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Original article

Multi-component synthesis and *in vitro* and *in vivo* anticancer activity of novel arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides

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ABSTRACT

A three component one-pot protocol has been investigated for the synthesis of arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides **1** from the commercially available materials. The title compounds **1** were also synthesized by a step-wise method and found to be identical with one-pot synthesis by spectral and analytical data. The newly synthesized compounds were evaluated for their *in vitro* anticancer activity against human cancer cell lines and *in vivo* anticancer activity on EAC-bearing mice. Compound **1a** was found to be the most active both in *in vitro* and *in vivo* cytotoxic studies.

1. Introduction

Multi-component reactions (MCRs) have proved to be remarkably successful in generating products in a single synthetic operation [1,2] and are of increasing importance in organic and medicinal chemistry [3–5]. In MCRs a high degree of molecular diversity can be introduced by variation of a single component at a time. Considering that, rapidity and diversity are key factors in modern drug discovery, MCR strategies offer significant advantages over conventional linear-type syntheses, owing to their exceptional synthetic efficiency [6]. MCRs contribute to the requirements of an environmentally friendly process by reducing the number of synthetic steps, energy consumption and waste production. Fused heterocycles are reported to show a wide variety of applications in medicinal chemistry [7,8]. Despite several reports on fused heterocycles, there is a continuing demand for development of new methods for synthesis of novel fused heterocycles due to their plethora of medicinal applications [9].

Nitric oxide (NO) is a highly reactive molecule involved in a number of physiological and pathological processes, which plays an important role in anticancer activity [10,11]. Nitric oxide acting as electron donor or electron acceptor, may participate in the reaction with inorganic molecules, DNA, proteins, consequently modifies enzymatic and transcriptional factor activities. Nitric oxide may be responsible for destruction of cellular components [12–14]. Owing to the potential activity of pyridine-*N*-oxides as anti-HIV and antiviral [15–17] agents, and isoxazole nitric oxide proved to act as anticancer molecule [18], we would like to introduce two isoxazolo[4,5-*b*] pyridine-*N*-oxide systems in a single molecular frame work to study the biological activity. Inspired by the potential activity of pyridine-*N*-oxides, and to develop new synthetic strategies, we performed a multi-component one-pot synthesis on 3,5-dimethyl-4-nitroisoxazole **5** to synthesize the title compounds. The present work is an extension of our on-going efforts towards the development and identification of new molecules with potential biological activity [19–21].

2. Results and discussion

2.1. Chemistry

3,5-Dimethyl-4-nitroisoxazole **5** represents a versatile building block bearing a number of different functionalities which can be selectively reacted to generate molecularly diverse products, and

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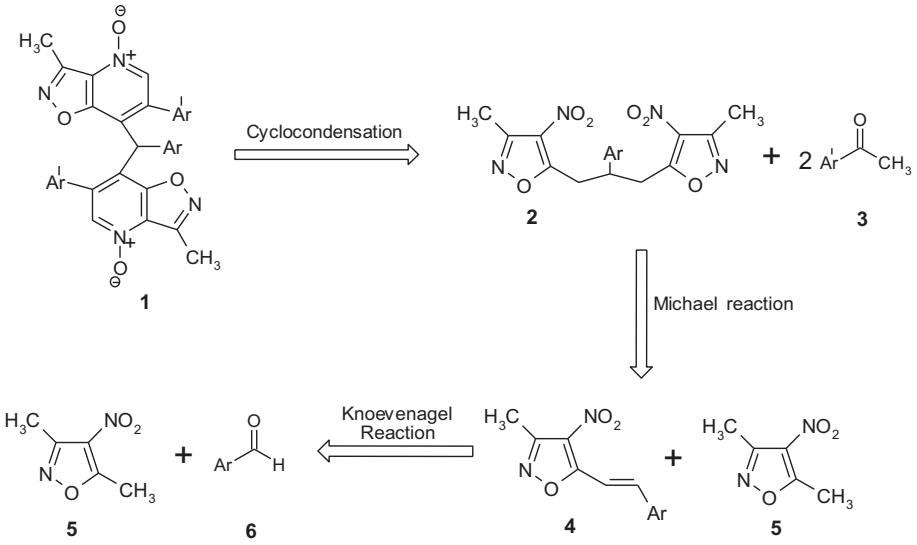


Fig. 1. A disconnection of compound **1**.

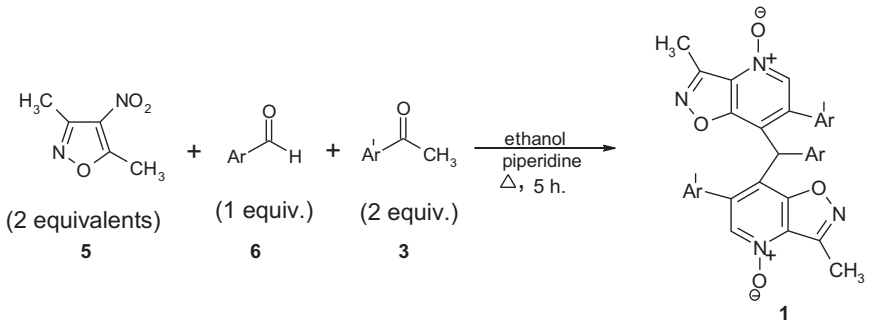
we reported many reactions by describing the reactivity of isoxazole **5** [22–24]. Our approach to the development of multi-component one-pot synthesis is based on the creation of building blocks containing several reactive centers, which can be selectively reacted. As a part of our endeavour to develop diversity-oriented synthesis using poly-functional scaffold such as 3,5-dimethyl-4-nitroisoxazole **5**, and products with high pharmacological activity, we now report a new one-pot, three-component procedure (actually involving 5 components, MCR-5) which allows the synthesis of arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides from commercially available materials.

We proposed the following retro-synthetic analysis for the target compounds (Fig. 1). This disconnection looked particularly attractive due to the commercial availability of 3,5-dimethyl-4-nitroisoxazole (**5**), aromatic aldehydes (**6**) and aromatic ketones (**3**) on the market. We reasoned that compound **1** could be obtained, when **2** reacts with **3** by cyclocondensation. Significantly, compound **2**, precursor of **1**, could be achieved from **4** and **5** by Michael type reaction. Finally **4** could arise by Knoevenagel condensation of **5** and **6**, where **5** is the building block in this multi-component synthesis.

Considering that, tandem synthesis involving sequential Knoevenagel and Michael processes [25], we anticipated that arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides could be prepared through a multi-component procedure, in which **4** could be generated first from **5**, and **6** by Knoevenagel condensation and these reacted *in situ* with **5** through Michael type addition to give **2**, which subsequently undergoes cyclocondensation with 2 mol of acetophenone **3** to give the title compounds **1**.

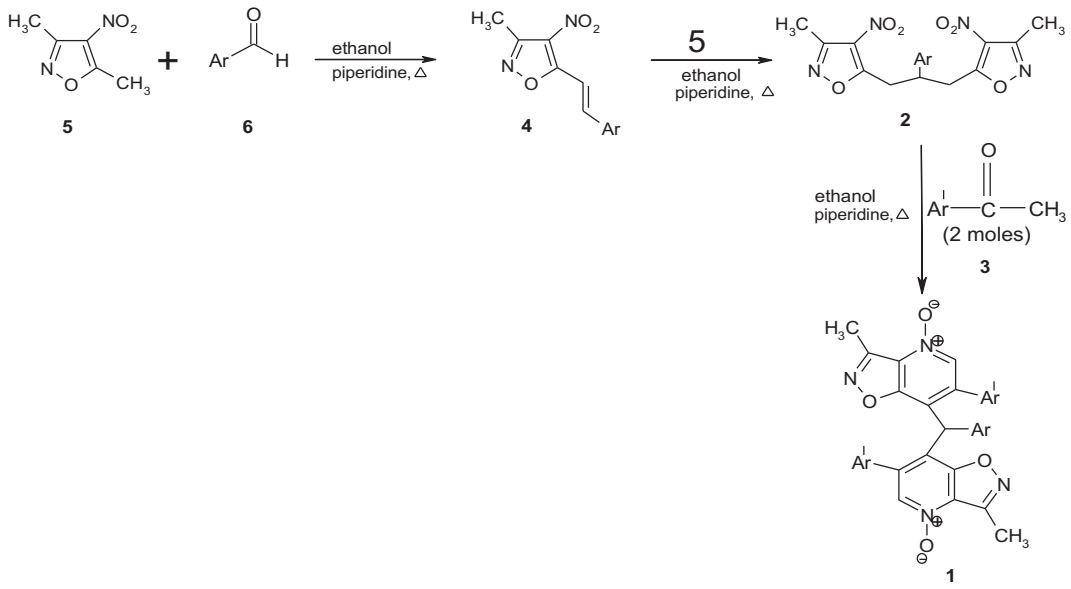
In a typical experiment, equimolar amounts of 3,5-dimethyl-4-nitroisoxazole **5**, aromatic aldehyde **6**, were reacted in the presence of piperidine in methanol for 1 h at 80 °C with stirring and one equivalent of **5** was added later and the refluxing continued for another 1 h at 80 °C, finally 2 equivalents of acetophenone **3** was added and the reaction was carried out for another 3 h at 80 °C. After completion of the reaction (monitored by TLC), the solvent was removed by vacuum distillation and the crude product that obtained was purified by recrystallization from ethanol. Compounds **1a**–**j**, were isolated in moderate to good yields. The products were identified as arylmethylene bis-isoxazolo[4,5-*b*] pyridine-*N*-oxides **1** on the basis of elemental analyses and by spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR and MS) (Scheme 1).

In order to study the scope of this reaction, different substituted aromatic aldehydes and acetophenones were utilized in this multi-component synthesis. The desired product was obtained in each case with moderate to good yield. Finally, the results indicate that



Ar =C_6H_5, 2-$CH_3C_6H_4$, 2-$CH_3OC_6H_4$, 2-ClC_6H_4, 4-ClC_6H_4, 4-$CH_3OC_6H_4$, Ar^1 = C_6H_5, 4-$CH_3C_6H_4$, 4-$CH_3OC_6H_4$, 2-OHC_6H_4 , 4-ClC_6H_4, 4-$NO_2C_6H_4$

Scheme 1. Multi-component synthesis of arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides **1**.



Scheme 2. Step-wise synthesis of arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides **1**.

our method is compatible with various functional groups present on benzene ring and the approach proved to be of general applicability. This synthetic strategy permits the introduction of a diverse array of substituents on to the benzene ring of aldehyde as well as on ketone.

The following findings were pivotal to the establishment of a one-pot procedure. In order to investigate the evidence for the formation of **1a**–**j**, we also carried out a step-wise synthesis. We reacted 3,5-dimethyl-4-nitroisoxazole **5** (1 mmol) with different aromatic aldehydes **6** (1 mmol) in alcohol under refluxing condition on a hot-water bath for 1 h in the presence of catalytic amount of piperidine, which led to the formation of nitrostyrylisoxazoles **4** by Knoevenagel condensation [26]. Compound **4** (1 mmol) was later reacted with **5** (1 mmol) in ethanol containing piperidine for 1 h at 80 °C. This reaction afforded Michael type adducts **2** in good yields [27]. Finally, compound **2** (1 mmol) was reacted with acetophenone **3** (2 mmol) in ethanol containing piperidine for 3 h at 80 °C, which resulted in the formation of title compounds **1a**–**j**. (Scheme 2). The compounds **1a**–**j**, obtained in a one-pot multicomponent synthesis and by a step-wise synthesis were found to be similar from mps, ^{1}H NMR, ^{13}C NMR and mass spectra and by microanalytical data.

To the best of our knowledge, this report is the first of its kind to construct arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxide in a single molecular framework from commercially available materials in a one-pot synthesis as well as in a step-wise synthesis.

2.2. Anticancer activity

The newly synthesized arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides **1a**–**j** were evaluated for *in vitro* anticancer activity against human cancer cells according to the procedures described in literature [28,29]. The tumor cell line panel consisted of HeLa, EAC and MCF-7 cells. Cisplatin (DDP) was used as the reference drug. The results are presented in Table 1. IC_{50} values were based on dose–response curves (IC_{50} value, defined as the concentration corresponding to 50% growth inhibition). As shown in Table 1, some of the compounds showed excellent activity against the tumor cells. All the compounds showed a moderate to good anticancer activity against three different cell lines and they are not selective towards any cell lines. While substitution of electron releasing groups such as methyl and methoxy on the benzene ring present at 6-position (**1f**, **1g**) increased the anticancer activity and has shown good activity, whereas the introduction of electron attracting chloro, nitro groups (**1i**, **1j**) on benzene ring decreased the activity and has shown moderate activity. Compounds **1a** and **1h** have more cytotoxic activity. The results suggested that, pyridine-*N*-oxides with unsubstituted and hydroxy substituted played a vital role in the modulation of cytotoxic activity. Among all the compounds tested **1a**–**j**, **1a** (bearing unsubstituted benzene) is the most cytotoxic in all the three cell lines.

Having obtained the excellent cytotoxic properties of **1a** and **1h** in *in vitro* studies, we evaluated the *in vivo* antitumor properties of these compounds in EAC-bearing mice by using liquid tumor model [30–32]. The effect of the compounds **1a** and **1h** 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, tumor cell count (viable cells) was studied and the results were presented in Table 2. The compound **1a** has significant activity ($p < 0.001$) in both doses, decreased the body weight of EAC-bearing mice, whereas the compound **1h** has the significant activity only at 10 mg/kg dose. **1a** significantly increased the mean survival time and deceased the tumor volume, packed cell volume, and viable tumor cell count in both doses, whereas **1h** only in higher dose

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

Compound	Ar	Ar[1]	HeLa	EAC	MCF-7
1a	C_6H_5	C_6H_5	28.61	26.57	31.87
1b	2-$CH_3C_6H_4$	C_6H_5	62.40	65.12	60.70
1c	2-$CH_3OC_6H_4$	C_6H_5	65.52	51.85	57.35
1d	2-ClC_6H_4	C_6H_5	79.03	70.37	72.06
1e	4-ClC_6H_4	C_6H_5	70.74	60.60	74.26
1f	C_6H_5	4-$CH_3C_6H_4$	42.04	48.42	44.29
1g	C_6H_5	4-$CH_3OC_6H_4$	47.91	41.70	45.96
1h	C_6H_5	2-OHC_6H_4	32.83	36.30	33.55
1i	C_6H_5	4-ClC_6H_4	70.22	78.35	79.05
1j	C_6H_5	4-$NO_2C_6H_4$	80.05	75.55	76.22
Cisplatin (DDP)	–	–	3.84	4.81	4.19

[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 arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides **1a** & **1h** on EAC-bearing mice.

Parameter	EAC control (5 × 106 cells)	Cisplatin (5 mg/kg)	**1a** (5 mg/kg)	**1a** (10 mg/kg)	**1h** (5 mg/kg)	**1h** (10 mg/kg)
Body weight (g)	12.20 ± 0.75	3.98 ± 0.43***	10.41 ± 0.39*	7.70 ± 0.49***	11.00 ± 0.27^{ns}	8.89 ± 0.35***
Mean survival time (days)	13.70 ± 0.03	29.30 ± 0.36***	16.40 ± 0.67**	22.10 ± 1.03***	14.80 ± 0.32*	18.00 ± 0.63***
% increase in life span (% ILS)	–	113.86***	19.70*	61.31***	8.02*	31.38**
Tumor volume (mL)	12.10 ± 0.89	3.01 ± 0.35***	7.61 ± 0.61***	4.50 ± 0.39***	10.69 ± 0.54^{ns}	8.29 ± 0.22**
Packed cell volume (mm)	2.75 ± 0.49	0.25 ± 0.05***	1.25 ± 0.23*	0.94 ± 0.11***	2.13 ± 0.33^{ns}	1.69 ± 0.28^{ns}
Viable tumor cell count ($\times 10^7$ cells/mL)	6.39 ± 0.51	0.16 ± 0.04***	4.05 ± 0.35***	3.27 ± 0.43***	5.21 ± 0.46^{ns}	4.07 ± 0.44***

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

Table 3
Effect of arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides **1a** & **1h** on biochemical and haematological parameters in EAC-bearing mice.

Treatment	Parameters					
	Haemoglobin (mg%)	RBC (million/mm^3)	WBC (10^3 cells/mm^3)	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)
Normal	13.10 ± 0.862	4.512 ± 0.084	7.160 ± 0.120	69 ± 1.31	29 ± 1.46	1 ± 0.29
Tumor control	5.750 ± 0.655	2.740 ± 0.081	20.60 ± 0.545	23 ± 0.567	73 ± 1.25	2.4 ± 0.12
Cisplatin (5 mg/kg)	11.50 ± 1.025***	4.190 ± 0.087***	9.160 ± 0.231***	63 ± 0.782***	30 ± 1.17***	1.2 ± 0.16***
1a (5 mg/kg)	8.080 ± 0.86*	3.020 ± 0.066**	18.48 ± 0.355^{ns}	31 ± 0.936**	54 ± 0.85*	2 ± 0.36*
1a (10 mg/kg)	9.30 ± 1.103**	3.880 ± 0.086**	13.92 ± 0.476***	49 ± 0.759**	39 ± 0.97***	1.6 ± 0.18**
1h (5 mg/kg)	5.960 ± 0.636^{ns}	2.720 ± 0.086^{ns}	19.90 ± 0.343^{ns}	26 ± 0.583*	65 ± 0.48*	2.3 ± 0.46^{ns}
1h (10 mg/kg)	7.020 ± 0.797*	3.300 ± 0.158*	18.80 ± 0.293**	40 ± 0.857**	51 ± 0.84**	1.9 ± 0.53*

All the values were expressed as mean ± SEM ($n = 10$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ & ns = nonsignificant, compared to tumor control.

(10 mg/kg) increased the mean survival time, decreased the tumor volume, packed cell volume and viable tumor cell count. On the day 14, the biochemical and haematological parameters, as regard to haemoglobin level, erythrocytes and leucocytes counts, were compared with EAC control groups, standard drug Cisplatin treated groups and the groups injected with the compounds **1a** and **1h**. As shown in Table 3, the biochemical and haematological parameters in the group treated with the compound **1a** have been nearly recovered completely to the normal values. Both derivatives namely **1a** and **1h** significantly decreased the ascetic fluid volume as compared to EAC control. These results could indicate macrophage activation and inhibition of vascular permeability, with the comparison of tumor control. The derivatives **1a** and **1h** increased the haemoglobin and RBC levels when compared with the tumor control, **1a** increased these levels more significantly than **1h**. The compounds **1a** and **1h** decreased the WBC levels when compared with the tumor control, **1a** decreased these levels more significantly than **1h**. Treatment with the **1a** and **1h** brought back the differential leukocyte count to normal levels. This indicates that the test drugs possess protective action on haemopoeitic system. These results suggest that compound **1a** is more active in *in vivo* cytotoxic studies and by doing simple modification in the structure a potent anticancer drug can be developed.

3. Conclusion

In conclusion, we reported the multi-component (MCR-3) one-pot protocol for the synthesis of arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides, 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 ease of purification compliments the one-pot synthesis, making the technology practical, easy to perform and facile. The newly synthesized novel arylmethylene bis-isoxazolo[4,5-*b*]pyridine-*N*-oxides **1a**–**j** were evaluated for their anticancer activity in *in vitro* and *in vivo*. Compound **1a** is proved to possess remarkable anticancer activity. It can be considered as future drug candidate for cancer therapy, by simple modification in the structure and requires further attempts to reveal the exact mechanism of action, so that, by structure–activity relationship a new potent analogue can be generated with the desired anticancer activity with good efficacy.

4. Experimental

All the melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Analytical TLC was performed on Merck precoated 60 F_{254} silica gel plates. Visualization was done by exposure to iodine vapour. IR spectra (KBr pellet) were recorded on a Perkin-Elmer BX series FT-IR spectrometer. ^{1}H NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. ^{13}C NMR spectra were recorded on a Bruker 75 MHz spectrometer. Chemical shift values are given in ppm (δ) with tetramethyl silane as an internal standard. Mass spectral measurements were carried out by EI method on a Jeol JMC-300 spectrometer at 70 eV. Elemental analyses were performed on a Carlo Erba 106 and Perkin-Elmer model 240 analyzers.

*4.1. General typical procedure for the multi-component synthesis of arylmethylene bis-isoxazolo[4,5-b]pyridine-N-oxides **(1a–j)***

To a stirred solution of **5** (1 mmol) in ethanol (10 mL) were added piperidine (1 mL) and distilled benzaldehyde **6** (1 mmol). The reaction mixture was refluxed with stirring at 80 °C for 1 h, and **5** (1 mmol) was added later to the reaction mixture, heating continued at 80 °C for another 1 h, and finally acetophenone **3** (2 mmol) was further added to it and the reaction was carried out at 80 °C for 3 h. After completion of the reaction (monitored by TLC), the solvent was removed by vacuum distillation and the crude product that obtained was purified by recrystallization from ethanol.

*4.1.1. 7,7′-(Phenylmethylene)bis(3-methyl-6-phenylisoxazolo[4,5-b] pyridine-N-oxide) **(1a)***

Yield 65%, mp 211–213 °C; IR (KBr) cm^{-1}: 1380 (=N$^+$–O$^-$), 1645 (C=N); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.42 (s, 6H, 2CH_3), 5.03 (s, 1H, benzylic–H), 6.81–8.04 (m, 15H, Ar-H), 9.01 (s, 2H, pyridine–H); ^{13}C NMR (75 MHz) δ 11.25, 40.08, 119.68, 120.65, 124.97, 127.31,

127.97, 129.08, 129.31, 129.61, 129.71, 130.04, 131.05, 134.21, 144.25, 144.66, 148.55, 152.12, 158.44, 164.69; ESI-MS (*m/z*): 540 (M^+); Anal. Calcd. for $C_{33}H_{24}N_4O_4$: C, 73.33; H, 4.44; N, 10.37. Found: C, 73.30; H, 4.47; N, 10.34%.

4.1.2. 7,7′-(2-Methylphenylmethylene)bis(3-methyl-6-phenyli-soxazolo[4,5-b]pyridine-N-oxide) ***(1b)***

Yield 65%, mp 195–197 °C; IR (KBr) cm^{-1}: 1380 (=N^+–O^-), 1640 (C=N); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.33 (s, 6H, $2CH_3$), 2.52 (s, 3H, CH_3), 4.90 (s, 1H, benzylic–H), 7.02–8.03 (m, 14H, Ar-H), 9.06 (s, 2H, pyridine–H); ^{13}C NMR (75 MHz) δ 11.45, 21.89, 41.05, 120.05, 120.85, 124.99, 127.36, 127.95, 129.22, 129.73, 130.05, 131.00, 132.08, 134.20, 135.26, 144.16, 145.05, 165.12; ESI-MS (*m/z*): 554 (M^+); Anal. Calcd. for $C_{34}H_{26}N_4O_4$: C, 73.64; H, 4.69; N, 10.10. Found: C, 73.62; H, 4.71; N, 10.13%.

4.1.3. 7,7′-(2-Methoxyphenylmethylene)bis(3-methyl-6-phenyli-soxazolo[4,5b]pyridine-N-oxide) ***(1c)***

Yield 75%; mp 200–202 °C; IR (KBr) cm^{-1}: 1375 (=N^+–O^-), 1640 (C=N)); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.34 (s, 6H, $2CH_3$), 3.82 (s, 3H, OCH_3), 5.04 (s, 1H, benzylic-H), 6.92–7.51 (m, 14H, Ar-H), 9.12 (s, 2H, pyridine-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.10, 42.10, 63.21, 120.01, 124.85, 126.54, 126.80, 127.15, 127.85, 129.11, 129.55, 129.85, 130.45, 131.15, 132.35, 134.01, 135.11, 144.28, 145.40, 158.75, 165.15; ESI-MS (*m/z*): 570 (M^+). Anal. Calcd. for $C_{34}H_{26}N_4O_5$: C, 71.57; H, 4.56; N, 9.82. Found: C, 71.59; H, 4.53; N, 9.84%.

4.1.4. 7,7′-(2-Chlorophenylmethylene)bis(3-methyl-6-phenyli soxazolo[4,5-b]pyridine-N-oxide) ***(1d)***

Yield 65%; mp 235–237 °C; IR (KBr) cm^{-1}: 1360 (=N^+–O^-), 1640 (C=N)); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.23 (s, 6H, $2CH_3$), 5.03 (s, 1H, benzylic-H), 6.81–7.52 (m, 14H, Ar-H), 9.14 (s, 2H, pyridine-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.01, 41.45, 119.25, 120.15, 125.15, 126.95, 127.15, 129.45, 129.95, 130.20, 130.95, 133.45, 134.90, 135.67, 143.96, 144.01, 148.11, 152.25, 158.10, 165.11; ESI-MS (*m/z*): 574 (M^+); Anal. Calcd. for $C_{33}H_{23}N_4O_4Cl$: C, 68.98; H, 4.00; N, 9.75. Found: C, 68.95; H, 4.03; N, 9.73%.

4.1.5. 7,7′-(4-Chlorophenylmethylene)bis(3-methyl-6-phenyli soxazolo[4,5-b]pyridine-N-oxide) ***(1e)***

Yield 60%; mp 222–224 °C; IR (KBr) cm^{-1}: 1370 (=N^+–O^-), 1650 (C=N); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.32 (s, 6H, $2CH_3$), 4.93 (s, 1H, benzylic-H), 6.82–7.83 (m, 14H, Ar-H), 9.03 (s, 2H, pyridine-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.95, 42.15, 118.95, 120.75, 124.98, 126.75, 126.85, 127.45, 129.15, 129.75, 130.45, 130.65, 133.15, 134.15, 143.25, 144.25, 148.35, 151.95, 158.35, 165.25; ESI-MS (*m/z*): 574 (M^+); Anal. Calcd. for $C_{33}H_{23}N_4O_4Cl$: C, 68.98; H, 4.00; N, 9.75. Found: C, 68.96; H, 4.02; N, 9.78%.

4.1.6. 7,7′-(Phenylmethylene)bis(3-methyl-6-(4-methylphenyl) isoxazolo[4,5-b]pyridine-N-oxide) ***(1f)***

Yield 70%; mp 190–192 °C; IR (KBr) cm^{-1}: 1365 (=N^+–O^-), 1640 (C=N)); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.34 (s, 6H, $2CH_3$), 2.52 (s, 6H, $2CH_3$), 4.91 (s, 1H, benzylic-H), 6.92–7.93(m, 13H, Ar-H), 9.12(s, 2H, pyridine-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.75, 22.15, 41.11, 119.26, 120.75, 124.80, 127.26, 129.25, 129.45, 129.66, 129.85, 130.20, 131.65, 134.45, 138.40, 144.15, 144.70, 148.32, 152.10, 158.20, 164.30; ESI-MS (*m/z*): 568 (M^+); Anal. Calcd. for $C_{35}H_{28}N_4O_4$: C, 73.94; H, 4.92; N, 9.85. Found: C, 73.97; H, 4.95; N, 9.83%.

4.1.7. 7,7′-(Phenylmethylene)bis(3-methyl-6-(4-methoxylphenyl) isoxazolo[4,5-b]pyridine-N-oxide) ***(1g)***

Yield 72%; mp; 180–182 °C; IR (KBr) cm^{-1}: 1370 (=N^+–O^-), 1640 (C=N); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.32 (s, 6H, $2CH_3$), 3.81 (s, 6H, $2OCH_3$), 5.02 (s, 1H, benzylic-H), 6.83–8.04(m, 13H, Ar-H), 9.02 (s, 2H, pyridine-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.35, 40.45, 62.75, 120.35, 120.65, 123.08, 124.55, 126.11, 127.55, 128.65, 129.01, 129.55, 130.15, 131.45, 132.55, 134.25, 135.25, 144.01, 145.11, 158.25, 165.20; ESI-MS (*m/z*): 600(M^+); Anal. Calcd. for $C_{35}H_{28}N_4O_6$: C, 70.00; H, 4.66; N, 9.33. Found: C, 70.02; H, 4.68; N, 9.37%.

4.1.8. 7,7′-(Phenylmethylene)bis(3-methyl-6-(2-hydroxyphenyl) isoxazolo[4,5-b]pyridine-N-oxide) ***(1h)***

Yield 70%; mp 216–218 °C; IR (KBr) cm^{-1}: 1365 (=N^+–O^-), 1630 (C=N); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.25 (s, 6H, $2CH_3$), 5.02 (s, 1H, benzylic-H), 6.92–7.86 (m, 13H, Ar-H), 9.05 (s, 2H, pyridine-H), 9.55 (bs, 2H, OH, D_2O exchangeable); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.20, 41.01, 119.25, 120.20, 125.01, 127.11, 127.80, 129.11, 129.70, 129.80, 130.00, 132.60, 134.10, 144.11, 144.35, 148.11, 152.30, 155.38, 158.01, 164.56; ESI-MS (*m/z*): 572 (M^+); Anal. Calcd. for $C_{33}H_{24}N_4O_6$: C, 69.23; H, 4.19; N, 9.79. Found: C, 69.27; H, 4.21; N, 9.76%.

4.1.9. 7,7′-(Phenylmethylene)bis(3-methyl-6-(4-chlorophenyl) isoxazolo[4,5-b]pyridine-N-oxide) ***(1i)***

Yield 60%; mp 240–242 °C; IR (KBr) cm^{-1}: 1370 (=N^+–O^-), 1650 (C=N); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.30 (s, 6H, $2CH_3$), 4.97 (s, 1H, benzylic-H), 6.85–7.89 (m, 13H, Ar-H), 9.09 (s, 2H, pyridine-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.50, 41.11, 118.98, 120.20, 125.20, 126.85, 127.10, 128.65, 129.30, 129.80, 130.15, 131.75, 133.10, 134.75, 143.80, 144.20, 148.33, 152.10, 158.20, 165.45; ESI-MS (*m/z*): 608 (M^+); Anal. Calcd. for $C_{33}H_{22}N_4O_4Cl_2$: C, 65.13; H, 3.61; N, 9.21. Found: C, 65.10; H, 3.58; N, 9.24%.

4.1.10. 7,7′-(Phenylmethylene)bis(3-methyl-6-(4-nitrophenyl) isoxazolo[4,5-b]pyridine-N-oxide) ***(1j)***

Yield 60%; mp 255–257 °C; IR (KBr) cm^{-1}: 1370 (=N^+–O^-), 1650 (C=N); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.23 (s, 6H, $2CH_3$), 5.02 (s, 1H, benzylic-H), 6.92–8.05 (m, 13H, Ar-H), 9.33 (s, 2H, pyridine-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.20, 40.15, 119.60, 120.55, 124.80, 127.15, 127.85, 129.15, 129.55, 129.80, 130.10, 132.27, 134,30, 144.45, 144.75, 148.40, 148.95, 152.10, 158.35, 164.50; ESI-MS (*m/z*): 630 (M^+); Anal. Calcd. for $C_{33}H_{22}N_6O_8$: C, 62.85; H, 3.49; N, 13.33. Found: C, 62.88; H, 3.46; N, 13.30%.

4.2. General procedure for step-wise synthesis of arylmethylene bis-isoxazolo[4,5-b]pyridine-N-oxides ***1***

4.2.1. 3-Methyl-4-nitro-5-styrylisoxazoles ***(4)***

3,5-Dimethyl-4-nitroisoxazole **5** (1 mmol) and freshly distilled aromatic aldehyde **6** (1 mmol) were refluxed in ethanol (10 mL) in the presence of piperidine (0.5 mL) for half an hour. The styrylisoxazole separated while the refluxing is in progress. The reaction mixture was cooled and the product on recrystallization from ethanol gave lemon yellow crystals.

4.2.2. Synthesis of 1,3-bis (3-methyl-4-nitroisoxazol-5-yl)-2-arylpropanes ***(2)***

3-Methyl-4-nitro-5-styrylisoxazole **4** (1 mmol) and 3,5-dimethyl-4-nitroisoxazole **5** (1 mmol), were taken in alcohol (10 mL) and piperidine (1 mL) was added to it and the contents are heated at 80 °C with stirring for 1 h. After the completion of the reaction (monitored by TLC), the solvent was distilled off and the resulting crude product was recrystallized from alcohol.

*4.2.3. Arylmethylene bis-isoxazolo[4,5-b]pyridine-N-oxides (**1a–j**)*

To a solution of Michael adduct **2** (1 mmol) in ethanol (10 mL), piperidine (1 mL) and acetophenone **3** (2 mmol) were added and the contents were refluxed at 80 °C with stirring for 3 h. After completion of the reaction (monitored by TLC), the solvent was removed by vacuum distillation and the crude product was purified

by recrystallization from ethanol. Compounds **1b**–**1j** were prepared by adopting the same procedure as for **1a**. The compounds **1a**–**j** obtained in this method were found to be similar to that of a multi-component one-pot synthesis by mps, ^{1}H NMR, ^{13}C NMR, Mass and elemental analyses.

4.3. Evaluation of anticancer activity

4.3.1. In vitro anticancer activity

The human cell cultures HeLa (cervical), Ehrlich Ascites Carcinoma (EAC) & MCF-7 (breast cancer) cell lines were obtained from National Center for Cancer 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 250,000 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 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 were added in to each well to dissolve formazan crystals, 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.

4.3.2. In vitro anticancer activity

Adult female Swiss albino mice (Mahaveer Enterprises, Hyderabad, India) of 8 weeks old at study start (mean weight in the range of 20–25 g) were selected and housed in polypropylene cages in a room where the congenial temperature was 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 **1a** and **1h** at doses of 5 mg/kg and 10 mg/kg respectively 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 sacrificed for the study of antitumor activity. The rest of the animal group was 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; ILS (%) = [(mean survival time of treated group/mean survival time of control group) − 1] × 100. The effect of compounds **1a** and **1h** 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.

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