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Synthesis and in vitro and in vivo anticancer activity of novel phenylmethylene bis-isoxazolo[4,5-*b*]azepines

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ABSTRACT

A series of novel phenylmethylene bis-isoxazolo[4,5-*b*]azepine derivatives (**10**) have been synthesized from 3-methyl-4-nitro-5-styrylisoxazoles **6**. The reaction of **6** with 3,5-dimethyl-4-nitroisoxazole (**7**) in piperidine afforded the Michael type adducts **8**, which on treatment with different substituted chalcones in the presence of piperidine gave the Michael adducts **9**. Compounds **9** underwent reductive cyclization on treatment with $SnCl_2$–MeOH to afford the title compounds **10**. Structure of these compounds was established on the basis of IR, ^{1}H NMR, ^{13}C NMR and Mass spectral data. The title compounds **10a–j** were evaluated for in vitro and in vivo anticancer activity. Compound **10j** exhibited good anticancer activity as that of standard drug Cisplatin.

Cancer is one of the main causes of death in the world, despite considerable progress in the understanding of its biology and pharmacology. The traditional therapeutic strategies for the treatment of cancer are surgery, radiotherapy, immunotherapy and chemotherapy. Of them, chemotherapy is effective, because it distributes anticancer drugs through the circulatory system.[1] The search for new drugs which can selectively target the tumor cells is today's goal of cancer therapy and is a never ending process, till the goal is reached. We are currently engaged in a program aimed at synthesizing novel heterocyclic compounds that inhibit the growth of cancer cells. The synthesis of isoxazolo[4,5-*b*]azepines have been reported from our laboratories.[2] Isoxazoloazepines have been a subject of much investigation, since they are a class of totally synthetic pharmacological agents with diverse action (Fig. 1). Azepine derivatives such as isoxazolo[4,5-*c*]azepine (**1**) is found to contain many pharmacological applications,[3] where as dibenzoisoxazolo[2,3-*a*]azepine (**2**) is a 5-$HT_{2A/2C}$ receptor antagonist.[4] Isoxazolo[4,5-*b*]azepine (**3**) and isoxazolo[5,4-*b*]azepine (**4**) are both active against Gram-positive and Gram-negative bacteria and exhibit cytotoxicity.[5–7] The bis-azepine **5** is found to possess in vitro anticancer activity and DNA binding affinity.[8]

Based on biological activity of isoxazoloazepines, it seemed that introduction of two isoxazoloazepine rings in a single molecular frame work may enhance the pharmacological activity of these compounds. Inspired by the anticancer activity of bis-azepine **5**, we embarked on synthesizing analogous of bis-azepine and study the in vitro and in vivo anticancer activity. As a sequel to our project on searching for new biologically active molecules possessing isoxazole moiety,[9–13] we, report the synthesis and anticancer activity of novel phenylmethylene bis-isoxazolo[4,5-*b*]azepines substituted with benzene rings at 5- and 7-positions and heterocyclic rings at 5-position. Two derivatives of bis-isoxazolo[4,5-*b*]azepines with furyl and thienyl rings at 5-position showed potential anticancer activity.

The synthesis of title compounds was accomplished by synthetic sequence shown in Scheme 1. 3-Mehtyl-4-nitro-5-styrylisoxazoles (**6**)[14] were reacted with 3,5-dimehtyl-4-nitroisoxazole (**7**) in refluxing ethanol in the presence of piperidine to afford Michael type adducts viz; 1,3-bis-(3-methyl-4-nitroisoxazol-5-yl)-2-phenyl propanes (**8**) in good yields.[15] Compound **8**, on treatment with chalcones in the presence of piperidine in alcohol under refluxing conditions, led to the formation of 4,6-di-(3-methyl-4-nitro-5-isoxazolyl)-1,3,5,7,9-penta-phenyl-1,9-nonanediones (**9**) by Michael addition.[16] Michael adducts **9** were further converted into phenylmethylene-bis-isoxazolo[4,5-*b*]azepines (**10**) by reductive cyclization on treatment with $SnCl_2$–MeOH.[17]

Ten new derivatives of both Michael adducts **9a–j** and phenylmethylene bis-isoxazolo[4,5-*b*]azepine derivatives **10a–j** were reported. The structures of newly synthesized compounds **9a–j** and **10a–j** were confirmed by analytical and spectral data (IR, ^{1}H NMR, ^{13}C NMR and ESI-MS).

In vitro cytotoxic bioassay: The human cell cultures HeLa (cervical), Ehrlich Ascites Carcinoma (EAC) and MCF-7 (breast

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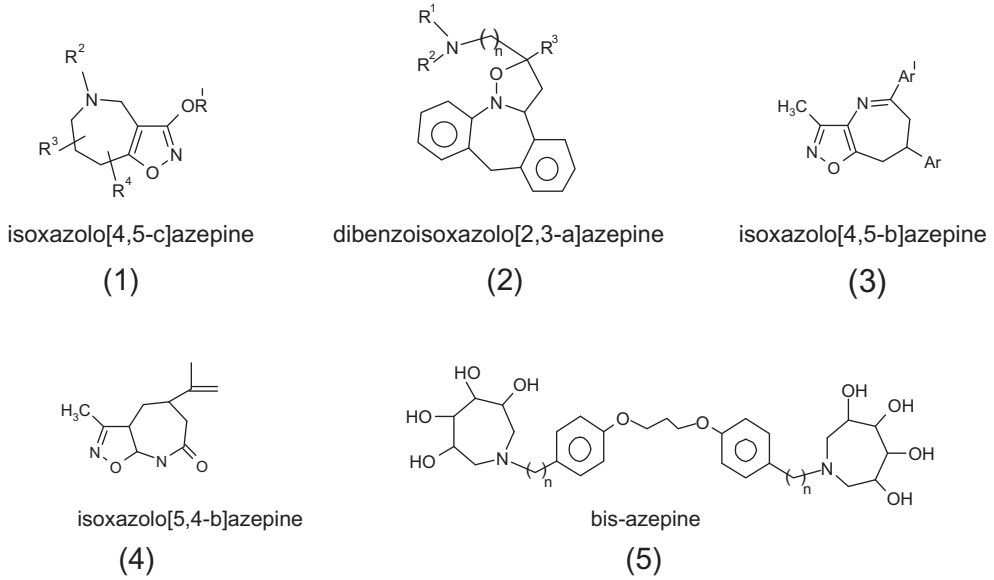
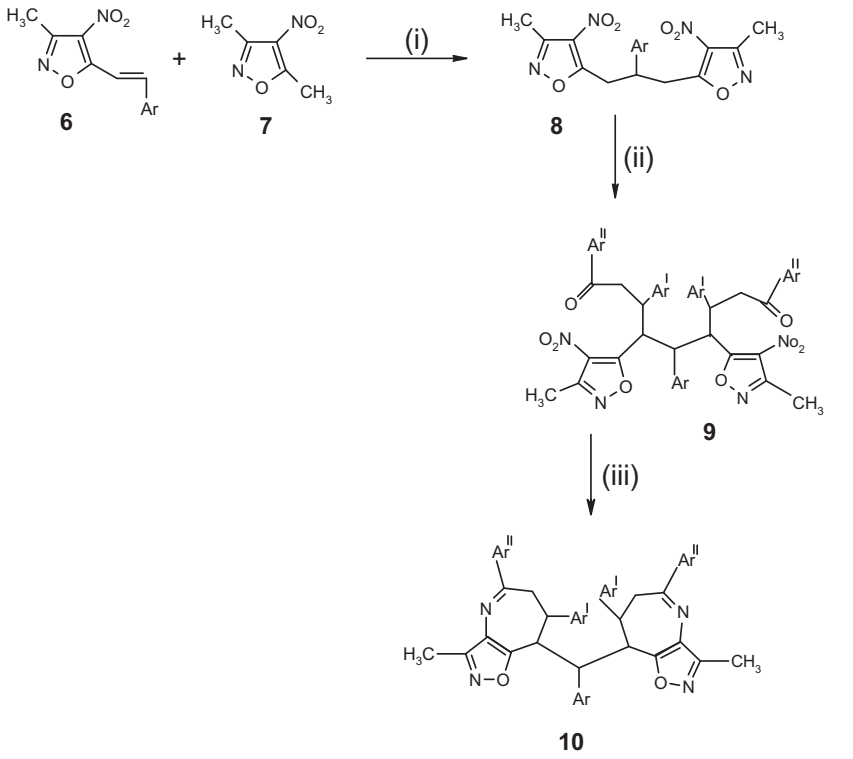


Figure 1. Structures of biologically active azepines as rational compounds design template.

cancer) cell lines, were obtained from National Centre for Cell Sciences (NCCS), Pune, India. These cell lines were grown in recommended media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin–streptomycin amphotericin B in a 5% CO_2 humidified atmosphere at 37 °C. Cells were seeded in 25 cm^2 tissue culture flasks (Tarsons, India) at 25,0000 cells/flask in a total volume of 9 mL. When confluent, all the cells were trypsinized (using Trypsin–EDTA, HiMedia, Mumbai, India), and seeded in 96-well plates (Tarsons, India). The cell suspension of 1×10^5 cells/mL was prepared in complete growth medium. Stock solutions of the compounds **10a–j** were prepared in DMSO. The stock solutions were serially diluted with complete growth medium containing 50 mg/mL of gentamycin to obtain working test solution of required concentrations (having <1% DMSO). The 100 μL of cell suspension was added to each well of the 96-well plates. The test materials in complete growth medium (100 μL) were added after 24 h incubation to the wells containing cell suspension. After 48 h of treatment with different concentrations of test compounds, the cells were incubated with MTT (2.5 mg/mL) for 2 h. The medium was then removed, and 100 μL of DMSO was added each well to dissolve formazan crystals, which is the metabolite of MTT. After thoroughly mixing, the plate was read at 490 nm for optical density that is directly correlated with cell quantity. The cytotoxic effects of the compounds were calculated as percentage inhibition in cell growth as per the formula. % Cytotoxicity = 1 − [(O.D. in sample well)/(O.D. in control well)] × 100.

In vivo cytotoxic bioassay: Adult female Swiss albino mice (Mahaveer Enterprises, Hyderabad, India) of 8 weeks old (mean weight in the range of 20–25 g) were selected and housed in polypropylene cages in a room, where the congenial temperature of 27 ± 1 °C and 12 h light and dark cycles were maintained. The animals were allowed to acclimatize to the environment for 7 days, and supplied with a standard pellet diet and water ad libitum. All procedures using animals were reviewed and approved by the Institutional Animal Ethical Committee of Kakatiya University. The animals were divided into seven groups (n = 10). The normal group was not inoculated with tumor cells, while six groups were injected with EAC cells (0.2 mL of 2×10^6 cells/mice) intraperitoneally. This was taken as day '0', and the experimental treatment started 24 h later. From the 1st day, 100 μL/mouse per day of sterile saline was administered intraperitoneally to the negative control group (EAC-bearing mice). Compounds **10i** and **10j** at doses of 5 mg/kg and 10 mg/kg were administered each day to the treated groups, and the standard drug Cisplatin at a dose of 5 mg/kg was administered to each animal from the positive control group. The pharmacological treatment lasted for 9 days. Fourteen days after the treatment, five mice from each group were killed for the study of antitumor activity. The rest of the animal groups were kept to check the mean survival time of EAC tumor bearing hosts. The antitumor effects of the extracts were determined by the change in body weight, mean survival time (MST) and percentage increased life span (% ILS). The MST of each group containing five mice was identified by recording the mortality on a daily basis for 30 days, and the % ILS was calculated using the following equations MST = (day of the first death + day of the last death)/2; and ILS (%) = [(mean survival time of treated group/mean survival time of control group) − 1] × 100. The effect of compounds **10i** and **10j** was also assessed by the determination of the body weight, tumor volume, packed cell volume and viable tumor cell count of EAC bearing mice by the Trypan blue incorporation method.

The cytotoxic activity of the newly synthesized phenylmethylene bis-ixoazolo[4,5-*b*] azepines **10a–j** was evaluated for in vitro anticancer activity against human cancer cell lines Hela, EAC and MCF-7 according to MTT assay method[18,19] by using Cisplatin (DDP) as a reference drug. The results are presented in Table 1. IC_{50} values were based on dose-response curves (IC_{50} values, defined as the concentration corresponding to 50% growth inhibition). From Table 1, it is clear that some of the compounds showed excellent activity against tumor cells. The compounds bearing benzene or substituted benzene rings at 5-position showed moderate to good anticancer activity against three different cell lines, and not selective towards any particular cell line. The substitution of the electron releasing groups such as methyl and methoxy on the phenyl ring present either at 5-position (**10c**) or 7-position (**10d** and **10e**) increased the anticancer activity. But the introduction of electron withdrawing chloro group (**10b**) decreased the activity moderately. The replacement of benzene by furan or thiophene ring at position-5 (**10i** and **10j**) enhanced the cytotoxic activity remarkably. These results suggest that bis-isoxazolo[4,5-*b*]azepines bearing furan and thiophene rings at position-5 played a vital role in the modulation of cytotoxicity among all the tested compounds **10a–j**. Compound **10j** (bearing thiophene ring) is most cytotoxic towards all the three cancer cell lines.



10a, Ar = C_6H_5, Ar′ = C_6H_5, Ar″ = C_6H_5

10b, Ar = C_6H_5, Ar′ = C_6H_5, Ar″ = 4-ClC_6H_4

10c, Ar = C_6H_5, Ar′ = C_6H_5, Ar″ = 4-$CH_3C_6H_4$

10d, Ar = C_6H_5, Ar′ = 4-$CH_3C_6H_4$, Ar″ = C_6H_5

10e, Ar = C_6H_5, Ar′ = 4-$CH_3OC_6H_4$, Ar″ = C_6H_5

10f, Ar = 4-$CH_3C_6H_4$, Ar′ = C_6H_5, Ar″ = C_6H_5

10g, Ar = 4-$CH_3OC_6H_4$, Ar′ = C_6H_5, Ar″ = C_6H_5

10h, Ar = 4-ClC_6H_4, Ar′ = C_6H_5, Ar″ = C_6H_5

10i, Ar = C_6H_5, Ar′ = C_6H_5, Ar″ = 2- furyl

10j, Ar = C_6H_5, Ar′ = C_6H_5, Ar″ = 2-thienyl

Scheme 1. Synthesis of bis-isoxazolo[4,5-*b*]azepines (**10a–j**). Reagents and conditions: (i) Ethanol, piperidine, reflux, 1 h. (ii) Ar′-CH=CH–CO-Ar″ (2 mol) ethanol, piperidine, reflux, 4 h. (iii) $SnCl_2$ (3 mol)–MeOH, reflux, 4 h.

Having obtained excellent cytotoxic properties for **10i** and **10j** in in vitro studies, we evaluated the in vivo antitumor activity of these compounds in EAC bearing mice by using liquid tumor model.[20–22] The effect of the compounds **10i** and **10j** in two different doses (5 mg/kg and 10 mg/kg) on body weight, mean survival time, % increase life span, tumor volume, packed cell volume and viable tumor cell count were studied. From Table 2, it is evident that compound **10j** has significant activity (P <0.001) in both the doses, and decreased the body weight of EAC-bearing mice, whereas the compound **10i** has the significant activity only at 10 mg/kg. **10j** significantly increased the mean survival time, and decreased the tumor volume, packed cell volume and viable cell count in both the doses, (5 mg/kg and 10 mg/kg), whereas, **10i** only in higher dose (10 mg/kg) has similar results. On the day 14, the biochemical and hematological parameters, with regard to hemoglobin level, erythrocytes and leukocytes counts were compared with EAC control groups, standard drug Cisplatin treated groups and the groups injected with the compounds **10i** and **10j**. As shown in Table 3, the biochemical and hematological parameters, in the group treated with the compound **10j**, have been recovered completely to the normal values. Both these compounds **10i**, and **10j**, significantly decreased the ascetic fluid volume compared to EAC control. These results could indicate macrophage activation and inhibition of vascular permeability with the comparison of tumor control. Compounds **10i** and **10j** increased the hemoglobin and RBC levels when compared with tumor control group (more significantly in case of **10j**) and the standard drug Cisplatin treated group. Similarly, **10i** and **10j** decreased the WBC levels, when compared with tumor control. Treatment with **10i** and **10j** brought back the differential leukocyte count more or less to the normal levels. This

Table 1
Cytotoxic activities of bis-isoxazolo[4,5-*b*]azepines **10a–j** on human cancer cell lines [in vitro[a] (IC_{50}, μg/mL[b])]

Compound	Ar	Ar^{I}	Ar^{II}	HeLa	EAC	MCF-7
10a	C_6H_5	C_6H_5	C_6H_5	68.7 ± 2.5	66.5 ± 2.8	68.9 ± 3.2
10b	C_6H_5	C_6H_5	4-ClC_6H_4	60.2 ± 3.2	58.5 ± 3.4	61.1 ± 2.9
10c	C_6H_5	C_6H_5	4-$CH_3C_6H_4$	55.1 ± 3.3	50.1 ± 2.5	55.9 ± 2.6
10d	C_6H_5	4-$CH_3C_6H_4$	C_6H_5	53.3 ± 2.8	51.6 ± 3.5	50.1 ± 3.1
10e	C_6H_5	4-$CH_3OC_6H_4$	C_6H_5	45.6 ± 2.8	40.1 ± 3.1	53.8 ± 2.1
10f	4-$CH_3C_6H_4$	C_6H_5	C_6H_5	38.4 ± 2.6	48.2 ± 3.1	41.9 ± 2.5
10g	4-$CH_3OC_6H_4$	C_6H_5	C_6H_5	65.9 ± 3.4	41.9 ± 2.3	57.3 ± 2.8
10h	4-ClC_6H_4	C_6H_5	C_6H_5	55.9 ± 2.7	60.4 ± 2.4	59.0 ± 2.4
10i	C_6H_5	C_6H_5	2-Furyl	32.7 ± 2.3	28.9 ± 2.6	35.9 ± 2.1
10j	C_6H_5	C_6H_5	2-Thienyl	28.5 ± 1.5	26.1 ± 1.2	24.1 ± 1.6
Cisplatin (DDP)	—	—	—	3.8 ± 0.1	4.8 ± 0.2	4.2 ± 0.1

$n = 4$, and all values are expressed as mean ± SEM.
[a] Cytotoxicity as IC_{50} for each cell line, is the concentration of compound which reduced by 50% the optical density of treated cells with respect to untreated cells using the MTT assay.
[b] Data represent the mean values of three independent determinations.

Table 2
Anticancer activity of phenylmethylene bis(isoxazolo[4,5-*b*]azepines **10i** and **10j** on EAC bearing mice

Parameters	EAC control (5 × 106 cells)	Cisplatin (5 mg/kg)	**10i** (5 mg/kg)	**10i** (10 mg/kg)	**10j** (5 mg/kg)	**10j** (10 mg/kg)
Body weight Grams ± SEM	12.2 ± 0.8	4.0 ± 0.4***	10.6 ± 0.3^{ns}	9.6 ± 0.4**	8.3 ± 0.5***	6.7 ± 0.4***
Mean survival time Days ± SEM	13.7 ± 0.4	29.3 ± 0.4***	14.9 ± 0.3*	18.3 ± 0.3***	17.8 ± 0.5***	23.8 ± 0.4***
% Increased in life span (% ILS)	—	113.9	8.8	33.6	29.9	73.7
Tumor volume (ml ± SEM)	12.1 ± 0.9	3.0 ± 0.4***	11.0 ± 1.0^{ns}	8.5 ± 0.4**	7.3 ± 0.6***	4.9 ± 0.6***
Packed cell volume (ml ± SEM)	2.8 ± 0.5	0.3 ± 0.1***	2.1 ± 0.4^{ns}	1.6 ± 0.4^{ns}	1.8 ± 0.5*	1.1 ± 0.2***
Viable tumor cell count ($\times 10^7$ cells/ml)	6.4 ± 0.5	0.2 ± 0.04***	6.1 ± 0.5^{ns}	4.9 ± 0.2*	4.2 ± 0.5**	3.2 ±0.4***

$n = 10$, and all values are expressed as mean ± SEM.
* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.001$.
^{ns} Nonsignificant, compared to tumor control.

Table 3
Effect of phenylmethylene bis-isoxazolo[4,5-*b*]azepines **10i** and **10j** on biochemical and hematological parameters in EAC bearing mice

Parameters	Normal	Tumor control	Cisplatin (5 mg/kg)	**10i** (5 mg/kg)	**10i** (10 mg/kg)	**10j** (5 mg/kg)	**10j** (10 mg/kg)
Hemoglobin (g %)	13.1 ± 0.8	5.8 ± 0.7	11.5 ± 1.0***	6.0 ± 0.5^{ns}	7.4 ± 0.7*	7.3 ± 0.7**	8.5 ± 0.6**
RBC (million/mm^3)	4.5 ± 0.1	2.7 ± 0.1	4.1 ± 0.1***	2.9 ± 0.1^{ns}	3.1 ± 0.1*	3.0 ± 0.1^{ns}	3.7 ± 0.1**
WBC (10^3cells/mm^3)	7.1 ± 0.1	20.6 ± 0.6	9.1 ± 0.2***	20.1 ± 0.3*	18.1 ±0.3**	18.7 ±0.6**	15.7 ± 0.4***
Lymphocytes (%)	69 ± 1.3	23 ± 0.6	63 ± 0.8***	29 ± 0.7^{ns}	46 ± 1.0*	35 ± 0.8**	51 ± 0.6**
Neurophils (%)	29 ± 1.5	73 ± 1.3	30 ± 1.2***	63 ± 0.8*	49 ± 0.4**	54 ± 0.6**	36 ± 0.4***
Monocytes (%)	1 ± 0.3	2.4 ± 0.1	1.2 ± 0.2***	2.1 ± 0.1*	1.7 ± 0.3*	1.9 ± 0.5*	1.4 ± 0.4**

$n = 10$, and all values are expressed as mean ± SEM.
* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.001$.
^{ns} Nonsignificant, compared to tumor control.

indicates that, these tested drugs possess protective action on haemopeitic system. These results suggest that compound **10j** is more active in in vivo cytotoxic studies. By effecting a simple modification in the structure, a potent anticancer drug can be developed.

In conclusion, we report the synthesis of novel phenylmethylene bis-isoxazolo[4,5-*b*] azepines, using inexpensive and commercially available materials with potential medicinal properties. This synthesis benefits from a simple method of purification, which does not require chromatography. This easiness of purification compliments the synthetic technology, which is practical, easy to perform and facile. The newly synthesized novel phenylmethylene bis-isoxazolo[4,5-*b*]azepines **10a–j** were evaluated for in vitro and in vivo anticancer activity. Compound **10j** is proved to possess remarkable anticancer activity. By effecting a simple modification in structure, a new potent analog can be generated with the desired anticancer activity with good efficacy. It also requires further studies to reveal structure–activity relationship.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.044.

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16. *General procedure for the preparation of 4,6-di-(3-methyl-4-nitro-5-isoxazolyl)-1,3,5,7,9-pentaphenyl-1,9-nonane diones: (**9a–j**)*: A mixture of **8** (1 mmol) and chalcone (2 mmol) in ethanol (20 mL) were refluxed in the presence of piperidine (0.5 mL) for 4 h. The reaction mixture on cooling gave the solid, which was filtered and washed with cold alcohol. Recrystallization from ethanol afforded pure Michael adducts **9**.
17. *General procedure for the preparation of bis-isoxazolo[4,5-b]azepines: (**10a–j**)*: The Michael adducts **9** (1 mmol) and $SnCl_2{\cdot}2H_2O$ (3 mmol) were dissolved in 20 mL of methanol and refluxed for 4 h. After completion of the reaction (monitored by TLC), solvent was removed in vacuum. The solid mass was decomposed with cold water and the reaction mixture was carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (2×20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated under vacuum and purified by recrystallization from ethanol.
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