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Synthesis, antibacterial, antifungal activity and DNA cleavage study of 3-(7-methoxy-benzofuran-2-yl)-5-aryl-*4H*-[1,2,4]triazoles

Hanumanagoud H. and K. M. Basavaraja*

Department of Industrial Chemistry, Vijayanagara Shri Krishnadevaraya University, Hospet Road, Vinayak Nagara, Bellary (Cant)-583 104, Karnataka, INDIA

ABSTRACT

*The key intermediate 7-methoxybenzofuran-2-carboxylic acid ethyl ester (**1**) was synthesized starting from ortho-vanillin and diethyl bromomalonate, which was converted into 7-methoxy-benzofuran-2-carboxylic acid hydrazide (**2**) by treating with hydrazine hydrate in presence of ethanol. Further the compound (**2**) in acetic acid medium got cyclised with substituted benzaldehyde in presence of ammonium acetate to form the 3-(7-methoxybenzofuran-2-yl)-5-aryl-4H-[1,2,4]triazole **3 (a-k)**. Structural assignments of these compounds have been made on the basis of elemental analysis, IR, ^{1}H NMR and GCMS spectral data. All the compounds synthesized have been screened for antimicrobial and DNA cleavage activities. Some of them gave encouraging results.*

Keywords: Benzofuran, Triazole, Antimicrobial, DNA cleavage.

INTRODUCTION

The benzofurans are important heterocyclic compounds, which not only act as key structural sub units in naturally occurring compounds that exhibit remarkable biological activities but also represent useful building blocks in the synthesis of natural products [1,2]. Moreover, many of the natural benzofurans have physiological, pharmacological and toxic properties [3]. Benzofuran derivatives have displayed wide range of biological activities such as antibacterial [4], analgesic [5, 6], anti-inflammatory [4, 6], anticancer [7] and cardiovascular [8]. In addition, benzofurans are used in cosmetic formulations [9] and have application as synthetic precursors for optical brighteners [10,11]. Triazoles occupy a unique position [12] amongst the potentially used bioactive compounds due to different potential activities such as antibacterial [13], antifungal [14], anticonvulsant [15], ant proliferative [16], antitumor [17], antitubercular [18], anticancer [19], anti-HIV [20] and antiviral [21]. It has been revealed that when one biodynamic heterocyclic system is coupled with another, a molecule with pronounced biological activity is produced. Prompted by these observations, as a part of our research program [22] we have aimed at developing a new biologically active heterocycles containing benzofuran and triazole. It was planned to synthesize various substituted benzofuranyltriazoles **3 (a-k).**

Various methods of construction of benzofuran nucleus were already reported from our laboratory [23]. In continuation of our synthetic work in search of benzofurans of pharmaceutical interest, in the present work we are reporting the synthesis of 7-methoxybenzofuran-2-carboxylic acid ethyl ester **(1)** and the title compounds **3 (a-k)**. The key intermediate (**1**) was synthesized by the condensation reaction of *ortho*-vanillin with diethyl bromomalonate in presence of anhydrous potassium carbonate and ethyl methyl ketone. The condensation and

cyclisation took place simultaneously by refluxing the reaction mixture. The ethanolic solution of compound (**1)** was refluxed with hydrazine hydrate to get 7-methoxybenzofuran-2-carboxylic acid hydrazide **(2)** which underwent cyclisation with substituted benzaldehydes in acetic acid medium in presence of ammonium acetate to form the title compounds **3 (a-k)** [24-27]. The structures of all the compounds synthesized in the present investigation were confirmed by the support of analytical data (table 1) and spectral data given in experimental section.

EXPERIMENTAL SECTION

Meting points were determined in open capillary tubes and are uncorrected. IR spectra (KBr disk) were recorded using a Perkin Elmer 237 spectrophotometer, ^{1}H NMR spectra were recorded on a Bruker Avance Spectrometer (at 400 MHz) using TMS as an internal standard. $CDCl_3$ and DMSO-d_6 as solvent, chemical shifts (δ) are given in ppm. The mass spectra (MS) were recorded on a Jeol GCmate GC-MS. Elemental analysis (C, H, N) was performed on Perkin Elmer 240 analyzer. The purity of the compounds were checked on silica gel G coated on aluminum plates by using ethyl acetate and petroleum ether (1:1) as the eluent and observed in UV light. All the chemicals used were of analytical grade.

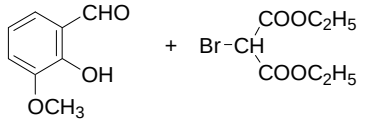
Preparation of 7-methoxybenzofuran-2-carboxylic acid ethyl ester (1).
A solution of *ortho*-vanillin (0.01 mol) and diethyl bromomalonate (0.013 mol) in ethyl methyl ketone (40 mL) was treated with anhydrous potassium carbonate (10 g). The reaction mixture was heated under reflux for 10 h on steam bath. Solvent was distilled off under reduced pressure and the residual salts were dissolved in about 200 mL of water and cooled in an ice bath and carefully acidified with dilute hydrochloric acid. The product (**1**) was extracted with ether and ethereal extract was washed with saturated sodium bicarbonate solution and dried over anhydrous calcium chloride. Removal of the solvent gave the colourless solid. IR (KBr): 1714 cm^{-1} due to carbonyl group of ester. ^{1}H NMR ($CDCl_3$) ppm: 1.39 (t, 3H, CH_3), 4.01 (s, 3H, OCH_3), 4.41 (quartet, 2H, OCH_2), 6.90-7.52 (m, 4H, Ar-H),. GCMS *m/z:* 220.

Preparation of 7-methoxybenzofuran-2-carboxylic acid hydrazide (2).
To a solution of 7-methoxybenzofuran-2-carboxylic acid ethyl ester (**1**) (0.01 mol) in ethanol (30 mL), hydrazine hydrate (25 mL) was added and the mixture was heated under reflux for 4 h on a water bath. Excess of ethanol was removed under reduced pressure and then diluted with water. The separated carbohydrazide (**2)** was collected and crystallized from ethanol as colorless needles. IR (KBr): 3400 and 3275 cm^{-1} due to NH.NH_2 group, 1670 cm^{-1} due to carbonyl group. ^{1}H NMR (DMSO-d_6) ppm: 3.95 (s, 3H, OCH_3), 4.58 (s, 2H, NH_2, D_2O exchangeable), 7.01-7.29 (m, 3H, Ar-H), 7.49 (s, 1H, C3-H), 10.01 (s, 1H, NH, D_2O exchangeable). GCMS *m/z:* 206.

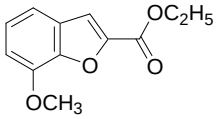
General procedure for the preparation of 3-(7-methoxybenzofuran-2-yl)-5-aryl-4H-[1,2,4]triazoles 3 (a-k).
To a solution of 7-methoxy-benzofuran-2-carboxylic acid hydrazide **(2)** (0.01mol) in acetic acid (20 mL) was added a pinch of ammonium acetate followed by the addition of substituted benzaldehyde (0.01 mol) and the mixture was stirred for 24 h at room temperature. The reaction mixture was then neutralized with ammonia solution and the solid **(3)** separated was filtered, washed with water and crystallized from suitable solvent.

Spectral data of representative compound 3-(7-methoxybenzofuran-2-yl)-5-(4-methoxy-phenyl)-4H-[1,2,4]triazole **3f** is as follows. IR(KBr) : 3221(NH); ^{1}H NMR ($CDCl_3$) ppm: 3.85 (s, 3H, OCH_3), 4.03 (s, 3H, OCH_3), 6.92-7.77 (m, 7H, Ar-H), 8.24 (s, 1H,C3-H), 9.65 (s, 1H, NH, D_2O exchangeable). GCMS *m/z:* 321.

Scheme-1

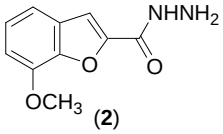


(i)



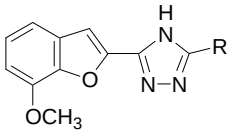
(**1**)

(ii)



(**2**)

(iii)



3 (a-k)

(i) $CH_3CH_2COCH_3$/ anhydrous K_2CO_3

(ii) $N_2H_4.H_2O$ / EtOH, Reflux

(iii) R-CHO / AcOH / CH_3COONH_4

3a, R = C_6H_5
3b, R = C_6H_4Cl (p)
3c, R = C_6H_4Br (p)
3d, R = $C_6H_4NO_2$ (p)
3e, R = C_6H_4OH (p)
3f, R = $C_6H_4OCH_3$ (p)
3g, R = C_6H_4Cl (m)
3h, R = C_6H_4Br (m)
3i, R = $C_6H_4NO_2$ (m)
3j, R = C_6H_4OH (m)
3k, R = $C_6H_4OCH_3$ (m)

The analytical data of the remaining compounds are summarized in table 1.

Table 1. Analytical data of synthesized compounds 1, 2 and 3(a-k)

Compound	R	Solvent for crystallization	Melting point (°C)	% Yield	Molecular formula	% Elemental analysis Found (cacld.)		
						C	H	N
1	-	Ethanol	85	80	$C_{12}H_{12}O_4$	65.42 (65.45)	5.46 (5.49)	-
2	-	Ethanol	180	85	$C_{10}H_{10}N_2O_3$	58.30 (58.25)	4.90 (4.89)	13.60 (13.59)
3a	C_6H_5	Ethanol	200	70	$C_{17}H_{13}N_3O_2$	70.55 (70.50)	4.51 (4.50)	14.48 (14.42)
3b	C_6H_4Cl(p)	aq. Dioxane	210	65	$C_{17}H_{12}ClN_3O_2$	62.70 (62.68)	3.72 (3.71)	12.93 (12.90)
3c	C_6H_4Br(p)	aq. Dioxane	195	70	$C_{17}H_{12}BrN_3O_2$	55.16 (55.15)	3.25 (3.27)	11.38 (11.35)
3d	$C_6H_4NO_2$(p)	Dioxane	205	65	$C_{17}H_{12}N_4O_4$	60.70 (60.71)	3.61 (3.60)	16.68 (16.66)
3e	C_6H_4OH(p)	Dioxane	186	75	$C_{17}H_{13}N_3O_3$	66.45 (66.44)	4.25 (4.26)	13.68 (13.67)
3f	$C_6H_4OCH_3$(p)	Dioxane	211	80	$C_{18}H_{15}N_3O_3$	67.30 (67.28)	4.70 (4.71)	13.10 (13.08)
3g	C_6H_4Cl(m)	Dioxane	215	70	$C_{17}H_{12}ClN_3O_2$	62.70 (62.68)	3.70 (3.71)	12.91 (12.90)
3h	C_6H_4Br(m)	Dioxane	180	68	$C_{17}H_{12}BrN_3O_2$	55.16 (55.15)	3.25 (3.27)	11.37 (11.35)
3i	$C_6H_4NO_2$(m)	Dioxane	203	78	$C_{17}H_{12}N_4O_4$	60.70 (60.71)	3.61 (3.60)	16.68 (16.66)
3j	C_6H_4OH(m)	Dioxane	209	65	$C_{17}H_{13}N_3O_3$	66.45 (66.44)	4.28 (4.26)	13.65 (13.67)
3k	$C_6H_4OCH_3$(m)	Dioxane	220	75	$C_{18}H_{15}N_3O_3$	67.26 (67.28)	4.72 (4.71)	13.09 (13.08)

(i) Antibacterial screening

Standard strains were procured from the microbial type culture collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. The anti-bacterial activity of the synthesized compounds **1, 2** and **3 (a-k)** were performed *in vitro* against (i) Gram-positive bacteria: *Streptococcus faecalis* (MTCC 3382), *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 297) and (ii) Gram-negative bacteria: *Pseudomonus aeruginosa* (MTCC 1034), *Klebsiella pneumoniae* (MTCC 3384) and *Escherichia coli* (MTCC 1089) by broth micro dilution method [28].

Table 2. Results of Antibacterial activities of compounds 1, 2, 3 (a-k) MICs (µg/mL)

Compound	R	Gram positive			Gram negative		
		S.faecalis	***S.aureus***	***B. subtilis***	***P.aeruginosa***	***K.pneumoniae***	***E.coli***
1	-	250	250	125	250	125	125
2	-	250	125	250	125	250	125
3a	C_6H_5	125	62.5	62.5	125	125	125
3b	C_6H_4Cl(*p*)	62.5	62.5	31.25	62.5	62.5	62.5
3c	C_6H_4Br(*p*)	31.25	31.25	62.5	31.25	31.25	31.25
3d	$C_6H_4NO_2$(*p*)	1	1	1	2	2	2
3e	C_6H_4OH(*p*)	16	16	8	8	16	8
3f	$C_6H_4OCH_3$(*p*)	8	8	16	16	8	16
3g	C_6H_4Cl(*m*)	125	125	62.5	125	125	125
3h	C_6H_4Br(*m*)	62.5	62.5	125	62.5	62.5	31.25
3i	$C_6H_4NO_2$(*m*)	2	2	2	4	4	4
3j	C_6H_4OH(*m*)	31.25	31.25	16	16	31.25	16
3k	$C_6H_4OCH_3$(*m*)	16	16	31.25	31.25	16	31.25
Ciprofloxacin	-	1	1	1	1	1	1

The MIC determination of the tested compounds was investigated in comparison with Ciprofloxacin. Double dilutions of the test compounds and reference drugs were prepared in Muller-Hinton agar. 10mg of each test compounds were dissolved in 1 ml of dimethylsulfoxide (DMSO) separately to prepare stock solution. Further,

progressive dilutions with melted Mueller-Hinton agar were performed to obtain the required concentrations of 500, 250, 125, 62.5, 31.25, 16, 8, 4, 2, 1 µg/ml. The Petri dishes were inoculated with 1-5 x 10^4 colony forming units (cfu/ml) and incubated at 37 ^oC for 18 hours. The minimum inhibitory concentration (MIC) was the lowest concentrations of the tested compound that yield no visible growth on the plate were recorded in Table 2. To ensure that the solvent had no effect on the bacterial growth, a control was performed with the test medium supplemented with DMSO at the same dilutions as used in the experiments.

(ii) Antifungal screening

The anti-fungal activity of the synthesized compounds **1, 2** and **3 (a-k)** were performed against the following standard fungal strains: *Candida albicans, Aspergillus fumigatus, Aspergillus niger, Penicillium chrysogenum, Mucor fuscus* and *Fusarium oxysporum* in DMSO by broth micro dilution method [29].

The MIC determination of the tested compounds was investigated in comparison with *Fluconazole* by broth micro dilution method. Double dilutions of the test compounds and reference drugs were prepared in Sabouraud's dextrose broth. 10 mg of each test compounds were dissolved in 1 ml of dimethylsulfoxide (DMSO) separately to prepare stock solution. Further progressive dilutions with Sabouraud's dextrose broth were performed to obtain the required concentrations of 500, 250, 125, 62.5, 31.25, 16, 8, 4, 2, 1 µg/ml. The petridishes were inoculated with 1-5 x 10^4 colony forming units (cfu / ml) and incubated at 25 ^oC for 48-72 hours. The minimum inhibitory concentration (MIC) was the lowest concentration of the tested compound that yield no visible growth on the plates. To ensure that the solvent had no effect on the fungal growth, a control was performed with the test medium supplemented with DMSO at the same dilutions as used in the experiments. The results are incorporated in Table 3.

Table 3. Results of Antifungal activities of compounds 1, 2 and **3 (a-k) MICs (µg/mL)**

Compound	R	*C. albicans*	*A. fumigatus*	*A. niger*	*P.chrysogenum*	*M. fuscus*	*F. oxysporum*
1	-	125	62.5	62.5	125	62.5	125
2	-	62.5	125	62.5	125	125	62.5
3a	C_6H_5	31.25	62.5	31.25	62.5	62.5	31.25
3b	$C_6H_4Cl(p)$	4	8	4	8	8	16
3c	$C_6H_4Br(p)$	8	4	8	4	4	8
3d	$C_6H_4NO_2(p)$	4	2	4	2	2	4
3e	$C_6H_4OH(p)$	4	8	4	8	16	4
3f	$C_6H_4OCH_3(p)$	8	4	8	8	4	4
3g	$C_6H_4Cl(m)$	8	16	8	16	16	16
3h	$C_6H_4Br(m)$	16	8	16	8	8	16
3i	$C_6H_4NO_2(m)$	8	4	8	4	4	8
3j	$C_6H_4OH(m)$	8	16	8	16	8	16
3k	$C_6H_4OCH_3(m)$	16	8	16	16	8	16
Fluconazole	-	8	8	8	8	8	8

(iii) DNA cleavage experiment

Preparation of culture media

DNA cleavage experiments were carried out according to the literature [30, 31]. Nutrient broth (peptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 10 g L^{-1}) was used for the culturing of *E. coli*. The 50mL medium was prepared and autoclaved for 15 min at 121 ^oC under 15-lb pressure. The autoclaved medium was inoculated with the seed culture. The *E. coli* was incubated for 24 h.

Isolation of DNA

The fresh bacterial culture (1.5 mL) was centrifuged to obtain the pellet, which was then dissolved in 0.5 mL of lysis buffer (100 mM Tris pH 8.0, 50 mM EDTA, 10 % sodium dodecyl sulphate (SDS)). To this, 0.5 mL of saturated phenol was added and incubated at 55 ^oC for 10 min. It was then centrifuged at 10,000 rpm for 10 min, and to the supernatant, an equal volume of chloroform: iso-amyl alcohol (24:1) and 1/20th volume of 3 M sodium acetate (pH 4.8) were added. Then, this solution was centrifuged at 10,000 rpm for 10 min and to the supernatant, 3 volumes of chilled absolute alcohol was added. The precipitated DNA was separated by centrifugation and the pellet was dried and dissolved in a TAE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and stored in cold conditions.

Agarose gel electrophoresis

Cleavage products were analyzed by the agarose gel electrophoresis method [30, 31]. Test samples (1 mg mL^{-1}) were prepared in DMF. The samples (25 µg) were added to the isolated DNA of *E. coli*. The samples were incubated

for 2 h at 37 ^{o}C. Then 20 µL of DNA sample (mixed with bromophenol blue dye at a 1:1 ratio) was loaded carefully into the electrophoresis chamber wells along with a standard DNA marker containing TAE buffer (4.84 g Tris base, pH 8.0, 0.5 MEDTA per 1 L) and finally loaded on agarose gel and a constant electricity of 50 V was passed for around 30 min. The gel was removed and stained with 10.0 µg mL^{-1} ethidium bromides for 10–15 min and the bands observed under Vilberlourmate Gel documentation system and photographed to determine the extent of DNA cleavage. Then, the results were compared with that of a standard DNA marker.

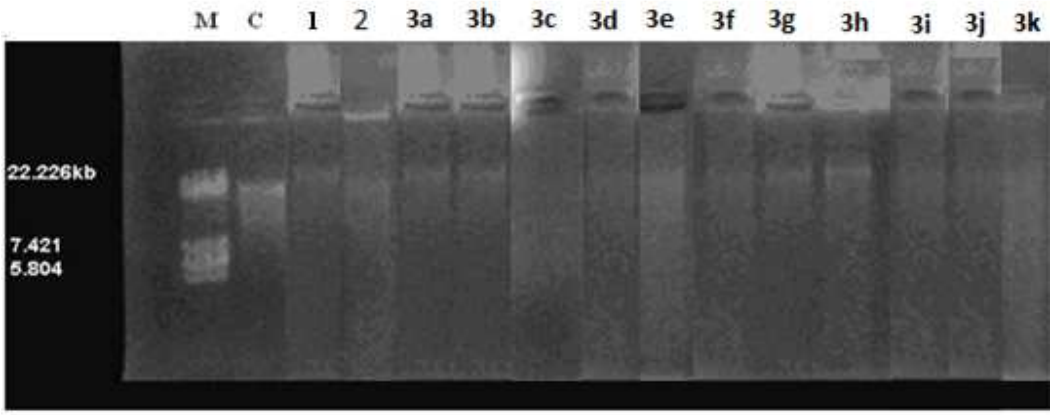


Figure 1. DNA cleavage on genomic DNA of *E. coli.*
***M**, standard molecular weight marker;*
***C**, control DNA of E. coli;*
*Lanes **1, 2** and **3 (a-k)** are E. coli DNA treated with compounds **1, 2** and **3(a-k)** respectively.*

RESULTS AND DISCUSSION

Antimicrobial studies

The antibacterial and antifungal data (Table 2 and 3) revealed that all synthesized compounds exhibited moderate to good activity. In the series, cyclised products i.e., title compounds **3 (a-k)** are more active than **1** and **2.** The compounds bearing nitro-group showed good activity. Among these, *p*-nitro compound (**3d**) is more active compared to *m*-nitro compound (**3i**).

DNA Cleavage study

The synthesized compounds **1, 2** and **3 (a-k)** were screened for their DNA cleavage activity by agarose gel electrophoresis method. Figure 1 shows the results of oxidative DNA cleavage experiments carried out against *E. coli*. Control experiments clearly revealed that, the untreated DNA does not show any cleavage (Lane-C), whereas all the compounds have exhibited cleavage activity on DNA. The difference in the migration was observed in the lane **1, 2 and 3(a-k)** for compounds **1, 2** and **3**(**a-k**) respectively, compared to the control DNA of *E. coli*. This shows that, the control DNA alone does not show any apparent cleavage whereas, **3d** and **3i** compounds shown. However, the nature of reactive intermediates involved in the DNA cleavage by the complexes has not been clear. The results indicated the important role of nitro group in isolated DNA cleavage reaction. From these results, we infer that the nitro compounds (**3d** and **3i**) act as a potent nuclease agents. As the compounds were observed to cleave the DNA, it can be concluded that, the compound inhibits the growth of the pathogenic organism by cleaving the genome. The gel containing *E. coli* DNA treated with compounds shows that after treatment, the intensity of all the treated DNA samples has diminished, possibly because of the cleavage of the DNA. The complete cleavage was observed with **3d** and **3i**.

CONCLUSION

A series of novel benzofuranyl-triazoles **3 (a-k)** have been synthesized in good yields and screened for their antibacterial, antifungal and DNA cleavage activities. In the series, the compounds bearing nitro-group **3d, 3i** showed good activity. Among these, *p*-nitro substituted compound **3d** exhibited the highest activity.

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