6.83 (d, 1H, imidazole CH), 6.83–7.45 (m, 4H, ArH), 5.28 (q, 1H, methine CH), 2.33 (s, 3H, *p*-tolyl CH_3), 1.54 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.58, 162.21, 138.40, 127.83, 122.04, 105.36, 137.52, 128.25, 126.22, 118.54, 60.33, 22.57, 19.66. MS m/z (%): 298 (M^+). *Anal*. Calcd. for $C_{15}H_{14}N_4O_3$: C, 60.40; H, 4.73; N, 18.78%. Found: C, 60.48; H, 4.80; N, 18.84%.

2-(4-Methylpiperazin-1-yl)-1-(3-p-tolylsydnon-4-yl)propan-1-one 4m. Obtained as brown solid (1 g) in 81% yield; mp 109–111°C. ^1H NMR (400 MHz, DMSO-d_6): δ 6.91–7.54 (m, 4H, ArH), 5.57 (q, 1H, methine CH), 2.82 (t, 4H, piperazine CH_2), 2.78 (t, 4H, piperazine CH_2), 2.31 (s, 3H, *N*-methyl), 2.24 (s, 3H, *p*-tolyl CH_3), 1.81 (d, 3H, CH_3). (100 MHz, DMSO-d_6): δ 171.74, 163.63, 138.73, 127.26, 125.15, 118.54, 105.44, 61.96, 60.41, 58.52, 42.15, 23.71, 19.24. MS m/z (%): 330 (M^+). *Anal*. Calcd. for $C_{17}H_{22}N_4O_3$: C, 61.80; H, 6.71; N, 16.96%. Found: C, 61.89; H, 6.81; N, 17.04%.

3-(1-Oxo-1-(3-p-tolylsydnon-4-yl)propan-2-yl)thiazolidin-2,4-dione 4n. Obtained as white solid (750 mg) in 78% yield; mp 115–117°C. ^1H NMR (400 MHz, DMSO-d_6): δ 7.25–7.65 (m, 4H, ArH), 5.20 (q, 1H, methine CH), 3.37 (s, 2H, methylene CH_2), 2.23 (s, 3H, *p*-tolyl CH_3), 1.42 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.51, 165.68, 151.34, 142.88, 139.45, 128.46, 125.38, 116.39, 106.14, 63.90, 60.54, 22.51, 20.37. MS m/z (%): 347 (M^+). *Anal*. Calcd. for $C_{15}H_{13}N_3O_5S$: C, 51.87; H, 3.77; N, 12.10%. Found: C, 51.93; H, 3.85; N, 12.18%.

General procedure for the preparation of 8a–d. Compound **7a–d** (0.001 mmol) was dissolved in acetone (15 mL) and stirred in a round bottom flask at 0°C. To the above reaction mixture anhydrous K_2CO_3 (1 mmol) and benzyltetrazole (0.001 mmol) were added and the reaction mixture stirred till completion (TLC). Then, the reaction mixture was filtered and the solvent was evaporated to get the crude product which further was purified by column chromatography (10% ethyl acetate: hexane as eluent system) to get regioisomers **8a–b** and **8c–d**. Similarly, regioisomers **5e–f** and **5g–h** were prepared using benzyl thiotetrazole.

2-(5-Benzyl-1H-tetrazol-1-yl)-1-(3-phenylsydnon-4-yl)propan-1-one 8a. Obtained as white solid (500 mg) in 75% yield; mp 119–121°C. ^1H NMR (400 MHz, DMSO-d_6): δ 7.11–7.45 (m, 10H, ArH), 6.06 (q, 1H, methine CH), 4.39 (s, 2H, methylene CH_2), 1.85 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 178.43, 164.46, 162.02, 115.77, 126.44, 126.20, 127.53, 128.64, 128.98, 129.44, 134.33, 104.45, 58.63, 29.40, 14.76. MS m/z (%): 376 (M^+). *Anal*. Calcd. for $C_{19}H_{16}N_6O_3$: C, 60.63; H, 4.28; N, 22.33%. Found: C, 60.70; H, 4.35; N, 22.40%.

2-(5-Benzyl-1H-tetrazol-1-yl)-1-(3-(4-methoxyphenyl)sydnon-4-yl)propan-1-one 8b. Obtained as pale yellow solid (450 mg) in 74% yield; mp 103–105°C. ^1H NMR (400 MHz, DMSO-d_6): δ 7.09–7.49 (m, 9H, ArH), 6.01 (q, 1H, methine CH), 4.32 (s, 2H, methylene CH_2), 3.84 (s, 3H, OCH_3), 1.73 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.44, 165.16, 161.99, 114.37, 126.65, 126.80, 127.09, 128.52, 128.65, 129.05, 134.31, 104.96, 57.93, 55.79, 28.45, 15.58. MS m/z (%): 406 (M^+). *Anal*. Calcd. for $C_{20}H_{18}N_6O_4$: C, 60.63; H, 4.28; N, 22.33%. Found: C, 60.69; H, 4.37; N, 22.41%.

2-(5-Benzyl-2H-tetrazol-2-yl)-1-(3-phenylsydnon-4-yl)propan-1-one 8c. Obtained as white solid (740 mg) in 84% yield; mp 135–137°C. ^1H NMR (400 MHz, DMSO-d_6): δ 6.91–7.32 (m, 10H, ArH), 6.29 (q, 1H, methine CH), 4.18 (s, 2H, methylene CH_2), 1.88 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 178.36, 165.82, 164.61, 136.84, 129.21, 128.33, 128.24, 128.28, 127.19, 126.53, 115.71, 105.20, 63.32, 30.5, 15.18. MS m/z (%): 376 (M^+). *Anal*. Calcd. for $C_{19}H_{16}N_6O_3$: C, 60.63; H, 4.28; N, 22.33%. Found: C, 60.69; H, 4.34; N, 22.42%.

2-(5-Benzyl-2H-tetrazol-2-yl)-1-(3-(4-methoxyphenyl)sydnon-4-yl)propan-1-one 8d. Obtained as brown solid (700 mg) in 83% yield; mp 96–98°C. ^1H NMR (400 MHz, DMSO-d_6): δ 7.11–7.30 (m, 9H, ArH), 6.36 (q, 1H, methine CH), 4.20 (s, 2H, methylene CH_2), 3.83, (s, 3H, OCH_3), 1.90 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.69, 165.44, 164.67, 136.82, 129.05, 128.78, 128.66, 128.48, 127.09, 126.63, 114.41, 105.27, 63.32, 55.77, 30.78, 15.18. LCMS m/z (%): 406 (M^+). *Anal*. Calcd. for $C_{20}H_{18}N_6O_4$: C, 59.11; H, 4.46; N, 20.68%. Found: C, 59.18; H, 4.53; N, 20.75%.

2-(5-(Benzylthio)-1H-tetrazol-1-yl)-1-(3-phenylsydnon-4-yl)propan-1-one 8e. Obtained as white solid (690 mg) in 78% yield; mp 99–101°C. ^1H NMR (400 MHz, DMSO-d_6): δ 7.29–7.73 (m, 10H, ArH), 5.91 (q, 1H, methine CH), 4.49 (s, 2H, methylene CH_2), 1.75 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.32, 165.77, 162.49, 136.91, 136.16, 134.49, 132.36, 129.43, 128.41, 127.43, 125.13, 105.35, 58.61, 35.44, 15.12. MS m/z (%): 408 (M^+). *Anal*. Calcd. for $C_{19}H_{16}N_6O_3S$: C, 55.87; H, 3.95; N, 20.58%. Found: C, 55.93; H, 3.99; N, 20.66%.

2-(5-(Benzylthio)-2H-tetrazol-2-yl)-1-(3-(4-methoxyphenyl)sydnon-4-yl)propan-1-one 8f. Obtained as white solid (1 g) in 77% yield; mp 104–106°C. ^1H NMR (400 MHz, DMSO-d_6): δ 7.20–7.68 (m, 10H, ArH), 6.37 (q, 1H, methine CH), 4.40 (s, 2H, methylene CH_2), 1.90 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.32, 165.32, 162.49, 134.60, 132.36, 129.62, 129.12, 128.53,

128.19, 127.43, 125.24, 105.48, 63.82, 35.44, 15.06. MS m/z (%): 438 (M^+). *Anal*. Calcd. for $C_{19}H_{16}N_6O_3S$: C, 54.79; H, 4.14; N, 19.17%. Found: C, 54.88; H, 4.19; N, 19.24%.

2-(5-(Benzylthio)-2H-tetrazol-2-yl)-1-(3-phenylsydnon-4-yl) propan-1-one 8g. Obtained as white solid (840 mg) in 77% yield; mp 104–106°C. ^{1}H NMR (400 MHz, 400 MHz, DMSO-d_6): δ 7.20–7.68 (m, 10H, ArH), 6.37 (q, 1H, methine CH), 4.40 (s, 2H, methylene CH_2), 1.90 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.32, 165.32, 162.49, 134.60, 132.36, 129.62, 129.12, 128.53, 128.19, 127.43, 125.24, 105.48, 63.82, 35.44, 15.06. MS m/z (%): 408 (M^+) *Anal*. Calcd. for $C_{19}H_{16}N_6O_3S$: C, 55.87; H, 3.95; N, 20.58%. Found: C, 55.94; H, 4.02; N, 20.66%.

2-(5-(Benzylthio)-2H-tetrazol-2-yl)-1-(3-(4-methoxyphenyl) sydnon-4-yl)propan-1-one 8h. Obtained as pale yellow solid (940 mg) in 82% yield; mp 123–125°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.03–7.69 (m, 9H, ArH), 6.34 (q, 1H, methine CH), 4.36 (s, 2H, methylene CH_2), 3.82 (s, 3H, methoxy CH_3), 1.82 (d, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.46, 164.36, 161.50, 134.67, 132.83, 129.62, 129.90, 128.73, 127.89, 127.03, 125.21, 105.23, 62.52, 58.14, 33.18, 15.12. MS m/z (%): 438 (M^+). *Anal*. Calcd. for $C_{20}H_{18}N_6O_4S$: C, 54.79; H, 4.14; N, 19.17%. Found: C, 54.86; H, 4.21; N, 19.23%.

BIOLOGICAL ASSAY

Methodology for *in vitro antitubercular assay.* The antimycobacterial activity of compounds was assessed against *M. tuberculosis* using microplate Alamar Blue assay [37]. This methodology is non-toxic, uses a thermally stable reagent, and shows good correlation with proportional and BACTEC radiometric method. Briefly, sterile deionized water (200 μL) was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 μL of the Middlebrook 7H9 broth, and serial dilution of compounds was made directly on plate. The final drug concentrations tested were 100 to 0.2 μg/mL. Plates were covered and sealed with parafilm and incubated at 37°C for 5 days. After this time, 25 μL of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% Tween 80 was added to the plate and incubated for 24 h. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as the lowest drug concentration that prevented the color change from blue to pink.

Methodology for *in vitro antifungal assay.* Nine dilutions of each drug have to be performed with brain heart infusion (BHI) for MIC. In the initial tube, 20 μL of drug was added into the 380 μL of BHI broth. For dilutions, 200 μL of BHI broth was added into the next nine tubes separately. Then from the initial tube, 200 μL was transferred to the first tube containing 200 μL of BHI broth. This was considered as 10^{-1} dilution. From 10^{-1} diluted tube, 200 μL was transferred to the second tube to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-9} dilution for each drug. From the maintained stock cultures of required organisms, 5 μL was taken and added into 2 mL of BHI broth. In each serially diluted tube, 200 μL of above culture suspension was added. The tubes were incubated for 24 h and observed for turbidity [38].

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