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Structure-guided discovery of 1,3,5-triazine–pyrazole conjugates as antibacterial and antibiofilm agent against pathogens causing human diseases with favorable metabolic fate ☆

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ABSTRACT

Impressed by the exceptional antibacterial activity exhibited by our earlier designed molecules originating from 1,3,5-triazine, the present study was undertaken to synthesize a novel series of 1,3,5-triazine–pyrazole conjugates to bring diversity around the core skeleton. The target analogues showed potent antibacterial activity against tested Gram-positive and Gram-negative microorganisms. The toxicity and metabolic site prediction studies were also held out to set an effective lead candidate for the future antibacterial drug discovery initiatives.

The overwhelming phenomenon of resistance towards the currently available antibiotics creates a significant lacuna in the health care for humankind. Alas, it can be ascribed to the widely misuse of these miracle drugs throughout the past 70 years and could weaken the major advances achieved in the treatment of infections.[1] Antimicrobial resistance (AMR) is now deemed as a global public health crisis which accounts for the death of 25 000 people and related costs of over €1.5 billion in healthcare expenses alone in European continent.[2] Paradoxically, in recent years, as the problems associated with the emergence of resistance to existing drug's increases, there has been gradually decline in the discovery of newer antimicrobial agents and drugs in the clinical pipeline.[3] The prevention of pharmaceutical industries on the investment of new projects related with discovery of antibiotics due to far too low incentives than lifestyle medication have made present situation catastrophic.[4] However, in part, technical difficulties associated with the identification of suitable novel compounds for development as candidate antibacterial make this situation complex. Regarding the fact, in 2010, Infectious Diseases Society of America (IDSA) outlines its '10 × '20' initiative, calling for a worldwide effort to acquire ten new antibiotics by 2020.[5]

The innovation of new antibacterial entity with low economic inputs has always a prolific option to cope with this state of affairs. Analogues derived from 1,3,5-triazine accommodated well with the above aim owing to its simple work-up and potent antibacterial activity. In our ongoing task to develop newer antimicrobial entity from 1,3,5-triazine, earlier, we had developed various hybrid analogues of 1,3,5-traizine clubbed with thiazole.[6] It was found that substitution of thiazole on pendant location makes the compound potent in comparison to non-substituent. In advancement of this observation and to optimize the pendant position, until now we have reported the various hybrid conjugates of 1,3,5-triazine with 1,3-thiazine,[7] piperazine,[8] and 1,3,4-thiadiazole,[9] 4-aminoquioline[10,11] and thiazolidin-4-one[12] Figure 1.

Our previous study has suggested that, the structure–activity relationship (SAR) could be exemplified on the nature of pendant substituent (i.e., pharmacophore), covalent bridge used to connect 1,3,5-triazine with pendant substituent, variety of fragment attached to the other two wings of 1,3,5-triazine and the nature of the substituent on the wings above. In our recent communication, inhibition of bacterial translation was disclosed as the mechanism of action of these 1,3,5-triazine conjugates.[7]

Present paper deals with the synthesis, antibacterial activity, antibiofilm, in silico toxicity, and metabolic site prediction of conjugates derived from 1,3,5-triazine and pyrazole. Moreover, this

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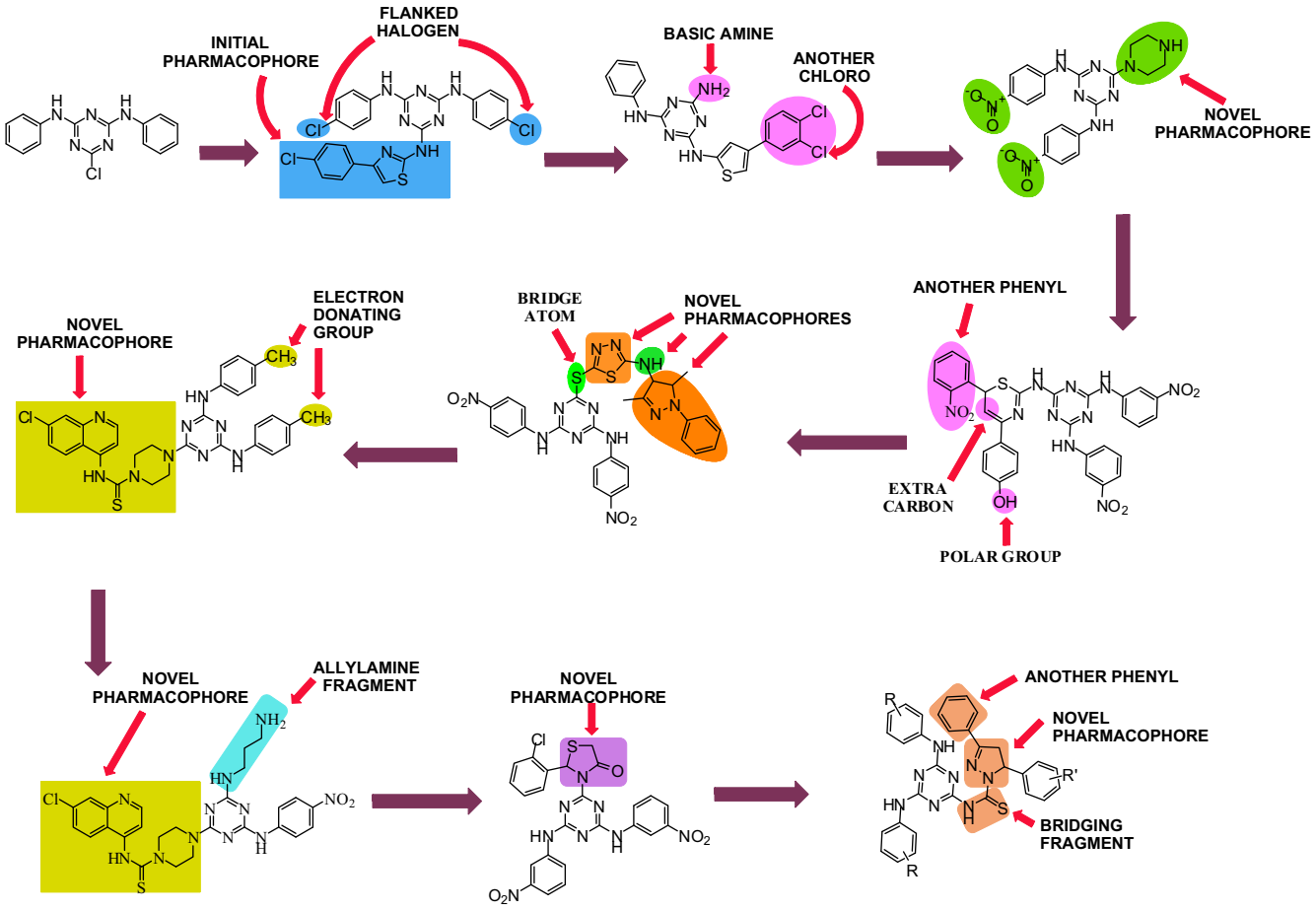
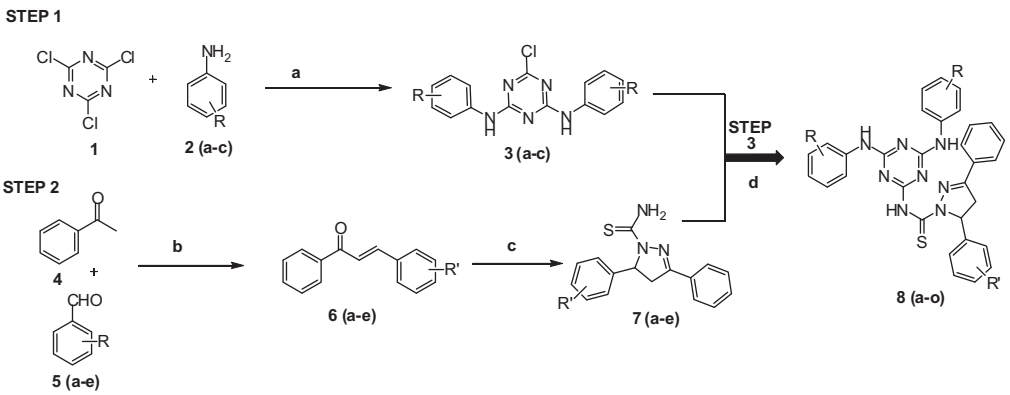


Figure 1. Structure-guided design of target 1,3,5-triazine–pyrazole conjugate.

study also provides the fresh insight and advancement about the SAR of hybrid 1,3,5-triazine conjugates.

The synthesis of title conjugates **8** (**a**–**o**) were accomplished via multi-step reaction as outlined in Scheme 1. The commercially available 2,4,6-trichloro-1,3,5-triazine (**1**) was treated with two equivalents of distinguished amines **2** (**a**–**c**) in the presence of $NaHCO_3$ followed by stirring and reflux at 40–45 °C to furnish di-substituted phenyl amines **3** (**a**–**c**) with the yield ranging from 71–89%, Step 1. The substituted chalcones **6** (**a**–**e**) was conveniently and efficiently obtained in 81–90% by crossed aldol condensation reaction between enolisable acetophenone (**4**) and substituted aldehydes **5** (**a**–**e**). Further, these substituted chalcones **6** (**a**–**e**) was then allowed to undergo Cyclo-condensation reaction in the presence of thiosemicarbazide to afford the next reaction intermediate in good yields (62–81%), 5-(substituted-phenyl)-3-phenyl-4,5-dihydro-pyrazole-1-carbothioic acid amide, **7** (**a**–**e**), Step 2. Finally, the target hybrid conjugates of 1,3,5-triazine–pyrazole **8** (**a**–**o**) was obtained through clubbing of mono chloro 1,3,5-triazine-2,4-diamine **3** (**a**–**c**) and 4,5-dihydro-pyrazole-1-carbothioic acid amide **7** (**a**–**e**) fragments in the presence of K_2CO_3 as activating base under vigorous condition. These analogues were synthesized in excellent to good yields (62–84%).



Code	R	R'
8a	4-Cl	H
8b	4-Cl	2-NO_2
8c	4-Cl	4-NO_2
8d	4-Cl	2-Cl
8e	4-Cl	4-Cl
8f	3-NO_2	H
8g	3-NO_2	2-NO_2
8h	3-NO_2	4-NO_2
8i	3-NO_2	2-Cl
8j	3-NO_2	4-Cl
8k	4-F	H
8l	4-F	2-NO_2
8m	4-F	4-NO_2
8n	4-F	2-Cl
8o	4-F	4-Cl

Scheme 1. Reagents and conditions: (a) $NaHCO_3$, 40–45 °C; (b) NaOH, stirring for 24 h; (c) thiosemicarbazide, ethanol, aq NaOH, reflux for 8 h; (d) 120–135 °C K_2CO_3, reflux.

The structures of the title compounds were ascertained on the basis of spectral analysis while the mechanism of reaction has been depicted in Figure 2.

Formation of the target product involve the tandem reactions as follows: (1) First step involves an acid–base reaction where hydroxide functions as a base and removes an acidic α-hydrogen from acetophenone (**i**) giving a reactive enolate (**ii**); (2) The nucleophilic enolate attacks the carbonyl carbon of benzaldehyde (**iii**) in a nucleophilic addition process giving an intermediate alkoxide (**iv**); (3) The alkoxide deprotonates (**iv**) a water molecule producing a hydroxide ion and a β-hydroxyketone, the aldol product (**v**); (4) The hydroxide acts as a base and removes an acidic β-hydrogen giving the reactive enolates. The electrons associated with a negative charge of the enolate are used to form a carbon–carbon double bond (C=C) and displace a leaving group, regenerating the hydroxide giving the product, the conjugated ketone (**vi**); (5) Nucleophilic attack by semicarbazide (**vii**) at the β-carbon of the α,β-unsaturated carbonyl system forms species (**viii**), in which the negatively charged is mainly accommodated by negative charged oxygen atom; (6) Proton transfer from nitrogen to negative oxygen produces an intermediate enol which simultaneously ketonises to ketoamine (**ix**); (7) Another intramolecular nucleophilic attack by the primary amino group of ketoamine on its carbonyl carbon followed by proton transfer from nitrogen to oxygen leads ultimately to carbonyl amine (**x**); (8) The later with a hydroxyl group and amino group on the same carbon lose water molecule to yield the pyrazolines (**xi**); (9) The last step corresponds to the nucleophilic reaction between chloro group of substituted 1,3,5-triazine derivatives and pyrazole-1-carbothioamide (**xi**) in the presence of K_2CO_3 as activating base under vigorous condition to afford target conjugates **8** (**a**–**o**).

The newly prepared compounds were screened for determination of their minimal inhibitory concentration (MIC) against selected Gram-positive organisms viz. *Bacillus subtilis* (NCIM-2063), *Bacillus cereus* (NCIM-2156), *Staphylococcus aureus* (NCIM-2079) and Gram-negative organism viz. *Escherichia coli* (NCIM-2065), *Proteus vulgaris* (NCIM-2027) and *Pseudomonas aeruginosa* (NCIM-2036) by broth microdilution (in tubes) method with minor modifications using Cefixime as standard.[13]

In the antibiofilm concentration experiment, overnight culture of bacterium (*S. aureus*, NCIM-2079) was diluted 1:10 in TSB (OD 600 = 0.6–0.8) and further diluted to 1:200 in Müeller-Hinton. The resulting bacterial suspension was further inoculated into the wells of sterile 96-well polystyrene Microtiter plates and incubated at 37 °C for 6 h. The plates with young biofilm were washed gently four times with sterile PBS before adding fresh TSB containing the various concentrations of compounds, and incubated at 37 °C for 16 h. The initiate dilution was 200 μM, while Vancomycin was used as standard.[14]

The antibacterial results in Table 1 showed that entire set of the title compounds were active against both Gram-positive and Gram-negative bacteria along with potent antibiofilm activity against *S. aureus*. The compounds **8** (**a**–**e**) having *p*-Cl on both the rings connected to 1,3,5-triazine core along with diversely substituted phenyl of pyrazole showed significant to substantial activity against tested pathogens. Especially, in the case of compound **8a**, having unsubstituted phenyl on pyrazole showed significant activity against entire test strains except moderately active against *E. coli* and *P. aeruginosa*. Introduction of 2-NO_2 (**8b**) on unsubstituted phenyl, led to a drastic decline in activity. Meanwhile, no significant change in activity was reported on isomeric replacement of NO_2 against the bacterial organisms (**8c**, 4-NO_2). In the next instance, on insertion of 2-Cl, a marked inhibition pattern was reported (**8d**) against the entire set of testing organisms except *P. vulgaris*. While no major shift in antibacterial activity was reported by changing the substitution pattern of Cl (**8e**), except it makes the compound two-fold more active against *B. cereus*. Conversely, the presence of 3-NO_2 on both the phenyl of 1,3,5-triazine showed improved antibacterial activity and render molecule potent against *S. aureus*, *B. cereus* and *P. vulgaris*, **8f**. While no change in activity was reported against *E. coli* and *P. aeruginosa* by the same test compound. In the next instance, compound **8g**, formed on the introduction of 3-NO_2 group at the phenyl of pyrazole makes the molecule almost inactive against tested microorganisms. No drastic change

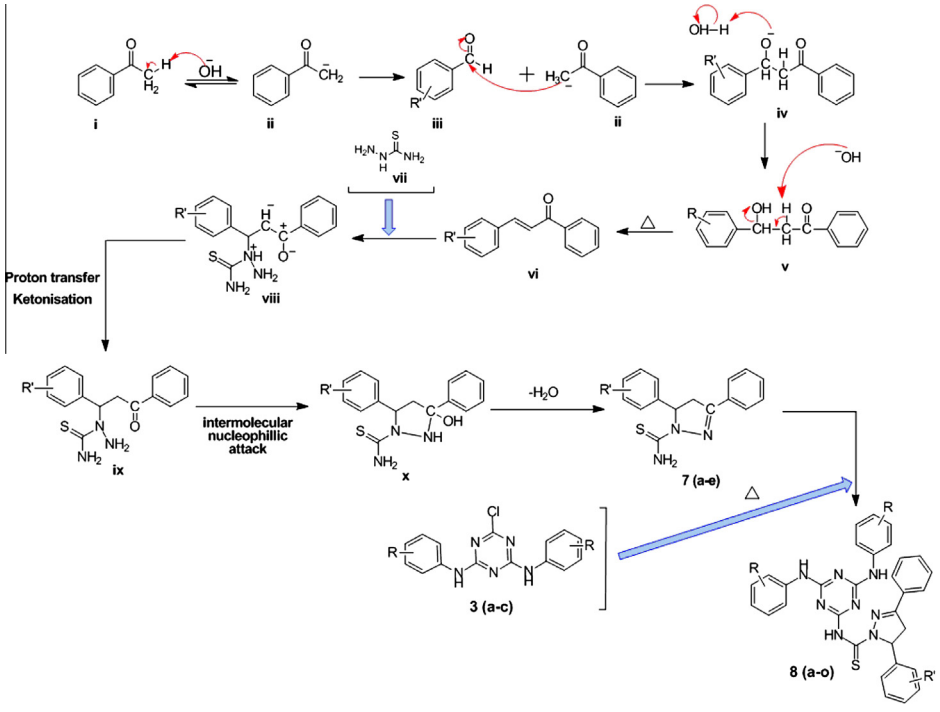


Figure 2. Reaction mechanism for the formation of target product **8** (**a**–**o**).

Table 1
Antibacterial activity of 1,3,5-triazine–pyrazole conjugates

Compound	MIC (minimum inhibitory concentration, in μg mL^{-1})						Antibiofilm activity (in μg mL^{-1})
	B. subtilis	*S. aureus*	*B. cerus*	*P. vulgaris*	*E. coli*	*P. aeruginosa*	*S. aureus*
8a	7.81	15.62	15.62	15.62	31.25	31.25	31.25
8b	15.62	62.5	31.25	62.5	125	125	125
8c	31.25	125	125	62.5	62.5	62.5	125
8d	3.91	15.62	62.5	15.62	15.62	15.62	31.25
8e	3.91	15.62	15.62	15.62	7.81	7.81	62.5
8f	15.62	7.81	7.81	7.81	7.81	7.81	31.25
8g	62.5	62.5	62.5	62.5	125	125	125
8h	31.25	31.25	125	62.5	62.5	62.5	62.5
8i	15.62	15.62	15.62	31.25	15.62	15.62	62.5
8j	7.81	15.62	7.81	15.62	31.25	31.25	15.62
8k	7.81	7.81	3.91	15.62	15.62	15.62	15.62
8l	31.25	31.25	62.5	31.25	31.25	31.25	31.25
8m	15.62	31.25	31.25	62.5	31.25	31.25	62.5
8n	7.81	15.62	15.62	7.81	15.62	15.62	62.5
8o	3.91	31.25	7.81	15.62	7.81	7.81	31.25
Cefixime	1.95	7.81	3.91	7.81	3.91	3.91	–
Vancomycin	–						3.91

in activity was observed upon isomeric replacement of NO_2 from second to fourth position, compound **8h**. Nevertheless, the introduction of 2-Cl (**8i**) disclosed improved antibacterial activity against tested strains with exceptionally threefold amplified against B. *cereus*. Further enhanced activity was disclosed in the case of compound **8j** against *B. subtilis* and *B. cereus* with no significant alteration against rest of the pathogens. Pronounced antibacterial with antibiofilm activity was reported against the entire set of the microorganism on the introduction of 4-Fluoro in both the phenyl tethered in 1,3,5-triazine core. On keeping the fragment of 4-Fluoro phenyl constant, while changing the substitution pattern on phenyl of pyrazole with electron withdrawing group led to decline in activity. Compounds **8l** and **8m** having NO_2 on second and fourth position, respectively, presented considerable activity against the entire set of pathogens. In the following example, marked increase in activity was observed in the introduction of Cl on second and fourth position of compound **8n** and **8o**, respectively.

The antibacterial results corroborated that the presence of the halogen atoms would drastically manipulate the activity of the molecules. More pronounced activity was disclosed by the analogues having the fluoro substitution on the phenyl ring connected to 1,3,5-triazine. The chloro group served as the next prominent molecule in the antibacterial assay. Least to moderate activity was observed by compounds substituted with NO_2 group. The strong electron withdrawing and lipophilic behavior of these groups may be responsible for the generation and escalation of activity via increased biological transportation and distribution to alter the integrity of the bacterial cell wall. It was surprising to find out that, substitution on pyrazole phenyl would hold a significant role on antibacterial activity. The most pronounced activity was kept with the intact phenyl, in contrast to their substituted counterparts (**8a**, **8f** and **8k**). The generation of steric hindrance on the probable binding site due to substitution could contribute to the lesser activity of substituted phenyl derivatives, Figure 3.

The prediction of metabolic and toxicity parameters of the molecule even before they are synthesized is considered as a critical process for the breakthrough of a new drug. While in silico methods used to predict these parameters are widely used in the present field of drug research. These advances are applied to interpret the attributes that are necessary to change leads into medicine in clinical practice, which increases the probability of success to reach the marketplace, thereby reducing attrition rates. The early identification of probable metabolic sites led to wider understanding about pharmacokinetics profile as well as to avoid the propagation

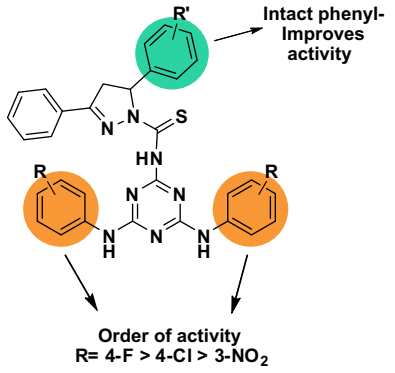


Figure 3. Structure–activity relationship (SAR) of 1,3,5-triazine–pyrazole conjugate.

of toxic metabolites by effective pharmacomodulation via chemically protecting the metabolic labile moieties in the drug candidate. Touching to the foregoing and to represent the lead like character of developing highly potent antibacterial agents, compound **8k** was subjected to in silico toxicity and metabolism prediction studies. These early determinations of above compound were taken into account to evaluate the suitability of the proposed molecule as a probable antibacterial drug candidate.

The toxicity risk assessment was predicted by OSIRIS property explorer and defined along the basis of mutagenic, tumorigenic, irritant and reproductive effects. In parliamentary law to evaluate the toxicity prediction's reliability, it operated a set of toxic compounds and a set of presumably non-toxic compounds through the prediction.[15] The prediction process relies on a pre-calculated set of structural fragment that give rise to toxicity alerts in case they are encountered in the structure currently drawn. These fragment lists were created by rigorously shredding all compounds of the RTECS database known to be dynamic in certain toxicity class (e.g., mutagenicity). During the shredding any molecule was first cut at every rotatable bonds, leading to a lot of core fragments. These in turn were used to reconstruct all possible bigger fragments being a substructure of the original particle. Subsequently, a substructure search process determined the occurrence frequency of any fragment (core and constructed fragments) within all compounds of that toxicity class. It also determined these fragment's frequencies within the structures of more than 3000 traded drugs. Established on the assumption that traded drugs are mostly

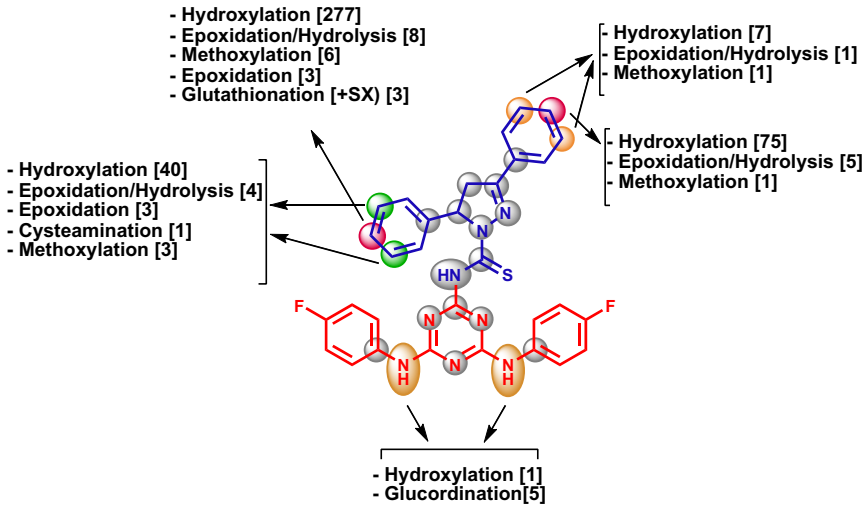


Figure 4. Possible ways of metabolic deactivation by MetaPrint-2D React of compound **8k**.

free of toxic effects, any fragment was considered a risk factor if it occurred frequently as a substructure of harmful compounds but never or rarely in trading drugs. As a result compound **8k** disclosed no toxicity to any of the predefined models of toxicity prediction.

MetaPrint2D-React, a metabolic product predictor developed by Unilever Cambridge, Centre for Molecular Science Informatics, University of Cambridge, UK. It is a tool for predicting the sites of a molecule based on historic metabolic data, described by circular fingerprints that are most likely to undergo Phase I metabolism, anchored in their similarity to known sites of metabolism and sites that are known not to be metabolized. The MetaPrint2D data is generated through processing of the transformations found in the Symyx® Metabolite database. For each transformation, the differences between the structure of the reactant and product are identified: groups added or eliminated, bonds broken or made and bonds whose order has changed. With the intention of simplify the results, only Phase I additions (defined as the addition of a single oxygen atom; covering hydroxylation, oxidation and epoxidation), and eliminations (e.g., dealkylation, ester and amide hydrolysis) are engaged. For an addition, the atom neighboring the added oxygen is marked as a reaction center. In the case of an elimination, a bond gets broken, and both atoms connected by the bond are considered to be reaction centres.[16,17] Compound **8k** was analysed through web server of MetaPrint2D-React for prediction of possible metabolic pathways and the predicted site of metabolism (only Human) has been outlined in Figure 4. The color highlighting an atom indicates its NOR (Normalized Occurrence Ratio). This NOR indicates the relative likelihood of each atomic site in a molecule being a center of metabolism, while making no prediction as to the absolute likelihood of the molecule undergoing metabolic transformation.

The NOR ratio for **8k** was observed as Red $0.66 \leqslant NOR \leqslant 1.00$, Orange $0.33 \leqslant NOR < 0.66$, Green $0.15 \leqslant NOR < 0.33$, White (No color) $0.00 \leqslant NOR < 0.15$, Grey Little/no data. A high NOR indicates a more frequently reported site of metabolism in the metabolite database. Whereas, the NOR does not show how likely a molecule is to be metabolized, but quite the relative probability of metabolism occurring at a particular site in the molecule, taking it is metabolized. It was indicated that skeleton of the target compounds were not prone to metabolic deactivation, which conform the utility of designed molecules.

In conclusion compound **8k** developed by structure-guided optimization from our previous work via facile synthetic protocol will act as prospective lead for further research. However, additional optimization work has been ongoing in our laboratory and their results will disclose subsequently.

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Supplementary data

Supplementary data (general procedures and analytical data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.05.103.

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