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Synthesis of some bisindolyl analogs for in vitro cytotoxic and DNA cleavage studies

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Abstract One-pot, three components, conventional and microwave-assisted synthesis of bisindolyl analogs is described. Michael addition of preformed 2,5-disubstituted indole-3-carboxaldehydes and 3-methyl-1*H*-pyrazol-5(4*H*)-one with 2,5-disubstituted indoles under solvent and catalyst-free conditions afforded the hitherto unreported 2,5-disubstituted bisindolyl analogs bearing a pyrazolone moiety in excellent yields. All the synthesized compounds were characterized using IR, ¹H NMR, mass spectral, and analytical data. The analogs were screened for in vitro cytotoxic and DNA cleavage studies. Among the screened compounds **4c**, **4f**, and **4h–4j** have emerged as most potent cytotoxic and **4c** and **4f–4h** as active DNA cleavage analogs.

Keywords Michael addition · Bisindolyl analogs · 3-Methyl-1*H*-pyrazol-5(4*H*)-one · Microwave-assisted synthesis · Cytotoxic activity

Introduction

Cancer is one of the main causes of deaths in the world despite the major breakthroughs in modern medicine over the past 100 years. Therefore, the development of new drugs against cancer continues to be the priority of development of science and fundamental research. Deoxyribonucleic acid (DNA) damage, mutation, and altered gene expression are key players in the process of carcinogenesis.

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B. S. Sasidhar e-mail: nayakbs7@rediffmail.com Thus, small molecules that bind to genomic DNA have proven to be effective antitumor, antiviral, and antibacterial agents. Hence, DNA cleavage agents form an important class of drugs in antitumor therapy (Brana *et al.*, 2001; Rangappa *et al.*, 2010; Ramya *et al.*, 2010).

Many natural and synthetic anticancer agents having the ability to interact with DNA have been discovered. Among them, indole derivatives have shown significant antitumor activity by possessing inhibitory properties of topoisomerase I- and II-mediated DNA cleavage activity (Jean *et al.*, 1993). Alongside, bisindolyl analogs inhibit the growth of bladder cancer, renal cell cancer, lung cancer, colon cancer, prostate cancer, proliferation process in breast tumor cells, and mammary tumors (Morteza *et al.*, 2010).

Within the frame of green chemistry, the search of innovative solutions for the reduction of chemical steps, wastes, and energy in organic processes has become a challenging task. In this context, multicomponent reactions (MCRs) represent a fascinating tool to achieve this goal (Virsodia et al., 2001; Atul et al., 2007; Kappe, 2004; Kappe and Dallinger, 2009). MCRs generate high levels of diversity, as they allow more than two building blocks to be combined in practical, time saving one-pot operations. Which gives rise to complex structures by simultaneous formation of two or more bonds (Bagley et al., 2002; Cui et al., 2005; Huang et al., 2005; Ramon and Yus, 2005; Domling, 2006; Domling et al., 2007). Achieving organic reactions under solvent- and catalyst-free conditions (Kidwai et al., 2005; Mu et al., 2006; Zhao et al., 2006) through a reaction cascade by two or more sequential reactions set off by a single trigger implies an elegant control over chemical transformations. On the other hand, solvent-, and catalyst-free conditions are especially suitable for microwave activation and several advantages are evident in this approach (Anastas and Warner, 1998; Pelle *et al.*, 2001; Cui *et al.*, 2005; Ramon and Yus, 2005; Mu *et al.*, 2006; Zhao *et al.*, 2006; Victoria *et al.*, 2010).

Michael addition of indoles to α,β -unsaturated system is an efficient approach to generate biologically potent indole analogs (Bartoli et al., 2003; Lin et al., 2005; Renzetti et al., 2008). The literature reveals several reports on Michael addition by, the combination of cerium(III) chloride heptahydrate and sodium iodide supported on silica gel (Bartoli et al., 2005), sulfamic acid catalyzed (An et al., 2007), p-TsOH catalyzed in acetonitrile (Chakrabarty et al., 2004), TiCl₄/Et₃N promoted (Renzetti et al., 2008), N-bromosuccinimide catalyzed in dichloromethane (Kuo et al., 2009), protic and Lewis acids (Ji and Wang, 2003; Zhan et al., 2005) for the C-3 alkylation of indoles. However, most of these protocols have significant drawbacks such as long reaction times, low yields, harsh reaction conditions, difficult workup and the use of expensive, environmentally toxic catalysts, and reagents.

Hence, as a part of our enduring efforts on the development of new routes for the synthesis of antitumor and DNA cleavage bioactive indole analogs by economic and environmentally benign techniques (Biradar *et al.*, 2008, 2010, 2011; Biradar and Sasidhar, 2011). Herein, we report bisindolyl analogs via one-pot Michael addition of indoles.

Results and discussion

Chemistry

The present method encompasses, synthesis of bisindolyl analogs by conventional and microwave irradiation using acetic acid as an energy-transfer medium and homogenizer to increase reaction temperature (Biradar *et al.*, 2008; Biradar and Sasidhar, 2011). An equimolar mixture of 2,5-disubstituted indole-3-carboxaldehydes (**1a–1c**) and 3-methyl-1*H*-pyrazol-5(4*H*)-one (**2**) undergo Knoevenagel condensation to form an intermediate **I**. 2,5-Disubstituted indoles (**3a–3d**) as nucleophile underwent Michael reaction with intermediate **I** to produce bisindolyl analogs (**4a–4k**). A reasonable mechanism for the formation of bisindolyl analogs is outlined in Scheme 1.

In microwave-assisted synthesis, under solvent- and catalyst-free conditions, the title compounds (4a–4k) obtained in excellent yields with high purity (Scheme 2). First, the Michael addition was optimized for both neat and utilizing acetic acid as energy-transfer medium and homogenizer. A controlled experiment without AcOH, has resulted in the formation of Michael adduct only in trace amounts due to the fractional conversion of reactants. When it is carried out with AcOH, it has produced excellent yields with high purity. The same reaction was carried

out in conventional heating. The products were obtained in moderate yields in 40–50 min (Table 1). However, further continuation of reaction (>60 min) has not shown any improvement on the yields. The reaction has suffered by moderate yields when carried out without energy-transfer medium (AcOH).

The reaction has been explored for variety of 2,5disubstituted indole-3-carboxaldehvdes and substituted indoles with 3-methyl-1H-pyrazol-5(4H)-one to look at the scope of the cascade Michael addition under optimized conditions. The representative results are shown in Table 1. The IR spectrum of 4-(bis(5-methyl-2-phenyl-1Hindol-3-yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one (4a) has shown strong absorption at 3.327 cm^{-1} corresponding to indole NH and absorption at 3,070 cm⁻¹ corresponds to pyrazolone NH. Absorptions at 1,655 and 1,578 cm^{-1} correspond to C=O and C=N stretching, respectively. ¹H NMR spectrum of 4a has exhibited a singlet at δ 11.50 due to indole NH (s, 1H, NH) and peak at δ 12.30 assigned to pyrazolone NH (s, 1H, NH) are D₂O exchangeable. A multiplet between δ 7.00–7.80 (m, 16H, Ar–H) integrating for 16 aromatic protons. A singlet at δ 1.90 and at δ 2.50 is for CH₃ protons in the pyrazolone and indoles, respectively. ¹³C NMR spectrum of **4a** has displayed a downfield signals at δ 167 for C=O carbon and less deshielded peak at δ 157 integrated for the C=N of the pyrazolone ring. Upfield signals at δ 18 and 22 correspond to the CH₃ carbon of pyrazolone and indoles, respectively. The mass spectrum of compound 4a has shown molecular ion peak at m/z 522 [M]⁺ which corresponds to its molecular weight.

Biological evaluations

In vitro cytotoxic studies

As a preliminary study, antitumor cytotoxicity for the synthesized compounds was performed against three tumor cell lines, A-549 (lung carcinoma), HEp-2 (laryngeal carcinoma), and HeLa (cervical carcinoma) by MTT assay (Skehan et al., 1990) with doxorubicin as positive reference. The results are presented in Table 2. As can be seen from Table 2, of all the synthesized compounds, 4f, 4h, and 4j have shown very effective cytotoxicity against all three cell lines. Compounds 4c against A-549 (IC₅₀) 1.80 µM), HEp-2 (IC₅₀ 3.50 µM), and 4i against A-549 (IC_{50} 1.60 μM), HeLa (IC_{50} 3.80 μM) have shown effective cytotoxicity compared with the standard drug. Compounds 4e and 4 k have also shown profound cytotoxic activity against all three cell lines. Interestingly, compounds 4c and 4f with 5-OCH₃ substitution and compounds **4h–4k** with 5-H substitution have their IC_{50} values falling



Scheme 1 Feasible mechanism for the Michael addition of indoles

Scheme 2 Michael addition of 2,5-disubstituted indoles



Table 1 Comparative data of conventional and MW methods for the synthesis of bisindolyl analogs (4a-4k)

Entry	Subst	ituents			Conventional	method	Microwave me	ethod		Mp (°C)
	R	R_1	R ₂	R ₃	Time (min)	Yield (%)	Time (min)	Power (%)	Yield (%)	
4a	Ph	Me	Ph	Me	40–50	60	7	70	87	209-210
4b	Ph	Me	Ph	Cl	40-50	63	7	70	90	240-242
4c	Ph	Me	Ph	OMe	40–50	57	7	70	80	190–192
4d	Ph	Me	Ph	Н	40-50	57	7	70	86	226-228
4e	Ph	Cl	Ph	Cl	40-50	60	7	70	85	236-238
4f	Ph	Cl	Ph	OMe	40-50	55	7	70	79	178-180
4g	Ph	Cl	Ph	Н	40-50	60	7	70	87	233-234
4h	Н	Н	Ph	Me	40-50	54	7	70	76	268-270
4i	Н	Н	Ph	Cl	40-50	64	7	70	80	201-202
4j	Н	Н	Ph	OMe	40-50	55	7	70	83	220-221
4k	Н	Н	Ph	Н	40-50	60	7	70	85	206-207

in the range of 3.20–6.70 with better activity than that of the drug doxorubicin (IC₅₀ 8.70 μ M) against HEp-2. In contrast, compounds **4a**, **4b**, **4g** have showed least activity, and **4d** has failed to show any tendency against all the three cell lines. The results clearly signify compounds **4c**, **4f**, and **4h–4k** with OCH₃ and H substitution, respectively, at the fifth position of the indole ring has improved and showed markedly strong cytotoxicity against all the three cell lines. Whereas, the "Cl" substitution has shown some tendency to improve the activity when compared with "CH₃" substituted bisindolyl analogs.

DNA cleavage activity

DNA cleavage activity was determined using gel electrophoresis by Sambrook et al. (1989). According to cell biology, DNA is the primary target molecule for most of the anticancer and antiviral therapies. Investigations of the interaction of DNA with small molecules are basic work in the design of new types of pharmaceutical molecules. When the compound is made to interacted with DNA could induce the breakage of DNA strands by appropriate methods. The gel after electrophoresis (Fig. 1) clearly

Table 2 In vitro cytotoxic studies of bisindolyl analogs (4a-4k)

Compounds	$IC_{50} (\mu M)$							
	A-549 (lung carcinoma)	HEp-2 (laryngeal carcinoma)	HeLa (cervical carcinoma)					
4a	37.0	NA	32.7					
4b	35.0	32.4	NA					
4c	1.80	3.50	NA					
4d	22.1	NA	NA					
4e	7.00	14.6	9.50					
4f	1.10	6.20	1.20					
4 g	31.3	41.7	29.8					
4h	1.30	3.20	2.40					
4i	1.60	NA	3.80					
4j	2.70	5.30	1.70					
4k	5.50	6.70	7.70					
Doxorubicin	0.70	8.70	0.71					

NA not active and having $IC_{50} > 100 \ \mu M$



Fig. 1 DNA cleavage analysis of bisindolyl analogs (4c and 4e–4k). Where M standard DNA molecular weight marker and C control E. *coli* DNA (untreated sample)

revealed that, the compounds tested did act on DNA as tailing in the bands can be observed in treated samples. The difference was observed in bands of all the compounds compared to the control DNA (C). This shows that the control DNA alone does not show any apparent cleavage as the compounds did. Compounds **4c** and **4f**–**4h** have shown more intense streaks, indicating the significant DNA cleavage ability of the molecules. However, the mechanism and the nature of the intermediates involved in the DNA cleavage by the compounds have not been clear. But, the results indicate the importance of bisindolyl analogs in these isolated DNA cleavage reactions. As the compounds **4c** and **4f**–**4h** were observed to cleave the DNA, it can be concluded that the compound inhibits the growth of the pathogenic organism by cleaving the genome.

Experimental section

Chemistry

All the chemicals and reagents were purchased from MERCK, Himedia and SD fine chemical companies and are used without further purification. Melting points of the synthesized compounds are determined in open capillaries and are uncorrected. Reactions are monitored by thin-layer chromatography (TLC) on silica gel 60 F₂₅₄ aluminium sheets (MERCK). The mobile phase was chloroform and benzene (3:1) and detection was made using UV light and iodine. IR spectra are recorded in KBr on Perkin-Elmer and FTIR spectrophotometer (v_{max} in cm⁻¹). ¹H NMR and ¹³C NMR spectra recorded on Bruker Avance II 400-MHz NMR spectrometer (chemical shift in δ ppm down field from TMS as an internal reference). The mass spectra are recorded on LC-MSD-Trap-SL instrument. The elemental analysis was determined on FLASH EA 1112 SERIES instrument. All the compounds gave C, H, and N analysis within ± 0.4 % of the theoretical values. Microwave reactions carried out in Onida 20STP21 800 W multimode microwave oven.

Typical experimental procedure for the synthesis of 2,5-disubstituted indole-3-carboxaldehydes (**1a–1c**)

The precursors 2,5-disubstituted indole-3-carboxaldehydes (**1a–1c**) were obtained from the Vilsmeier–Haack formylation reaction of 2,5-disubstituted indoles (Biradar, 1982).

Synthesis of 3-methyl-1H-pyrazol-5(4H)-one (2)

Ethylacetoacetate (0.01 mol) in ethanol (10–15 ml) was cyclized with hydrazine hydrate (0.01 mol) by stirring at room temperature for about 2–3 h. Solid separated out was filtered washed with ethanol and recrystallized from ethanol (Biradar and Sasidhar, 2011).

General procedure for the synthesis of bisindolyl analogs (4a-4k)

Conventional method

A mixture of 2,5-disubstituted indole-3-carboxaldehyde (1a-1c) (0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one(2) (0.5 mmol), and 2,5-disubstituted indole (**3a–3d**) (0.5 mmol) in acetic acid (2 ml) were stirred for 40–50 min at 160–170 °C. After completion [TLC, chloroform–benzene (3:1)] the reaction mixture was allowed to cool at room temperature, washed with aqueous ethanol and filtered off to get the crude

product. On further crystallization with ethanol will produce 4a-4k.

Microwave-assisted synthesis

Neat reaction A mixture of 2,5-disubstituted indole-3carboxaldehyde (**1a–1c**) (0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (**2**) (0.5 mmol), and 2,5-disubstituted indole (**3a–3d**) (0.5 mmol) were mixed, introduced into open borosil glass tube and irradiated for 7 min at 160–170 °C under microwave power (Table 2). After completion [TLC, chloroform–benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the titled compounds (**4a–4k**).

With AcOH A mixture of 2,5-disubstituted indole-3-carboxaldehyde (1a) (0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)one (2) (0.5 mmol), and 2,5-disubstituted indole (3a) (0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160–170 °C under microwave power (Table 2). After completion [TLC, chloroform–benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get **4a–4k** with high purity (TLC).

Synthesis of 4-(bis(5-methyl-2-phenyl-1H-indol-3yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one (4a)

A mixture of 5-methyl-2-phenyl-1*H*-indole-3-carboxaldehyde (1a) (0.117 g, 0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (2) (0.050 g, 0.5 mmol), and 5-methyl-2-phenyl-1*H*-indole (3a) (0.103 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4a as pale yellow solid. Yield: 87 %; m.p.: 209-210 °C; IR (KBr) v_{max} (cm⁻¹): 3327, 3187, 3070, 1655, 1578; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 11.50 (s, 2H, indole NH), 12.30 (s, 1H, pyrazolone NH), 7.00-7.80 (m, 16H, Ar-H), 4.30 (m, 2H, 2-CH), 2.50 (s, 3H, CH₃), 1.90 (s, 6H, 2-CH₃); ¹³C NMR (DMSO- d_6 + CDCl₃) δ (ppm):167, 157, 147, 146, 145, 144, 140, 138, 137, 136, 130, 129, 127, 122, 118, 116, 48, 35, 25, 22; MS: m/z = 522 [M]⁺; Anal. calcd. for C₃₅H₃₀N₄O: C, 80.43; H, 5.79; N, 10.72. Found: C, 80.37; H, 5.82; N, 10.69.

Synthesis of 4-((5-chloro-2-phenyl-1H-indol-3-yl)(5methyl-2-phenyl-1H-indol-3-yl)methyl)-3-methyl-1Hpyrazol-5(4H)-one (**4b**)

A mixture of 5-methyl-2-phenyl-1H-indole-3-carboxaldehyde (1a) (0.117 g, 0.5 mmol), 3-methyl-1H-pyrazol-5(4H)-one (2) (0.050 g, 0.5 mmol), and 5-chloro-2-phenyl-1H-indole (3b) (0.113 g, 0.5 mmol) in acetic acid (2 ml) were made to paste. introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4b as pale yellow solid. Yield: 90 %; m.p.: 240-242 °C; IR (KBr) $v_{\rm max}$ (cm⁻¹): 3438, 3410, 3190, 3013, 1670, 1600; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 11.90 (s, 1H, indole NH), 11.20 (s, 1H, indole NH), 12.24 (s, 1H, pyrazolone NH), 6.80-7.50 (m, 16H, Ar-H), 4.40 (m, 2H, 2-CH), 2.10 (s, 3H, CH₃), 1.70 (s, 3H, CH₃); ¹³C NMR (DMSO- d_6 + CDCl₃) δ (ppm): 162, 151, 146, 145, 144, 143, 140, 138, 137, 136, 134, 133, 130, 129, 127, 125, 125, 118, 117, 50, 27, 25, 20; MS: $m/z = 543 \text{ [M]}^{+}$; Anal. calcd. for C₃₄H₂₇ClN₄O: C, 75.20; H, 5.01; N, 10.32. Found: C, 75.10; H, 5.11; N, 10.18.

Synthesis of 4-((5-methoxy-2-phenyl-1H-indol-3-yl)(5methyl-2-phenyl-1H-indol-3-yl)methyl)-3-methyl-1Hpyrazol-5(4H)-one (**4c**)

A mixture of 5-methyl-2-phenyl-1H-indole-3-carboxaldehyde (1a) (0.117 g, 0.5 mmol), 3-methyl-1H-pyrazol-5(4H)-one (2) (0.050 g, 0.5 mmol), and 5-methoxy-2-phenyl-1*H*-indole (3c) (0.111 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4c as pale yellow solid. Yield: 80 %; m.p.: 190-192 °C; IR (KBr) v_{max} (cm⁻¹): 3300, 3121, 3020, 3013, 1651, 1600. ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 9.30 (s, 1H, indole NH), 10.00 (s, 1H, indole NH), 12.23 (s, 1H, pyrazolone NH), 7.00-8.00 (m, 16H, Ar-H), 4.70 (m, 2H, 2-CH), 3.20 (s, 3H, OCH₃), 2.42 (s, 3H, CH₃) 2.10 (s, 3H, CH₃); ¹³C NMR (DMSO- d_6 + CDCl₃) δ (ppm): 165, 155, 148, 147, 146, 145, 138, 137, 136, 134, 133, 131, 130, 129, 125, 124, 123, 121, 52, 47, 32, 23, 20; MS: m/z = 538 [M]⁺; Anal. calcd. for C₃₅H₃₀N₄O₂: C, 78.04; H, 5.61; N, 10.40. Found: C, 77.92; H, 5.66; N, 10.33.

Synthesis of 3-methyl-4-((5-methyl-2-phenyl-1H-indol -3-yl)(2-phenyl-1H-indol-3-yl)methyl)-1H-pyrazol-5(4H)-one (**4d**)

A mixture of 5-methyl-2-phenyl-1H-indole-3-carboxaldehyde (1a) (0.117 g, 0.5 mmol), 3-methyl-1H-pyrazol-5(4H)one (2) (0.050 g, 0.5 mmol), and 2-phenyl-1H-indole (3d) (0.096 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160–170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4d as colorless solid. Yield: 86 %; m.p.: 226-228 °C; IR (KBr) v_{max} (cm⁻¹): 3367, 3271, 3100, 2941, 1647, 1590; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 9.70 (s, 1H, indole NH), 10.50 (s, 1H, indole NH), 11.90 (s, 1H, pyrazolone NH), 7.10-8.20 (m, 17H, Ar-H), 4.50 (m, 2H, 2-CH), 2.40 (s, 3H, CH₃), 2.00 (s, 3H, CH₃); ¹³C NMR $(DMSO-d_6 + CDCl_3) \delta$ (ppm): 161, 151, 147, 146, 145, 144, 143, 138, 137, 136, 135, 134, 133, 131, 130, 129, 125, 124, 123, 120, 118, 117, 58, 29, 25, 18; MS: m/z = 509 $[M+1]^{+}$; Anal. calcd. for C₃₄H₂₈N₄O: C, 80.29; H, 5.55; N, 11.02. Found: C, 80.19; H, 5.59; N, 10.88.

Synthesis of 4-(bis(5-chloro-2-phenyl-1H-indol-3yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one (**4e**)

A mixture of 5-chloro-2-phenyl-1H-indole-3-carboxaldehyde (**1b**) (0.127 g, 0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (2) (0.050 g, 0.5 mmol), and 5-chloro-2-phenyl-1*H*-indole (3b) (0.113 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160–170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4e as yellow solid. Yield: 85 %; m.p.: 236-238 °C; IR (KBr) v_{max} (cm⁻¹): 3321, 3162, 3097, 3037, 1679, 1602; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 9.90 (s, 2H, indole NH), 12.50 (s, 1H, pyrazolone NH), 7.20-8.10 (m, 16H, Ar-H), 4.00 (m, 2H, 2-CH), 2.60 (s, 3H, CH_3); ¹³C NMR $(DMSO-d_6 + CDCl_3) \delta$ (ppm): 167, 155, 147, 146, 145, 144, 138, 137, 136, 135, 133, 132, 130, 125, 124, 123, 120, 118, 55, 27, 24; MS: $m/z = 564 [M+1]^+$; Anal. calcd. for C₃₃H₂₄Cl₂N₄O: C, 70.34; H, 4.29; N, 9.94. Found: C, 70.23; H, 4.40; N, 9.82.

Synthesis of 4-((5-chloro-2-phenyl-1H-indol-3-yl)(5methoxy-2-phenyl-1H-indol-3-yl)methyl)-3-methyl-1Hpyrazol-5(4H)-one (**4f**)

A mixture of 5-chloro-2-phenyl-1H-indole-3-carboxaldehyde (1b) (0.127 g, 0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (2) 0.050 g, (0.5 mmol), and 5-methoxy-2-phenyl-1H-indole (3c) (0.111 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4f as yellow solid. Yield: 79 %; m.p.: 178-180 °C. IR (KBr) v_{max} (cm⁻¹): 3309, 3171, 2993, 2923, 1665, 1581; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 10.00 (s, 1H, indole NH), 10.70 (s, 1H, indole NH), 11.40 (s, 1H, pyrazolone NH), 6.30-8.60 (m, 16H, Ar-H), 4.30 (m, 2H, 2-CH), 3.50 (s, 3H, OCH₃), 2.20 (s, 3H, CH₃); 13 C NMR (DMSO- d_6 + CDCl₃) δ (ppm): 162, 153, 146, 145, 144, 137, 136, 134, 132, 130, 125, 124, 47, 40, 27, 21; MS: $m/z = 561 [M+2]^+$; Anal. calcd. for C₃₄H₂₇ClN₄O₂: C, 73.05; H, 4.87; N, 10.02. Found: C, 73.27; H, 4.92; N, 10.28.

Synthesis of 4-((5-chloro-2-phenyl-1H-indol-3-yl)(2phenyl-1H-indol-3-yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one (**4g**)

A mixture of 5-chloro-2-phenyl-1H-indole-3-carboxaldehyde (1b) (0.127 g, 0.5 mmol), 3-methyl-1H-pyrazol-5(4H)one (2) (0.050 g, 0.5 mmol), and 2-phenyl-1H-indole (3d) (0.096 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4g as colorless solid. Yield: 87 %; m.p.: 233-234 °C; IR (KBr) v_{max} (cm⁻¹): 3222, 3137, 3007, 2901, 1643, 1597; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 10.40 (s, 1H, indole NH), 10.90 (s, 1H, indole NH), 11.70 (s, 1H, pyrazolone NH), 7.30-8.70 (m, 17H, Ar-H), 4.10 (m, 2H, 2-CH), 2.70 (s, 3H, CH₃); ¹³C NMR (DMSO- d_6 + CDCl₃) δ (ppm): 161, 153, 144, 143, 137, 136, 135, 132, 130, 131, 123, 122, 120, 118, 43, 27, 23; MS: m/z = 530 [M]⁺; Anal. calcd. for C₃₃H₂₅ClN₄O: C, 74.92; H, 4.76; N, 10.59. Found: C, 74.98; H, 4.66; N, 10.87.

Synthesis of 4-((1H-indol-3-yl)(5-methyl-2-phenyl-1Hindol-3-yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one (**4h**)

A mixture of indole-3-carboxaldehyde (1c) (0.073 g, 0.5mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (2) (0.050 g, 0.5 mmol), and 5-methyl-2-phenyl-1*H*-indole (3a) (0.103 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4h as pale yellow solid. Yield: 76 %; m.p.: 268–270 °C; IR (KBr) v_{max} (cm⁻¹): 3343, 3211, 3180, 2907, 1697, 1598; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 9.90 (s, 1H, indole NH), 10.40 (s, 1H, indole NH), 11.00 (s, 1H, pyrazolone NH), 7.50-8.60 (m, 13H, Ar-H), 4.20 (m, 2H, 2-CH), 2.70 (s, 3H, CH₃), 2.10 (s, 3H, CH₃); ¹³C NMR (DMSO $d_6 + \text{CDCl}_3$ δ (ppm): 167, 157, 146, 145, 144, 136, 135, 134, 133, 126, 125, 124, 120, 118, 117, 45, 37, 25, 20; MS: m/ $z = 432 \text{ [M]}^+$; Anal. calcd. for C₂₈H₂₄N₄O: C, 77.75; H, 5.59; N, 12.95. Found: C, 77.62; H, 5.76; N, 12.75.

Synthesis of 4-((5-chloro-2-phenyl-1H-indol-3-yl)(1H-indol-3-yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one (**4i**)

A mixture of indole-3-carboxaldehyde (1c) (0.073 g, 0.5 mmol), 3-methyl-1H-pyrazol-5(4H)-one (2) (0.050 g, 0.5 mmol), and 5-chloro-2-phenyl-1H-indole (**3b**) (0.113 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4i as yellow solid. Yield: 80 %; m.p.: 201–202 °C; IR (KBr) v_{max} (cm⁻¹): 3323, 3200, 3101, 2911, 2923, 1667, 1583; ¹H NMR (DMSO $d_6 + \text{CDCl}_3 \delta$ (ppm): 10.70 (s, 1H, indole NH), 11.70 (s, 1H, indole NH), 12.30 (s, 1H, pyrazolone NH), 7.00-8.30 (m, 13H, Ar-H), 4.70 (m, 2H, 2-CH), 2.40 (s, 3H, CH₃); ¹³C NMR $(DMSO-d_6 + CDCl_3) \delta$ (ppm): 163, 150, 145, 144, 143, 136, 135, 134, 132, 131, 125, 124, 123, 122, 119, 52, 37, 25; MS: m/ $z = 452 \text{ [M]}^{+}$; Anal. calcd. for C₂₇H₂₁ClN₄O: C, 71.60; H, 4.67; N, 12.37. Found: C, 71.37; H, 4.72; N, 12.55.

Synthesis of 4-((1H-indol-3-yl)(5-methoxy-2-phenyl-1Hindol-3-yl)methyl)-3-methyl-1H-pyrazol-5(4H)-one (**4j**)

A mixture of indole-3-carboxaldehyde (1c) (0.073 g, 0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (2) (0.050 g, 0.5

mmol), and 5-methoxy-2-phenyl-1H-indole (3c) (0.111 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4i as pale yellow solid. Yield: 83 %; m.p.: 220-221 °C; IR (KBr) v_{max} (cm⁻¹): 3323, 3277, 3024, 2930, 1685, 1581; ¹H NMR (DMSO- d_6 + CDCl₃) δ (ppm): 10.10 (s, 1H, indole NH), 10.80 (s, 1H, indole NH), 11.60 (s, 1H, pyrazolone NH), 6.50-7.50 (m, 13H, Ar-H), 4.20 (m, 2H, 2-CH), 3.00 (s, 3H, OCH₃), 2.50 (s, 3H, CH₃); ¹³C NMR (DMSO- d_6 + CDCl₃) δ (ppm): 162, 153, 146, 145, 144, 137, 136, 134, 132, 130, 125, 124, 50, 45, 27, 22; MS: $m/z = 450 [M+2]^{+}$; Anal. calcd. for C₂₈H₂₄N₄O₂: C, 74.98; H, 5.39; N, 12.49. Found: C, 74.92; H, 5.47; N, 12.65.

Synthesis of 4-((1H-indol-3-yl)(2-phenyl-1H-indol-3-yl) methyl)-3-methyl-1H-pyrazol-5(4H)-one (**4**k)

A mixture of indole-3-carboxaldehyde (1c) (0.073 g, 0.5 mmol), 3-methyl-1*H*-pyrazol-5(4*H*)-one (2) (0.050 g, 0.5 mmol), and 2-phenyl-1*H*-indole (3d) (0.096 g, 0.5 mmol) in acetic acid (2 ml) were made to paste, introduced into open borosil glass tube and irradiated for 7 min at 160-170 °C under microwave power (Table 2). After completion [TLC, chloroform-benzene (3:1)] the reaction mixture was brought to room temperature, washed with aqueous ethanol and filtered off to get the crude product. On further crystallization with ethanol will produce compound 4k as colorless solid. Yield: 85 %; m.p.: 206–207 °C; IR (KBr) v_{max} (cm⁻¹): 3287, 3171, 2993, 2923, 1665, 1581; ¹H NMR (DMSO $d_6 + \text{CDCl}_3$) δ (ppm): 9.80 (s, 1H, indole NH), 10.40 (s, 1H, indole NH), 11.00 (s, 1H, pyrazolone NH), 6.60-7.30 (m, 14H, Ar-H), 4.10 (m, 2H, 2-CH), 2.60 (s, 3H, CH₃); ¹³C NMR (DMSO- d_6 + CDCl₃) δ (ppm): 160, 154, 144, 143, 142,137, 136, 135, 134, 132, 130, 125, 124, 123, 120, 119, 44, 33, 25; MS: $m/z = 420 [M+2]^+$; Anal. calcd. for $C_{27}H_{22}$ N₄O: C, 77.49; H, 5.30; N, 13.39. Found: C, 77.54; H, 5.21; N, 13.45.

In vitro cytotoxic studies

Cell lines and culture condition

A-549 (lung carcinoma), HEp-2 (laryngeal carcinoma), and HeLa (cervical carcinoma) cells were cultured in RPMI 1640 (Himedia, India) medium supplemented with 10 % FCS, containing penicillin 100 U/ml and streptomycin 100 μ g/ml at 37 °C in a CO₂ incubator with 5 % CO₂.

MTT assay

Briefly, the test compounds were diluted in DMSO (0–100 μ g/ml) and cytotoxic activity of the compounds on A-549, HEp-2, and HeLa cells (1 × 10⁵ cells/well) were tested by using the cell quantity MTT cell viability assay kit. The wells with only culture medium served as control and the graph is plotted with cell viability against the time period in hours at increasing concentrations of secondary metabolite. The IC₅₀ values were calculated by nonlinear regression analysis from three independent experiments (Skehan *et al.*, 1990).

DNA cleavage activity

Preparation of culture media

DNA cleavage experiments were done according to the literature (Sambrook *et al.*, 1989). Nutrient broth [peptone 10, yeast extract 5, NaCl 10, in $(\mu g/l)$] was used for culturing of *Escherichia coli*. 50 ml media was prepared, autoclaved for 15 min at 121 °C under 15 lb pressures. The autoclaved media were inoculated for 24 h at 37 °C.

Isolation of DNA

The fresh bacterial culture (1.5 ml) is centrifuged to obtain the pellet which is then dissolved in 0.5 ml of lysis buffer (100 mM tris pH 8.0, 50 mM EDTA, 10 % SDS). To this 0.5 ml of saturated phenol was added and incubated at 55 °C for 10 min, then centrifuged at 10,000 rpm for 10 min and to the supernatant, equal volume of chloroform:isoamyl alcohol (24:1) and 1/20th volume of 3 M sodium acetate (pH 4.8) was added. Centrifuge at 10,000 rpm for 10 min and to the supernatant, three volumes of chilled absolute alcohol is added. The precipitated DNA was separated by centrifugation and the pellet was dried and dissolved in TAE buffer (10 mM tris pH 8.0, 1 mM EDTA) and stored in cold condition.

Agarose gel electrophoresis

Cleavage products were analyzed by agarose gel electrophoresis method. Test samples (1 μ g/ml) were prepared in DMF. The samples (25 μ g) were added to the isolated DNA of *E. coli*. The samples were incubated for 2 h at 37 °C and then 20 ml of DNA sample (mixed with bromophenol blue dye at 1:1 ratio) was loaded carefully into the electrophoresis chamber wells along with standard DNA marker containing TAE buffer (4.84 g tris base, pH 8.0, 0.5 M EDTA/1 l) and finally loaded on agarose gel and passed the constant 50 V of electricity for 30 min. Removing the gel and stained with 10.0 mg/ml ethidium bromide for 10–15 min, the bands were observed under Vilber Lourmat gel documentation system and then photographed to determine the extent of DNA cleavage. The results are compared with standard DNA marker.

Conclusions

In conclusion, we have demonstrated the synthesis of bisindolyl analogs via one-pot, three-component reactions in solvent- and catalyst-free condition. In our preliminary cytotoxic studies, 5-OCH₃ and 5-H-substituted bisindolyl analogs 4c, 4f and 4h-4k, respectively, have shown profound cytotoxic potentialities. In DNA cleavage activity, compounds 4c and 4f-4h have shown promising ability in DNA cleavage. The above-mentioned Michael addition has provided new types of potential anticancer compounds originating from indole. Consequently, this method will provide a great impact on organic chemists and biochemists for further investigations in the innovation of economic and greener methodologies in synthesizing "drug-like" molecules. Based on results, selected compounds are being screened for in vivo anticancer activity which will be reported in due course.

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