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Synthesis and *in-vitro* Cytotoxic Screening of Some Novel 3, 4-diphenyl-2, 5-diaza-bicyclo [4.3.1] Decane Derivatives

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Abstract: The bicyclo compounds possess a wide variety of activities so, in the present study we have develop a novel and simple method for the synthesis of such 3, 4-diphenyl-2, 5-diaza-bicyclo [4.3.1] decane derivatives. All the compounds synthesized were characterized by running TLC, IR, NMR and LCMS spectra. All the title compounds were screened for the determination of Cytotoxicity by MTT and SRB assay methods on Vero cell line (African green monkey kidney normal cell line). The compound D and compound C showed potent cytotoxicity as compared to compound E, compound A and compound B in both the assay performed.

Key words: Vero cell line · Amino acids · MTT assay and SRB assay

INTRODUCTION

Cancer is a major public health problem. Cancer statistics in India suggests that one in every 12 women and one in every 12 men upto 64 years of age are expected to get some form of cancer in their lifetimes and preventive remedies against many cancers have not been well developed. Current research is focused on some synthetic origin drugs for cancer research and chemotherapy of different malignancies because of their purity, specificity compared to the drugs of natural origin for the study of different cancers [1]. The analogues of bicyclic systems, 8, 10-diza-bicyclo [4.3.1] decane and 3, 10-diza-bicyclo [4.3.1] decane bicyclo compounds, have been found to possess a wide range of biological activities, which include analgesic-antiinflammatory [2-4], muscarinic receptor antagonist [5], antibacterial [6], antiviral [7], antiprotozoal [8] and antispasmolytic [9].

The reason to incorporate biologically friendly amino acids into pharmacologically active moiety is not only to minimize the side effects of the metabolites of the parent compound upon metabolism in the body but also to protect the vulnerable acidic moiety present in the nucleus and in order to enhance the hydrophilicity of the synthesized candidates, like bicyclo compounds may exhibit potent cytotoxic activity [10]. In the same context, our objective of the study was to synthesize such compounds by novel and convenient method and to further evaluate these synthesized candidates, like bicyclo compounds that may exhibit potent cytotoxic activity.

EXPERIMENTAL

Melting points (m.p.) were determined in open glass capillary tubes and are uncorrected. Infrared (IR) spectra were recorded on an FT-IR Bruker Tensor 27 spectrometer and are expressed in cm^{-1}. The NMR spectra of the compounds were recorded on Bruker DRX-300 spectrometer. The chemical shifts were reported as parts per million (δ ppm) using tetramethylsilane (TMS) as an internal standard. The LC mass spectra of the compounds were recorded on Shimadzu 8201PC spectrometer. The progress of the reaction was monitored on precoated silica gel 60 F254 plates (Merck) using different solvent systems. Spectral data (IR, NMR and LC mass spectra) confirmed the structures of the synthesized compounds. Synthesis and analytical studies of the title compounds were carried out using laboratory grade and analytical grade as the case may be.

General Procedure:

Synthesis of 2-(3, 5-dinitrobenzamido) acetic acid (X1): A solution of 3, 5-dinitrobenzoyl chloride (1 mole) in 1,

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4-dioxan was added to glycine (1.2mole) in 0.1 N sodium hydroxide (10 ml) and refluxed for 6 hrs. The reaction mixture was allowed to cool and poured into 1N hydrochloric acid and crushed ice. The crude product was filtered, dried, recrystallized with methanol and column chromatographed on silica gel (60-120 mesh) eluting with methanol: ethyl acetate (8:2). Mol. Formula: $C_9H_7N_3O_7$, Yield 67%, m.p. 182°C.

All the other compounds X2-X5 were prepared by the same procedure using the corresponding amino acid.

General Procedure:

Synthesis of 2-(3,5-diamino-benzamido) acetic acid (Y1): A suspension of 2-(3,5-dinitrobenzamido)acetic acid (1 mole) and zinc dust (2.5 mole) in methanol was stirred with 5 ml of 90% formic acid at room temperature for 5 hrs. After completion of the reaction (monitored by TLC), the reaction mixture was filtered off. The organic layer was evaporated and the residue was dissolved in ether and washed with saturated sodium chloride solution (5 times) to remove of ammonium formate. Then the etheral layer was evaporated to dryness [11]. The crude product was recrystallized with ethanol and purified by column chromatography on silica gel (60-120 mesh) eluting with chloroform: ethyl acetate (9:1). Mol. Formula: $C_9H_{11}N_3O_3$, Yield 65%, m.p. 173°C.

All the other compounds Y2-Y5 were prepared similarly.

General Procedure:

Synthesis of 2-[(3, 4-diphenyl-2, 5-diaza-bicyclo [4.3.1] deca-1(9), 2, 4, 6(10), 7-pentaene-8-carbonyl) -amino]-pentanedioic acid (A):- 2-(3, 5-Diamino-benzamido) acetic acid (1 mole) was dissolved in 0.1N sodium hydroxide solution. To this, a mixture of benzil (1.1mole) and sodium ethoxide (2.3 mole) in ethanol was dissolved with continuous stirring and refluxed for 55hrs. The reaction mixture was then allowed to cool and poured into 1N hydrochloric acid and crushed ice. The content was kept over night at room temperature, filtered, dried and recrystallized with methanol. The completion of the reaction was monitored by TLC and purified by column chromatography on silica gel (60-120 mesh) eluting with methanol: ethyl acetate (8:2). Mol. Formula: $C_{23}H_{17}N_3O_3$, Yield 48%, m.p. 83°C; IR (cm^{-1}): 3712(-OH), 3670 ($-NH_2$), 2858($-CH_2$), 1681(C=O), 2968(CH-Ar), 1530(C=N); ^{1}H NMR(DMSO): δ 2.5 (d, 2H, CH_2), 9.03 (t, 1H, NH), 8.86 (s, 1H, COOH), 7.60-7.93 (m, 13H, ArH); LCMS: m/z $[M+1]^+$384.4, $[M+2]^+$ 385.4.

2-[(3, 4-diphenyl-2, 5-diaza-bicyclo[4.3.1]deca-1(9), 2, 4, 6(10),7-pentaene-8-carbonyl)-amino]-pentanedioic Acid (B): Mol. Formula: $C_{26}H_{21}N_3O_5$, Yield 43%, m.p. 68°C; IR (cm^{-1}): 3737(-OH), 3528($-NH_2$), 2888($-CH_2$), 1675, 1699(C=O), 3083(CH-Ar), 1588(C=N); ^{1}H NMR(DMSO): δ 1.21 (s, 1H, CH), 8.66 (s, 1H, COOH), 2.5 (m, 4H, $(CH_2)_2$), 8.88 (t, 1H, NH), 9.10 (s, 1H, COOH), 7.53-7.93 (m, 13H, ArH); LCMS: m/z $[M+1]^+$ 456.

1-(3, 4-diphenyl-2, 5-diaza-bicyclo[4.3.1]deca-1(9), 2, 4, 6(10), 7-pentaene-8-carbonyl)-pyrrolidine-2-carboxylic Acid (C): Mol. Formula: $C_{26}H_{21}N_3O_3$, Yield 47%, m.p. 55°C; IR (cm^{-1}): 3602(-OH), 2798($-CH_2$), 1707, 1546(C=O), 3098(CH-Ar), 1493(C=N), 1336(tert.N); ^{1}H NMR(DMSO): δ 1.20 (t, 1H, CH), 3.75 (m, 4H, $(CH_2)_2$ pyrrolidine ring), 2.5 (m, 2H, CH_2), 9.0 (s, 1H, COOH), 7.23-8.02 (m, 13H, ArH); LCMS: m/z $[M+1]^+$424.4.

2-[(3, 4-diphenyl-2, 5-diaza-bicyclo[4.3.1]deca-1(9), 2, 4, 6(10), 7-pentaene-8-carbonyl)-amino]-3-mercapto Propionic Acid (D): Mol. Formula: $C_{24}H_{19}N_3O_3S$, Yield 50%, m.p. 73°C; IR (cm^{-1}): 3506(-OH), 3399($-NH_2$), 2877($-CH_2$), 1711, 1626(C=O), 3084(CH-Ar), 1532(C=N), 2637(-SH); ^{1}H NMR(DMSO): δ 1.20 (s, 1H, CH), 8.65 (s, 1H, COOH), 2.5 (d, 2H, CH_2), 9.09 (t, 1H, NH), 8.87 (s, 1H, SH), 7.27-7.93 (m, 13H, ArH); LCMS: m/z $[M-1]^+$428.3.

3, 4-diphenyl-2, 5-diaza-bicyclo[4.3.1]deca-1(9), 2, 4, 6(10),7-pentaene-8-carboxylicAcid(5-amino-6-hydroxy-6-oxo-hexyl)-amide (E): Mol. Formula: $C_{27}H_{26}N_4O_3$, Yield 42%, m.p. 77°C; IR (cm^{-1}): 3726 (-OH), 3647($-NH_2$), 2864($-CH_2$), 1678(C=O), 2924(CH-Ar), 1569(C=N); ^{1}H NMR(DMSO): δ 1.21 (t, 1H, CH), 8.66 (s, 1H, COOH), 2.5 (m, 2H, CH_2), 9.09 (t, 1H, NH), 8.88 (d, 2H, NH_2), 3.58 (m, 6H, CH_2) 7.60-7.93 (m, 13H, ArH); LCMS: m/z $[M+1]^+$455.4, $[M-1]^+$452.8.

***In-vitro* Cytotoxicity Screening:**

Chemicals: 3-(4,5-Dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide(MTT) and sulphorhodamine B (SRB) were obtained from Sigma Chemical Co., Mo, U.S.A., RPMI-1640 and antibiotics were obtained from Hi-media Ltd., Mumbai. Trichloro acetic acid (TCA) and tris buffer were obtained from SD-Fine Chemicals Pvt. Ltd., Boisar. Dimethyl sulphoxide (DMSO), glacial acetic acid and propanol were obtained from E. Merck Ltd., Mumbai.

Cell Line and Culture Medium: Vero (Normal, African green monkey kidney) cell culture was obtained from Pasteur Institute of India, Coonoor, India and was cultured in RPMI-1640 and 10% heat activated New born calf serum with antibiotics (1000 I.U./ml Penicillin, 100 µg/ml Streptomycin and 25 µg/ml Amphotericin B). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured twice a week.

Determination of Mitochondrial Synthesis by Microculture Tetrazolium (MTT) Assay: The monolayer cell culture was trypsinized and the cell count was adjusted to 3.0 x 10^5 cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added and kept for 24 hrs in incubator at 37°C in 5% CO_2 atmosphere for cell monolayer formation. After 24 hrs, when a partial monolayer was formed at the bottom of the well, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations i.e. title compounds (A-E) were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and observations recorded every 24 hrs. After 72 hrs, the sample solution in the wells was flicked off and 50 ml of MTT dye was added to each well, plates were gently shaken and incubated for 4hrs at 37°C in 5% CO_2 incubator. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 490 nm.

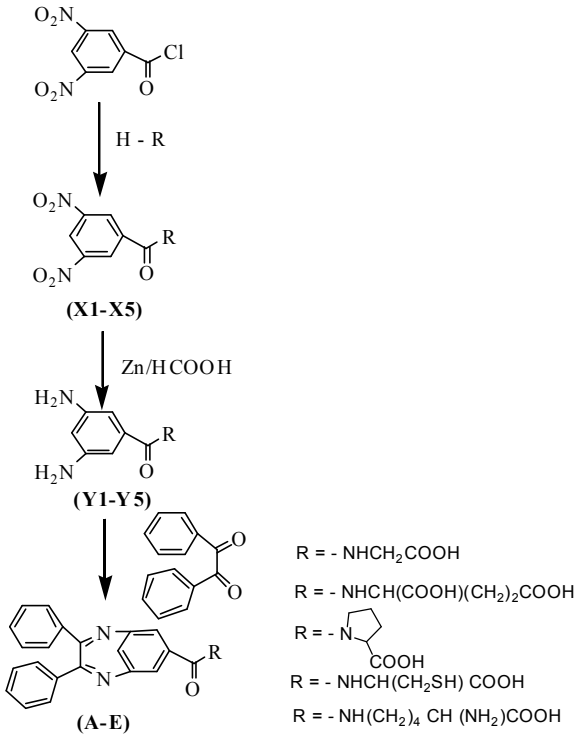
Determination of Total Cell Protein Content by Sulphorhodamine B (SRB) Assay: The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 10^5 cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added and kept for 24hrs in incubator at 37°C in 5% CO_2 atmosphere for cell monolayer formation. After 24 hrs, when a partial monolayer was formed at the bottom of the well, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations i.e. title compounds (A-E) were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and observations recorded every 24 hrs. After 72 hrs, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the drug dilutions to form a over all concentration of 10%. The plates were incubated at 4°C for one hour. After that, plates were flicked and washed five times with tap water to remove traces of medium, drug and serum and were then air-dried. The air dried plates were stained with 100ml of SRB and kept for 30 minutes at room temperature. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100µl of 10mM tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5minutes. The absorbance was measured using microplate reader at a wavelength of 540 nm [12-13]. The percentage growth inhibition was calculated using the formula below:

% Growth Inhibition = [1-Mean O.D. of Individual Test Group / Mean O.D. of Individual Control Group] x 100

RESULTS AND DISCUSSION

All the title compounds were synthesized as per scheme-1 and were characterized by running TLC, determining m.p., IR, NMR and LCMS spectra and were

Scheme 1:



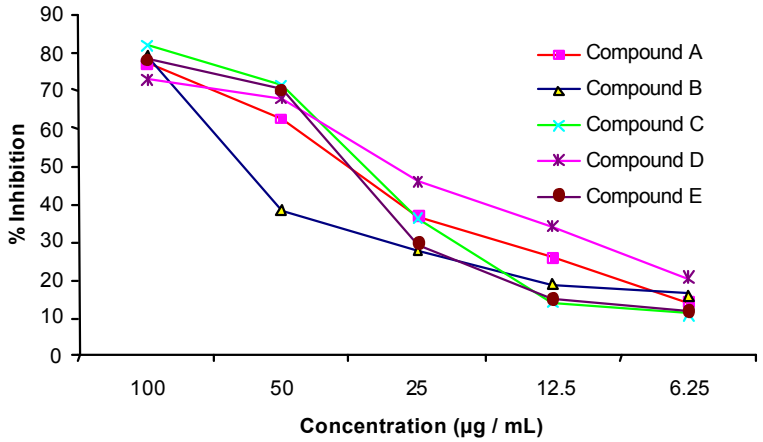


Fig. 1: Determination of Cytotoxicity by MTT Assay in Vero Cell Line

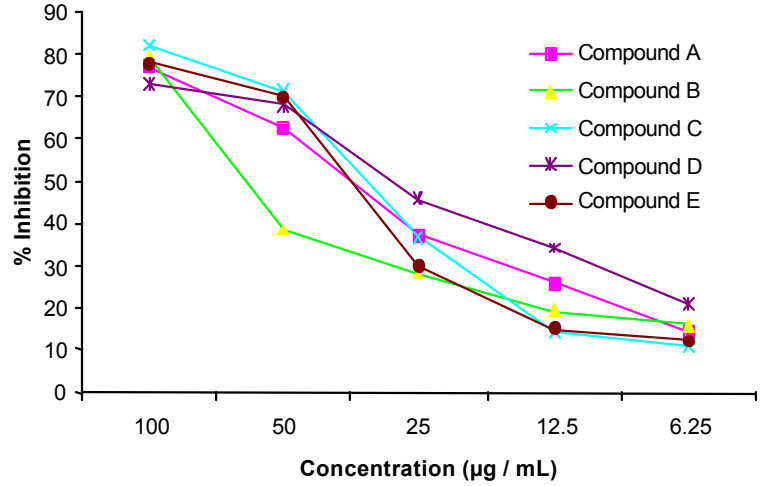


Fig. 2: Determination of Cytotoxicity by SRB Assay in Vero Cell Line

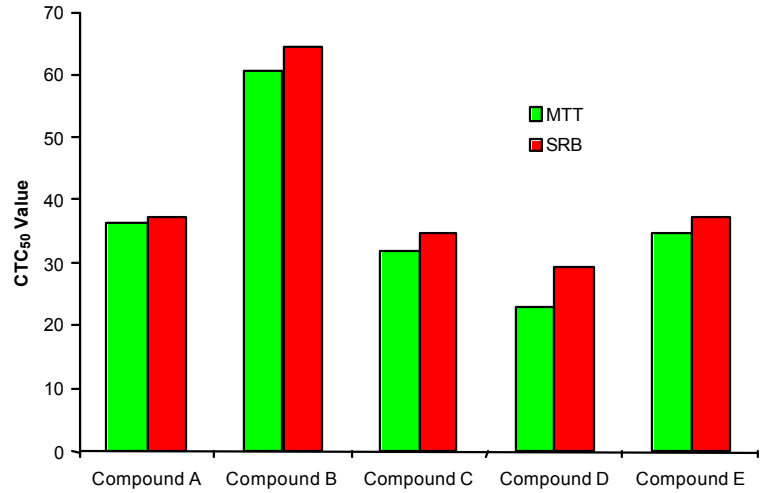


Fig. 3: Comparison of Average of CTC_{50} Value of MTT and SRB Assays

Table 1: Determination of Cytotoxicity by MTT Assay in Vero Cell Line

Compound No.	Concentration (µg / ml)	Absorbance	% Inhibition	CTC_{50} (µg / ml)
Compound A	100	0.101	79.34	36.50
	50	0.164	66.42	
	25	0.319	34.72	
	12.5	0.373	23.72	
	6.25	0.4	18.20	
Compound B	100	0.104	78.73	60.50
	50	0.287	41.30	
	25	0.362	25.97	
	12.5	0.425	13.09	
	6.25	0.446	8.79	
Compound C	100	0.055	88.75	32.00
	50	0.137	71.98	
	25	0.287	41.31	
	12.5	0.415	15.13	
	6.25	0.443	9.40	
Compound D	100	0.067	86.29	23.00
	50	0.134	72.59	
	25	0.237	51.53	
	12.5	0.284	41.92	
	6.25	0.343	29.85	
Compound E	100	0.091	81.39	35.00
	50	0.161	67.07	
	25	0.298	39.06	
	12.5	0.405	17.17	
	6.25	0.423	13.49	
Cell Control	--	0.489	--	--

Table 2: Determination of Cytotoxicity by SRB Assay in Vero Cell Line

Compound No.	Concentration (µg / ml)	Absorbance	% Inhibition	CTC_{50} (µg / ml)
Compound A	100	0.513	76.98	37.50
	50	0.835	62.54	
	25	1.404	37.01	
	12.5	1.651	25.93	
	6.25	1.917	13.99	
Compound B	100	0.468	79.00	64.25
	50	1.371	38.49	
	25	1.605	27.99	
	12.5	1.806	18.97	
	6.25	1.873	15.97	
Compound C	100	0.403	81.92	34.75
	50	0.637	71.42	
	25	1.415	36.51	
	12.5	1.917	13.99	
	6.25	1.984	10.99	
Compound D	100	0.602	72.99	29.50
	50	0.714	67.96	
	25	1.203	46.03	
	12.5	1.471	34.00	
	6.25	1.761	20.99	
Compound E	100	0.492	77.92	37.50
	50	0.671	69.89	
	25	1.563	29.87	
	12.5	1.897	14.89	
	6.25	1.961	12.02	
Cell Control	--	2.229	--	--

tested for cytotoxic activity against a normal vero cell culture at different concentrations by determining CTC_{50} (cytotoxic concentration at which 50% of the cells are dead after 72hrs of drug exposure) with the help of MTT and SRB assay. The results obtained from MTT and SRB assay shows that the percentage growth inhibition was found to be increasing with increasing concentration of the test compounds. The cytotoxic range was 23 to 60.5 µg/ml MTT assay and 29.5 to 64.25 µg/ml in SRB assay respectively.

The results are tabulated in Table 1-2 & Figure 1-3. Thus from the data it can be concluded that all test compounds are potent cytotoxic agents because of higher CTC_{50} a lower concentrations and moreover, the compound D and compound C were found to be most potent agent among all the compounds tested.

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