Chemistry Europe
European Chemical Societies Publishing

WILEY VCH

Excellence in Chemistry Research

Announcing our new flagship journal

- Gold Open Access
- Publishing charges waived
- Preprints welcome
- Edited by active scientists

Meet the Editors of *ChemistryEurope*

Luisa De Cola
Università degli Studi di Milano Statale, Italy

Ive Hermans
University of Wisconsin-Madison, USA

Ken Tanaka
Tokyo Institute of Technology, Japan

Medicinal Chemistry & Drug Discovery

Assessment of Dihydro[1,3]oxazine-Fused Isoflavone and 4-Thionoisoflavone Hybrids as Antibacterials

Ankit Lathwal,[a] Asghar Ali,[b] Amad Uddin,[b] Nashra Shareef Khan,[c] Gerard Sheehan,[d, e] Kevin Kavanagh,[d] Qazi Mohd. Rizwanul Haq,[b] Mohammad Abid,*[b] and Mahendra Nath*[a]

A series of isoflavone functionalized 3,4-dihydro-1,3-oxazine hybrids was synthesized in good to excellent yields through a Mannich-type condensation cyclization reaction of 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromen-4-one or 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromene-4-thione with formaldehyde and primary amines. After spectroscopic characterization, these newly prepared hybrids were evaluated for their antibacterial activities against two of each Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains. Among the screened compounds, dihydro[1,3]oxazine-fused 4-thionoisoflavones(**9 b**and**9 c**) exhibited potent inhibitory activity against all the tested bacterial species. Moreover, compound **9 b** possessed most promising antibacterial activity against *P. aeruginosa and B. subtilis*with MIC 16 μg/mL and *S. aureus*and *E. coli*with MIC 32 μg/mL. Further, **9 b**demonstrated better efficacy (MIC = 16 μg/mL) than the standard drug ampicillin (MIC = 32 μg/mL) against *P. aeruginosa*and it also found to be equipotent (MIC = 16 μg/mL) as ampicillin against *B. subtilis*. Considering the disk diffusion and synergistic studies, **9 b**emerged asmost active compound showing potent activity against all the tested bacterial strains. In addition, no significant hemolysis or cytotoxicity was observed towards human embryonic kidney (HEK293)cells as well as *Galleria mellonella*larvae (in vivo). Hence, compound **9 b**has potential to be further explored alone and in combination with ampicillin as a next generation antibacterial agent.

Introduction

Bacterial pathogens are majorly responsible for a wide range of life threatening infections and their treatment is becoming more difficult due to the emergence of multi-drug resistant (MDR) strains. In the current scenario, these bacterial strains are identified as a major cause of morbidity and mortality.[1,2] In fact, the patients suffering from infections caused by MDR pathogens often have very little hope to recover because of the lack of effective antibiotics.[3–6] Particularly, the uprising of methicillin resistant *S. aureus* strains and other drug-resistant Gram positive pathogens, vancomycin-resistant enterococci and penicillin-resistant *S. pneumonia*has further aggravated the situation.[7] Hence, there is an urgent need to explore new therapies by developing potent antibacterial agents and/or antibiotic adjuvants[8] that can inhibit the resistant mechanism and control the rapid emergence of deadly pathogens.

In past years, various synthetic and naturally occurring isoflavone derivatives containing hydroxyl and allyl functionalities (Figure 1) have been discovered to exhibit significant antibacterial[9] and anti-mycobacterial properties.[10] On the other hand, a number of biological studies revealed that dihydro-1,3-oxazines are privileged heterocyclic motifs with a wide range of remarkable pharmacological activities such as antitumor,[11] anti-HIV[12] and antidiabetic agents.[13] The compounds containing 1,3-oxazine scaffold such as PD-102807 are reported as a selective M_4 muscarinic acetylcholine receptor antagonist.[14] Similarly, other 1,3-oxazine based molecules are being currently used for the treatment of HIV infections,[15] Alzheimer's disease[16] and Parkinson's disease.[17] In addition, 6-arylbenzoxazines have been applied as potent nonsteroidal progesterone receptor agonists.[18] Besides, 1,3-oxazine heterocycles (Figure 1) exhibited good antimicrobial and antitubercular efficacy against various bacterial, mycobacterial and fungal strains.[19–21]

By considering the antimicrobial potential of isoflavones and oxazines, a novel series of hybrid heterocycles was designed as potential antibacterial agents by incorporating both the pharmacophores in a single molecular framework. Furthermore, the presence of various sulfur-derived functional

[a] *A. Lathwal, Prof. M. Nath*
Department of Chemistry, University of Delhi,
North Campus, Delhi-110 007, India
Tel: +91-11-27667794 Extn: 186
E-mail: mnath@chemistry.du.ac.in
[b] *A. Ali, A. Uddin, Q. M. R. Haq, Dr. M. Abid*
Department of Biosciences,
Faculty of Natural Sciences, Jamia Millia Islamia,
New Delhi-110 025, India
E-mail: mabid@jmi.ac.in
[c] *N. S. Khan*
Department of Biotechnology,
Faculty of Natural Sciences, Jamia Millia Islamia,
New Delhi-110 025, India
[d] *G. Sheehan, K. Kavanagh*
Department of Biology, Maynooth University,
Maynooth, Co. Kildare, Ireland
[e] *G. Sheehan*
Institute of Microbiology and Infection,
School of Biosciences, University of Birmingham, Edgbaston,
Birmingham, B15 2TT, UK

Supporting information for this article is available on the WWW under https://doi.org/10.1002/slct.202101364

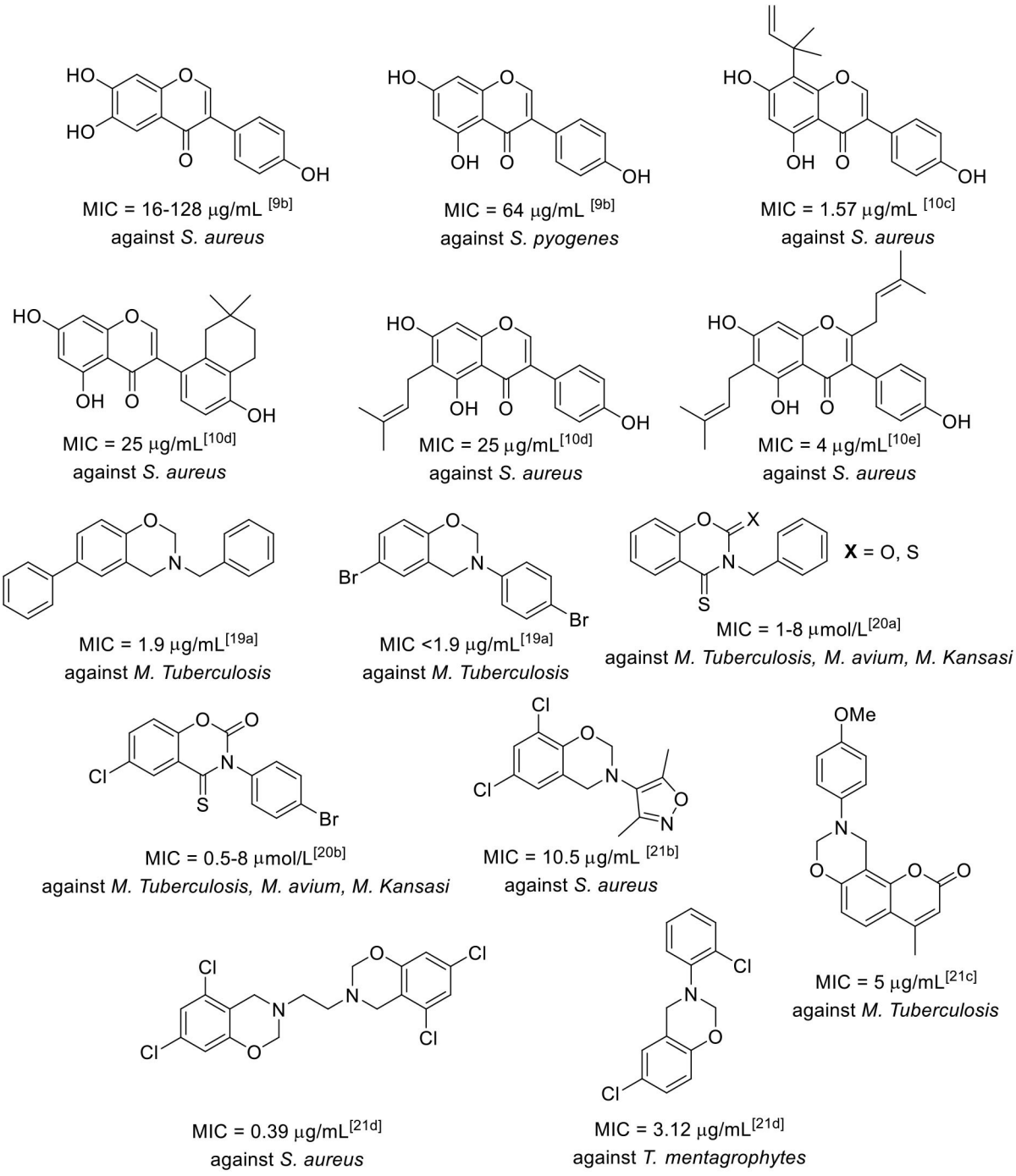


Figure 1. Structures of some representative antimicrobial isoflavone and 1,3-oxazine derivatives.

groups in a wide range of pharmaceuticals and FDA approved drugs[22] prompted us to contemplate the synthesis of 4-thionoisoflavone derivatives of newly designed isoflavone-fused 3,4-dihydro-1,3-oxazine hybrids. These hybrid heterocycles have been synthesized by using an environmentally benign protocol in appreciable yields for the study of their antibacterial efficacy and synergistic effects with standard antibacterial drug ampicillin. The synthesis, spectroscopic characterization and antibacterial activity results along with in vitro and in vivo cytotoxicity studies of novel 3,4-dihydro-1,3-oxazine-fused isoflavone analogues are presented in this paper.

Results and Discussion

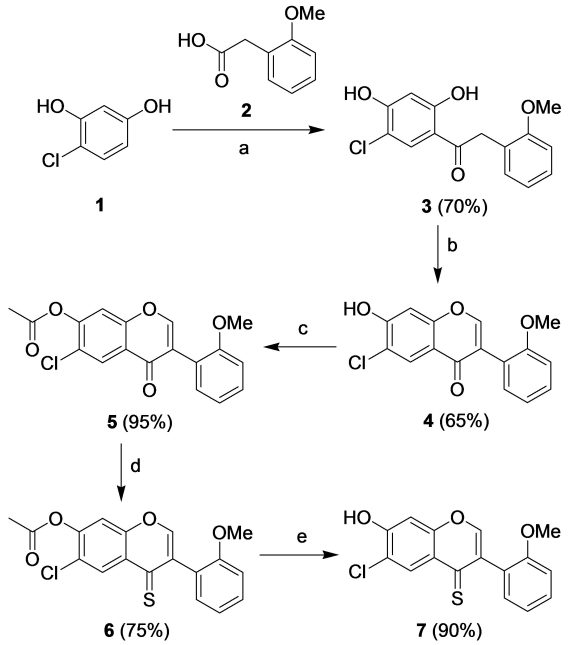
Chemistry

For the synthesis of desired isoflavone and 4-thionoisoflavone functionalized dihydro-1,3-oxazine hybrids (**8a–j** and **9a–d**), 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromen-4-one (**4**) and 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromene-4-thione (**7**) were synthesized as precursors by following the literature procedures.[23–26] Initially, Friedel-Craft acylation of 4-chlororesorcinol (**1**) was carried out by using 2-methoxyphenylacetic acid (**2**) in presence of BF_3-OEt_2 at 100–110 °C to afford corresponding deoxybenzoin (**3**) which on treatment with mesyl chloride in DMF, afforded a key starting material 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromen-4-one (**4**). On acetylation using acetic anhydride, chromone (**4**) afforded 7-acetoxy-6-chloro-3-(2-methoxy-phenyl)-chromen-4-one (**5**) which after thionation using Lawesson's reagent in refluxing toluene for 3 hours produced 7-acetoxy-6-chloro-3-(2-methoxy-phenyl)-chromene-4-thione (**6**). Finally, the desired precursor 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromene-4-thione (**7**) was obtained in excellent yield after the deacetylation of compound (**6**) using triethylamine in methanol at ambient temperature (Scheme 1).

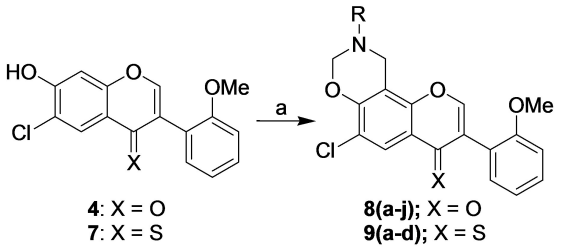
After having the desired starting materials in hand, the targeted isoflavone and 4-thionoisoflavone based dihydro-1,3-oxazines (**8a–j** and **9a–d**) were synthesized in good to excellent yields (60–88%) using an eco-friendly procedure which involves one-pot Mannich-type condensation reaction of 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromen-4-one (**4**) or 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromene-4-thione (**7**) with formaldehyde and primary amines in 50% aqueous ethanol at 80–90 °C for 2 hours (Scheme 2).

The structures of all the synthesized compounds were established on the basis of spectral data. The IR spectrum of a representative compound **8b** showed a carbonyl stretching at 1635 cm^{-1} and two characteristic absorption peaks at 1232 and 1026 cm^{-1} corresponding to the C–O–C asymmetric and C–O–C symmetric stretching, respectively due to the formation of oxazine ring. In the ^{1}HNMR spectrum of **8b**, a peak at δ 3.82 ppm as a singlet was assigned to methoxy protons. The appearance of two characteristic singlets at δ 4.81 and 5.50 ppm for two methylene groups of the 1,3-oxazine moiety and one singlet at δ 8.16 ppm due to the presence of a pyran ring proton confirmed the assigned structure of compound (**8b**). Similarly, the characteristic peaks at δ 47.00, 80.97 and 175.32 ppm in the ^{13}CNMR spectrum of **8b** were assigned to the methylene carbons of the oxazine ring and carbonyl carbon of the fused pyran ring, respectively. The mass spectrum of 10-chloro-3-(3,4-dichlorophenyl)-7-(2-methoxyphenyl)-3,4-dihydro-2*H*-1,5-dioxa-3-aza-phenanthren-8-one (**8b**) displayed a molecular ion peak $[M+H]^+$ at m/z = 488.0233 corresponding to the molecular formula $C_{24}H_{17}Cl_3NO_4$ which further supported the formation of desired compound (**8b**).

The plausible mechanistic pathway for the formation of newly designed isoflavone and 4-thionoisoflavone functionalized dihydro-1,3-oxazine hybrids (**8a–j** and **9a–d**) is depicted in Scheme 3. On the basis of our hypothesis, the reaction is believed to proceed *via* the formation of Schiff base intermediate by the reaction of formaldehyde and primary amines. On reaction with 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromen-4-one (**4**) or 6-chloro-7-hydroxy-3-(2-methoxy-phenyl)-chromene-4-thione (**7**), the imines generated *in-situ* afford key intermediates (**10** or **11**) which react with another mole of formaldehyde followed by deprotonation to produce desired isoflavone and 4-thionoisoflavone based 1,3-oxazine hybrids (**8a–j** and **9a–d**).

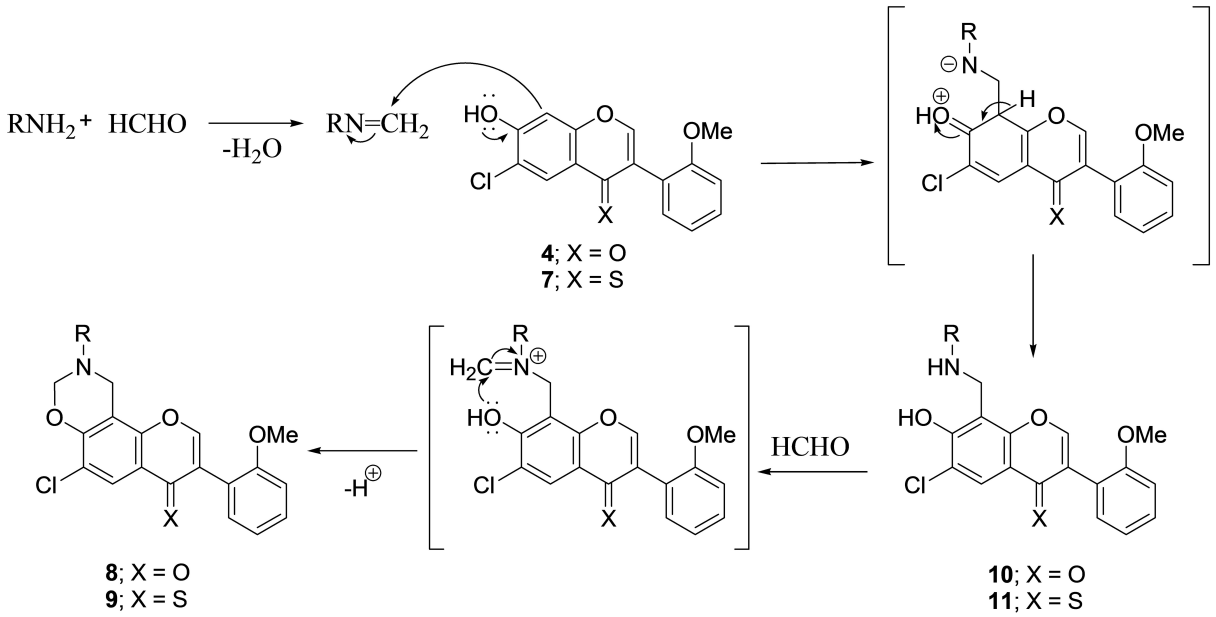


Scheme 1. Reagents and conditions: (a) BF_3-OEt_2, 100–110 °C, 4 h; (b) DMF, mesyl chloride, 50–90 °C, 4 h; (c) acetic anhydride, $NaHCO_3$, EtOAc, rt, 12 h; (d) Lawesson's Reagent, toluene, reflux, 3 h; (e) MeOH, Et_3N, rt, 24 h.



8a: R = C_6H_5, (65%)
8b: R = 3,4-$Cl_2C_6H_3$, (82%)
8c: R = 3-Cl-4-FC_6H_3, (85%)
8d: R = 4-IC_6H_4, (70%)
8e: R = 4-BrC_6H_4, (68%)
8f: R = 4-ClC_6H_4, (72%)
8g: R = 4-FC_6H_4, (60%)
8h: R = $CH_2C_6H_5$, (78%)
8i: R = 2,4-$Cl_2C_6H_3$, (68%)
8j: R = 3,4-$F_2C_6H_3$, (62%)
9a: R = C_6H_5, (68%)
9b: R = 3,4-$Cl_2C_6H_3$, (85%)
9c: R = 3-Cl-4-FC_6H_3, (88%)
9d: R = 4-IC_6H_4, (71%)

Scheme 2. Reagents and conditions: (a) RNH_2, HCHO, 50% aq. ethanol, 80–90 °C, 2 h.



Scheme 3. Proposed mechanistic pathway for the formation of isoflavone and 4-thionoisoflavone fused dihydro-1,3-oxazine hybrids.

Evaluation of in-vitro antibacterial efficacy

Two of each, Gram positive (*Bacillus subtilis*MTCC736 and *Staphylococcus aureus*MTCC902) and Gram negative (*Escherichia coli* MTCC443, and *Pseudomonas aeruginosa*MTCC2453) bacterial strains were used to check the inhibitory potential of the synthesized compounds (**8a**–**j** and **9a**–**d**). Initially, 200 μg/mL as a single highest concentration was used to start in vitro screening to classify potent compounds and those compounds which are ineffective at this concentration were precluded. Most of the compounds exhibited significant inhibition against *S. aureus, P. aeruginosa*and *E. coli*bacterial strains. However, little significant inhibition was observed against *B. subtilis*after the treatment with any of the test compounds. Compounds **8a**,**8f**,**8i** and **9d** did not show any significant antibacterial potential while compound **8e** and **8g** showed selective inhibition against *S. aureus* and *E. coli*bacterial strains. Furthermore, *P. aeruginosa* and *E. coli*. showed 100% growth inhibition when treated with the compound **8h**. The compounds **8j**, **9b and 9c**showed best results with 100% growth inhibition of all bacterial strains except *E. coli*in case of **8j**at 200 μg/mL. The activity results are presented in Table 1.

In general, the biological results revealed that the replacement of carbonyl functionality with thiocarbonyl group in chromone system enhances the efficacy of 4-thionoisoflavone based dihydro-1,3-oxazine hybrids against most of bacterial strains used in the present study. Further, the antibacterial activity of these compounds is also influenced by the nature of

Table 1. Growth inhibition profile of dihydro[1,3]oxazine-fused isoflavones and 4-thionoisoflavones (**8a**–**j** and **9a**–**d**).

Compounds	Growth inhibition (%) at 200 μg/mL			
	P. aeruginosa	*B. subtilis*	*S. aureus*	*E. coli*
8a	42.5	34.11	61.23	45.2
8b	95.02	42.88	86.3	100
8c	78.9	29.36	85.03	100
8d	44.32	32.12	100	93.10
8e	56.44	88.08	100	100
8f	16.03	29.77	98.23	95.5
8g	26.21	34.69	100	100
8h	100	39.00	85.33	100
8i	60.63	14.22	93.33	91.30
8j	100	100	100	64.3
9a	84.67	69.33	100	82.55
9b	100	100	100	100
9c	100	100	100	100
9d	77.22	89.32	92.5	77.8
Amp[a]	100	100	100	100

[a] Amp (ampicillin).

Table 2. Minimum inhibitory concentration (MIC) of compounds (**8j**, **9b** and **9c**) in μg/mL.

Bacterial species	MIC value in μg/mL			
	8j	**9b**	**9c**	Amp
E. coli (MTCC443)	256	32	64	1
B. subtilis (MTCC736)	64	16	16	16
S. aureus (MTCC902)	64	32	64	1
P. aeruginosa (MTCC2453)	128	16	16	32

halogen atom present on the aromatic ring connected to the 1,3-oxazine scaffold. It is interesting to note that the presence of more electronegative halogen atom on *meta*- and *para*-positions of aryl group increases the antibacterial efficacy of 4-thionoisoflavone-fused 1,3-oxazines.

Determination of minimum inhibitory concentration(MIC)

The minimum inhibitory concentration (MIC) was examined only for those compounds which showed 100% growth inhibition at 200 μg/mL against specific bacterial strains. Compound **8j** showed poor to moderate activity with MIC values ranging from 256 to 64 μg/mL among tested isolates. Interestingly, compounds **9b** and **9c**emerged as the most potent inhibitors among all with a broad spectrum antibacterial property. They showed excellent inhibitory properties against *P. aeruginosa*and *B. subtilis*with the same MIC values 16 μg/mL. Moreover, **9b** and **9c** showed good to moderate activity against *S. aureus*and *E. coli*with MIC values 32 and 64 μg/mL, respectively. The results were also compared with the standard antibiotic drug ampicillin (Table 2). Compound **9b** was found more effective than **9c** against *S. aureus*and *E. coli*.It was also found to be equally effective inhibitor of *B. subtilis* cells as ampicillin with MIC 16 μg/mL. It was interesting to note that compounds **9b** and **9c** exhibited better antibacterial potential than ampicillin against *P. aeruginosa*. Thus, compounds **9b** and **9c** were selected as lead inhibitors for further pharmacological investigations.

Disk diffusion assay

Disk diffusion assay was performed to determine the antibacterial properties of test compounds **9b** and **9c** on a solid nutrient agar medium at the concentration corresponding to $^{1/2}$MIC, MIC, and 2MIC concentrations. In the assay, the zones of clearance (dose-dependent) were observed in the presence of various concentrations of both the test compounds. On treatment with **9b**,clear zone of inhibition (ZOI) ranging from 12 to 22 mm was measured around the disk with all four bacterial cultures (Table 3). Thereby, the best ZOI are obtained at 2MIC of **9b** and **9c**against *P. aeruginosa, B. subtilis, S. aureus* and *E. Coli*(Table 3 and 4).

Fractional inhibitory concentration index (FICI)

The assessment of compounds **9b**and**9c**by in vitro antibacterial activity including combination effect with standard drug ampicillin (Amp) against all the four tested bacterial strains was also performed. Many fold decrease in MIC values of compounds **9b** and**9c** along with ampicillin were noticed whenever used in combination. It was observed that antibacterial activity of **9b** was significantly increased (synergistic effect) when used in combination with **9c** against all the tested bacterial strains while **9b** in combination with ampicillin showed synergistic as well as indifferent FICI values. Similarly, **9c** also showed good combination effect with ampicillin and showed synergy against *E. coli* and *B. subtilis* and showed indifferent against *P. aeruginosa*and *S. aureus* bacterial strains. The results are presented in Table 5, 6 and 7. These results suggest that compound **9b** has good synergistic effect with **9c** and ampicillin which may be useful for treating resistant bacterial strains using combination therapy.

Table 3. Zone of inhibition (in mm) measured around the well of $^{1}/_{2}$MIC, MIC and 2MIC concentrations of compound**9b.**

Bacterial species	Zone of Inhibition at different concentrations of **9b**		
	$^{1}/_{2}$MIC	MIC	2MIC
E. coli (MTCC443)	12	16	20
B. subtilis (MTCC736)	12	17	22
S. aureus (MTCC902)	12	15	19
P. aeruginosa (MTCC2453)	12	15	20

Table 4. Zone of inhibition (in mm) measured around the well of $^{1}/_{2}$MIC, MIC and 2MIC concentrations of compound**9c**.

Bacterial species	Zone of Inhibition at different concentrations of **9c**		
	$^{1}/_{2}$MIC	MIC	2MIC
E. coli (MTCC443)	10	14	20
B. subtilis (MTCC736)	10	12	16
S. aureus (MTCC902)	14	18	20
P. aeruginosa (MTCC2453)	15	18	20

Growth kinetics assay

Growth kinetics study was performed to determine the effect of compound **9b** on the growth of test organisms. The study was performed against *E. coli, B. subtilis, S. aureus*and *P. aeruginosa* bacterial strains (Figure 2a–d). As a positive and negative control, we took ampicillin treated cells and untreated cells, respectively. The results showed the growth curve of untreated bacterial cells with clear lag, exponential or log, brief stationary, and decline phases of the cell cycle. At MIC concentrations of compound **9b**, no growth was appeared

Table 5. Synergistic effect of compound **9b** with standard antibacterial drug ampicillin.

Bacterial species	MIC alone (μg/mL)		MIC in combination (μg/mL)		FICI	Mode of Interaction*
	9b	AMP	**9b**	AMP		
E. coli (MTCC443)	32	1	4	0.125	0.25	Synergistic
B. subtilis (MTCC736)	16	16	4	4	0.5	Synergistic
S. aureus (MTCC902)	32	1	4	0.125	0.25	Synergistic
P. aeruginosa (MTCC2453)	16	32	16	8	1.25	Indifferent

*Synergy and antagonism were defined by FIC indices of <0.5 and >4, respectively. An FIC index result of >0.5 but <4 was considered indifferent

Table 6. Synergistic effect of compound **9c** with standard antibacterial drug ampicillin.

Bacterial species	MIC alone (μg/mL)		MIC in combination (μg/mL)		FICI	Mode of Interaction*
	9c	AMP	**9c**	AMP		
E. coli (MTCC443)	64	1	16	0.125	0.375	Synergistic
B. subtilis (MTCC736)	16	16	4	4	0.5	Synergistic
S. aureus (MTCC902)	64	1	4	1	1.0625	Indifferent
P. aeruginosa (2453)	16	32	16	16	1.5	Indifferent

*Synergy and antagonism were defined by FIC indices of <0.5 and >4, respectively. An FIC index result of >0.5 but <4 was considered indifferent.

Table 7. Synergistic effect of compound **9b** in combination with **9c**.

Bacterial species	MIC alone (μg/mL)		MIC in combination (μg/mL)		FICI*	Mode of Interaction*
	9b	**9c**	**9b**	**9c**		
E. coli (MTCC443)	32	64	4	8	0.25	Synergistic
B. subtilis (MTCC736)	16	16	0.125	4	0.257	Synergistic
S. aureus (MTCC902)	32	64	8	16	0.5	Synergistic
P. aeruginosa (MTCC2453)	16	16	2	4	0.375	Synergistic

*Synergy and antagonism were defined by FIC indices of <0.5 and >4, respectively. The FIC index result of >0.5 but <4 was considered indifferent.

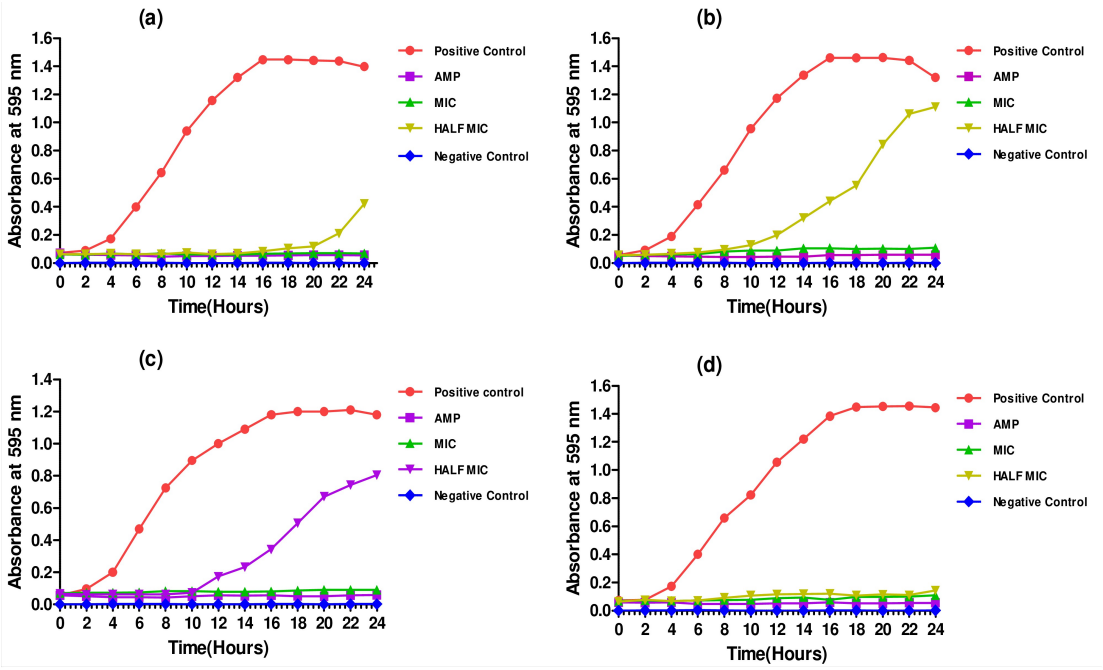


Figure 2. Growth kinetics in the presence of different concentration of synthesized compound **9b**(a)*E. coli*(b)*B. subtilis*(c)*S. aureus*(d)*P. aeruginosa*.

even after 24 h in all tested bacterial strains. At $^{1/2}$MIC concentration of **9b**, growth appeared in *E. coli*, *B. Subtilis*and *S. aureus* after 20 h, 10 h and 12 h, respectively, which is still delayed as compared to untreated cells. However, no growth was observed in *P. aeruginosa* stain at $^{1/2}$MIC concentrations till 24 h. Thus the results clearly showed the bactericidal nature of compound **9b** against all the tested strains.

Time-kill curve assay

The bacteriostatic or bactericidal nature of lead compound **9b** against *P. aeruginosa, B. subtilis, S. aureus* and *E. coli* was determined by time-kill curve study. Two different concentrations equivalent to MIC and 4MIC were used to determine dose dependent response of the test compound and ampicillinwas used as a reference. A significant decline in $\log_{10}$ CFU/mL with respect to time in hours was observed in treated samples. Complete eradication of viability of *E. coli*and *P. aeruginosa*cells was found at 12 h and 16 h, respectively after treating with 4MIC concentrations of compound **9b**. At 4MIC concentrations of compound **9b**against*E. coli*and *P. aeruginosa*, the antibacterial effect on bacterial viability was almost similar to 16 μg/mL concentration of ampicillin. The compound **9b** also displayed significant activity against *B. subtilis*and *S. aureus*at 4MIC concentration, although here some of the colonies were observed after 16 h.The complete eradication in case of *E. coli*and *P. aeruginosa*bacterial population suggested a bactericidal nature of compound **9b** (Figure 3a–d).

Cytotoxicity studies using human (HEK293) normal cells

Human embryonic kidney (HEK293) cells were used to assess the cytotoxic effect of compound **9b**.The screening was done using standard MTT assay in the concentration range of 0–200 μM, for 48 h. Interestingly, the results showed that the treatment of **9b** doesn't affect the viability of HEK293 cells even at 200 μM concentration. These results clearly suggested that compound **9b** is non-cytotoxic to HEK293 cells in the tested concentration range (Figure 4a). On the basis of cell viability results, we proposed that **9b** acts as a vital lead molecule against selective bacterial strains, as in the studied sub-micromolar concentration range, it does not possess toxicity against normal cells, but selectively inhibited the bacterial cells.

Hemolytic assay

The toxicity effect of compound **9b** was also determined by hemolytic assay with human red blood cells (*h*RBCs) using concentrations ranging from 200 to 1.56 μg/mL. Ampicillin was used as the standard drug for comparison (Figure 4b). Compound **9b**at its MIC concentration (16 μg/mL) showed less than 4% RBCs lyses owing negligible toxicity. Even at 100 μg/mL concentration, only 8% lyses of *h*RBCs occurred. Although, it showed more toxicity at higher concentrations as compared to ampicillin but can be considered safe due to negligible effect

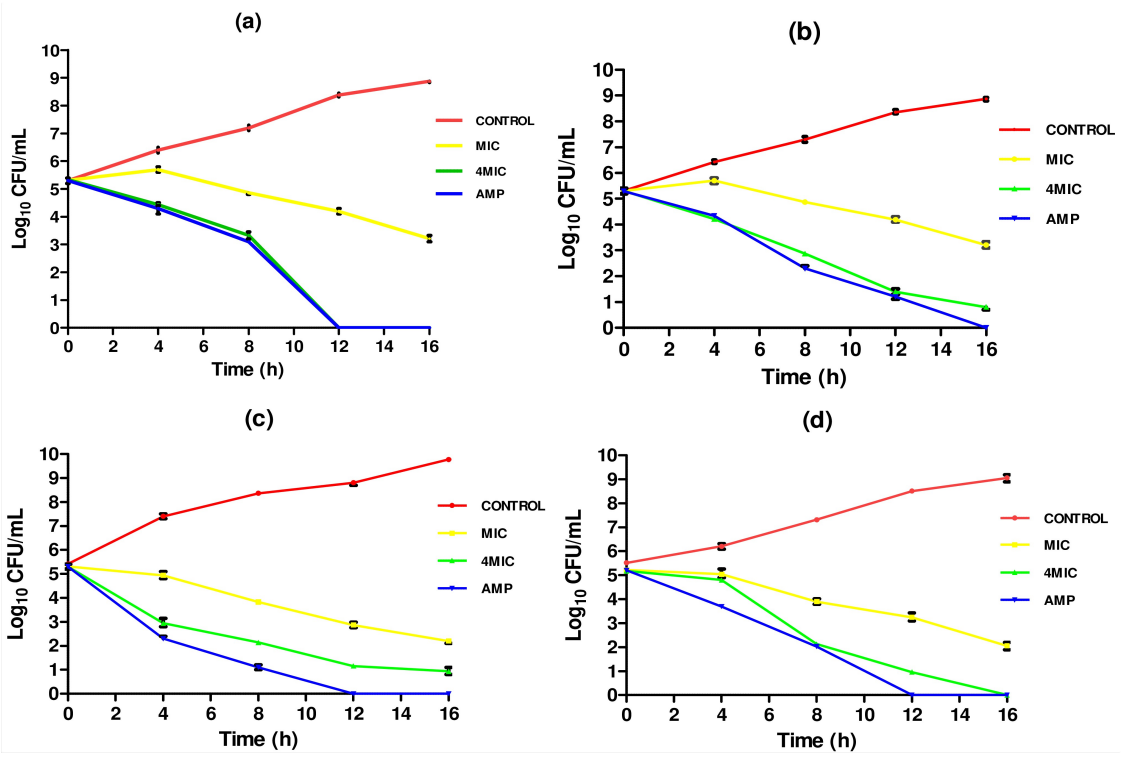


Figure 3. Time-dependent killing of bacterial cells by compound **9b**(a)*E. coli* showed complete inhibition of cells at 4MIC concentration after 12 h. (b) *B. subtilis* did not show complete inhibition cells at MIC and 4MIC concentrations after 16 h (c)*S. aureus* did not show complete inhibition of cells at MIC and 4MIC concentrations after 16 h(d)*P. aeruginosa* showed complete inhibition of cells at 4MIC concentration after 16 h.

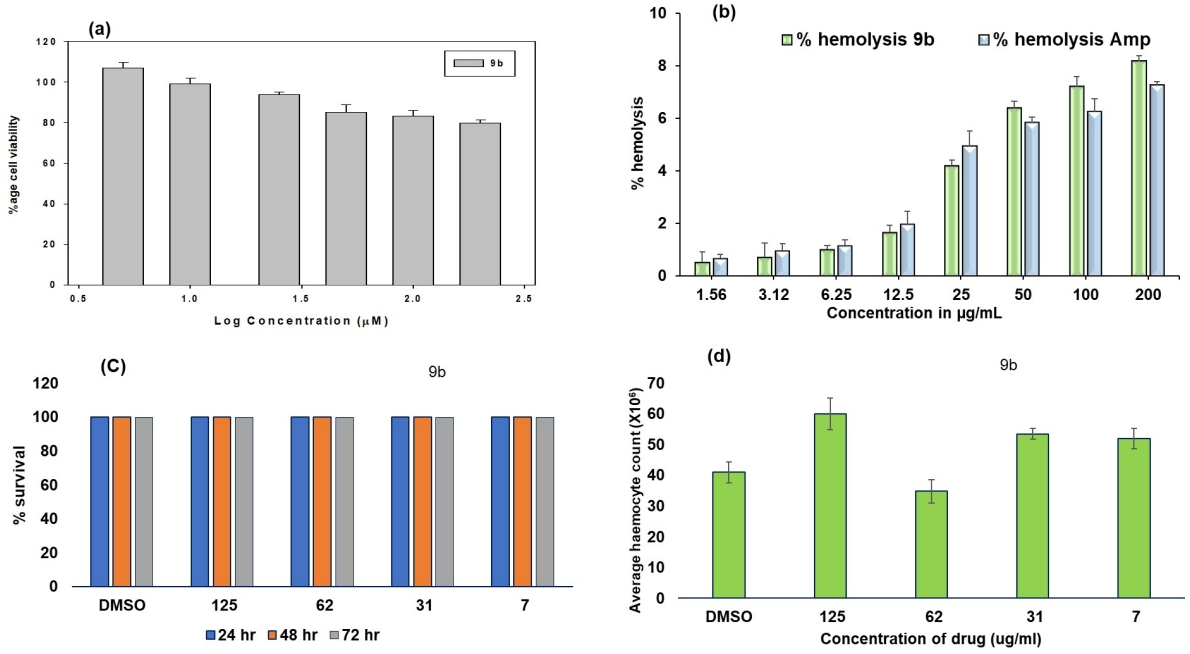


Figure 4. (a) Cytotoxic effect ofcompound **9b** using HEK293 cells and (b) Hemolytic assay of compound **9b** with standard drug ampicillin on human red blood cells (*h*RBCs). (c) In vivo toxicity of compound **9b** and (d) Changes in hemocyte density 24 h after injected with **9b**at 37 °C.

at its effective antibacterial concentration range *i.e.*less than 8 μg/mL.

In vivo toxicity in *G. mellonella* larvae

The in vivo toxicity of compound **9b** was determined on *G. mellonella*larvae at 7, 31, 62 and 125 μg/mL concentrations along with DMSO as the negative control. 100% survival of larvae occurred even after 72 h at maximum concentration (Figure 4c). Alterations in circulation hemocyte density were determined in order to assess the immune modulatory effects on *G. mellonella* larvae. There were no significant changes in circulating hemocyte density following injection of larvae with compound **9b**at 24 h post injection (Figure 4d).

Conclusion

Conclusively, a novel small library of various 3,4-dihydro-2*H*-1,5-dioxa-3-aza-phenanthren-8-ones and 3,4-dihydro-2*H*-1,5-dioxa-3-aza-phenanthren-8-thiones was synthesized using an environmentally benign protocol and evaluated for their antibacterial efficacy. The biological results revealed that most of the compounds exhibited moderate to significant activity against most of the tested strains. Among all the synthesized compounds, 10-chloro-3-(3,4-dichloro-phenyl)-7-(2-methoxy-phenyl)-3,4-dihydro-2*H*-1,5-dioxa-3-aza-phenanthren-8-thione (**9b**) was found to be the most potent antibacterial agent. It selectively inhibited *S. aureus*and *E. coli*bacterial cellswith MIC values 32 μg/mL and *P. aeruginosa and B. subtilis*with MIC 16 μg/mL. The growth kinetic studies showed that the compound **9b** effectively inhibited the growth of selective bacterial strains hence possess bactericidal nature. Further, **9b** when used in combination with **9c** or ampicillin, showed synergistic effect as enhanced antimicrobial activity was observed against most of the tested bacterial strains. Moreover, compound **9b** showed no cytotoxic effect on normal human cells (HEK293), *h*RBCs as well as to *G. mellonella*larvae when checked in vivo. Overall, the results suggested that 4-thionoisoflavone based 1,3-oxazine derivative (**9b**) could be considered as a promising antimicrobial agent for further synthetic alterations and biological studies towards antimicrobial infections.

Supporting Information Summary

Details of the experimental procedures, pharmacology and characterization data including ^{1}H and ^{13}CNMR spectra of synthesized compounds are provided in supporting information.

Acknowledgements

Authors are thankful to University of Delhi for providing the DU-DST PURSE grant to complete this work. The authors are also thankful to the HOD, Biosciences, JMI for providing the facilities for biological studies. AL is grateful to UGC, India for providing the Senior Research Fellowship. GS is the recipient of a Maynooth University Doctoral studentship.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Antibacterial activity · Cytotoxicity · Dihydro-1,3-oxazines · Eco-friendly synthesis, Heterocycles · Synergistic effect.

[1] S. Rachakonda, L. Cartee, *Curr. Med. Chem.* **2004**, *11*,775–793.
[2] M. Ellis, *Mol. Immunol.* **2002**, *38*,947–957.
[3] R. M. Klevens, J. R. Edwards, C. L. Richards, T. C. Horan, R. P. Gaynes, D. A. Pollock, *Public Health Rep.* **2007**, *122*, 160–166.
[4] A. Y. Peleg, D. C. Hooper, *N. Engl. J. Med.* **2010**, *362*,1804–1813.
[5] J. M. Conly, *Can. J. Infect. Dis. Med. Microbiol.* **2004**, *15*,249–251.
[6] M. Vaara, *Curr. Opin. Pharmacol.* **2009**, *9*, 571–576.
[7] D. Jabes, *Curr. Opin. Microbiol.* **2011**, *14*, 564–569.
[8] M. M. Cadelis, S. A. Li, M. L. B. Kondracki, M. Blanchet, H. Douafer, J. M. Brunel, B. R. Copp, *ChemMedChem* **2021**, *16*, 513–523.
[9] a) M. Miski, A. Ulubelen, C. Johansson, *J. Nat. Prod.* **1983**, *46*, 874–875; b) J. Hummelova, J. Rendevaldova, A. Balastikova, O. Lipcik, O. Kokoska, *Lett. Appl. Microbiol.* **2014**, *60*, 242–247; c) H. Hong, M. R. Landauer, M. A. Foriska, G. D. Ledney, *J. Basic Microbiol.* **2006**, *46*, 329–335; d) A. C. Abreu, A. Coqueiro, A. R. Sultan, N. Lemmens, H. K. Kim, R. Verpoorte, W. J. B. van Ramel, M. Simoes, Y. H. Choi, *Sci. Rep.* **2017**, *7*, 3777; e) R. Pereira, A. L. Pereira, M. M. Ferreira, R. O. S. Fontenelle, S. Saker-sampaio, H. S. Santos, P. N. Bandeira, M. A. Vasconcelos, J. A. N. Queiroz, R. Braz-filho, E. H. Teixeira, *An. Acad. Bras. Cienc.* **2019**, *91*, e20180204; f) T.-M. Shao, H.-X. Liao, X.-B. Li, G.-Y. Chen, X.-P. Song, C.-R. Han, *Nat. Prod. Res.* **2021**, https://doi.org/10.1080/14786419.2020.1864368; g) N. J. Sadgrove, T. B. Oliveira, G. P. Khumalo, S. F. van Vuuren, B.-E. van Wyk, *Antibiotics* **2020**, *9*, 223.
[10] a) A. Sen, S. O. Turan, L. Bitis, *Pharm. Biol.* **2017**, *55*, 541–546; b) A. Mahmood, H. Z. Alkhathlan, *Acad. J. Med. Plants* **2019**, *7*, 252–260; c) M. M. Rahman, A. I. Gray, P. Khondkar, S. D. Sarker, *Pharm. Biol.* **2008**, *46*, 356–359; d) S. G. Dastidar, A. Manna, K. A. Kumar, K. Mazumdar, N. K. Dutta, A. N. Charkrabarty, N. Motohashi, Y. Shirataki, *Int. J. Antimicrob. Agents* **2004**, *23*, 99–102; e) W. Mahabusarakam, S. Deachathai, S. Phongpaichit, C. Jansakul, W. C. Taylor, *Phytochemistry* **2004**, *65*, 1185–1191.
[11] a) D. S. Zinad, A. Mahal, R. K. Mohapatra, A. K. Sarangi, M. R. F. Pratama, *Chem. Biol. Drug Des.* **2019**, *95*, 16–47; b) S. Putatunda, A. Chakraborty, *Chem. Heterocycl. Compd.* **2015**, *51*, 763–768; c) V. Botla, N. Pilli, D. Koude, S. Misra, C. Malapaka, *Arch. Pharm. Chem. Life Sci.* **2017**, *350*, e1700169.
[12] a) O. S. Pedersen, E. B. Pedersen, *Synthesis* **2000**, *4*, 479–495; b) A. J. Cocuzza, D. R. Chidester, B. C. Cordova, S. Jeffrey, R. L. Parsons, L. T. Bacheler, S. E. Viitanen, G. L. Trainor, S. S. Ko, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1177–1179; c) R. Gawali, J. Trivedi, S. Bhansal, R. Bhosale, D. Sarkar, D. Mitra, *Eur. J. Med. Chem.* **2018**, *157*, 310–319.
[13] G. R. Madhavan, R. Chakrabarti, K. A. Reddy, B. M. Rajesh, V. Balraju, P. B. Rao, R. Rajagopalan, J. Iqbal, *Bioorg. Med. Chem.* **2006**,*14*,584–591.
[14] a) T. M. Bohme, C. E. Augelli-Szafran, H. Hallak, T. Pugsley, K. Serpa, R. D. Schwarz, *J. Med. Chem.* **2002**, *45*, 3094–3102.
[15] R. E. Ziegler, B. K. Desai, J. A. Jee, B. F. Gupton, T. D. Roper, T. F. Jamison, *Angew. Chem. Int. Ed. Engl.* **2018**, *57*, 7181–7185.
[16] A. C. Arai, M. Kessler, G. Rogers, G. Lynch, *Mol. Pharmacol.* **2000**, *58*, 802–813.
[17] a) M. J. Millan, B. D. Cara, M. Hill, M. Jackson, J. N. Joyce, J. Brotchie, S. McGuire, A. Crossman, L. Smith, P. Jenner, A. Gobert, J.-L. Peglion, M. Brocco, *J. Pharmacol. Exp. Ther.* **2004**, *309*, 921–935; b) J. N. Joyce, S. Presgraves, L. Renish, S. Borwege, T. Osredkar, D. Hagner, M. Replogle, M. PazSoldan, M. J. Millan, *Exp. Neurol.* **2003**, *184*, 393–407.
[18] P. Zhang, E. A. Terefenko, A. Fensome, Z. Zhang, Y. Zhu, J. Cohen, R. Winneker, J. Wrobel, J. Yardley, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 787–790.
[19] a) J. B. Chylinska, M. Janowiec, T. Urbanski, *Br. J. Pharmacol.* **1971**, *43*, 649–657; b) A. N. Mayekar, H. S. Yathirajan, B. Narayana, B. K. Sarojini, N. Suchetha Kumari, W. T. A. Harrison, *Int. J. Chem.* **2011**, *3*, 74–86; c) N. C. Desai, N. B. Bhatt, S. B. Joshi, D. V. Vaja, *Synth. Commun.* **2017**, *47*, 2360–2368; d) K. M. Hannath, M. Chandran, K. Krishnakumar, *Int. J. Pharm. Sci. Rev. Res.* **2020**, *63*, 102–106; e) A. Hamza, H. A. El-Sayed, M. G. Assy, N. H. Ouf, M. E. Farhan, *World Appl. Sci. J.* **2018**, *36*, 637–645; f) A. Lathwal, B. P. Mathew, M. Nath, *Curr. Org. Chem.* **2021**, *25*, 133–174.
[20] a) K. Waisser, E. Petrlikova, M. Perina, V. Klimesova, J. Kunes Jr., K. Palat, J. Kaustova, H. M. Dahse, U. Mollmann, *Eur. J. Med. Chem.* **2010**, *45*, 2719–2725; b) K. Waisser, J. Gregor, L. Kubicova, V. Klimesova, J. Kunes, M. Machacek, J. Kaustova, *Eur. J. Med. Chem.* **2000**, *35*, 733–741.
[21] a) W. M. Duffin, I. M. Rollo, *Br. J. Pharmacol.* **1957**, *12*, 171–175; b) E. Rajanarendar, G. Mohan, A. S. R. Reddy, *Ind. J. Chem.* **2008**, *47B*, 112–116; c) B. P. Mathew, R. Tandon, N. Batra, D. Agarwal, M. Bose, R. D. Gupta, M. Nath, *Ind. J. Chem.* **2017**, *56B*, 1237–1242; d) B. P. Mathew, A. Kumar, S. Sharma, P. K. Shukla, M. Nath, *Eur. J. Med. Chem.* **2010**, *45*, 1502–1507.
[22] E. A. Ilardi, E. Vitaku, J. T. Njardarson, *J. Med. Chem.* **2014**, *57*, 2832–2842.
[23] A. Goel, A. Kumar, Y. Hemberger, A. Raghuvanshi, R. Jeet, G. Tiwari, M. Knauer, J. Kureel, A. K. Singh, A. Gautam, R. Trivedi, D. Singh, G. Bringmann, *Org. Biomol. Chem.* **2012**, *10*,9583–9592.
[24] F. N. Lugemwa, K. Shaikh, E. Hochstedt, *Catalysts* **2013**, *3*, 954–965.
[25] M. Ferraroni, F. Carta, A. Scozzafova, C. T. Supuran, *J. Med. Chem.* **2016**, *59*, 462–473.
[26] T. Furuta, H. Takeuchi, M. Isozaki, Y. Takahashi, M. Kanehara, M. Sugimoto, T. Watanabe, K. Noguchi, T. M. Dore, T. Kurahashi, M. Iwamura, R. Y. Tsien, *ChemBioChem* **2004**, *5*, 1119–1128.

Submitted: May 11, 2021
Accepted: July 21, 2021