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Synthesis, Anti-Tumor, Anti-Diabetic, and Anti- Asthmatic Activitives of Some Novel Benzimidazole Derivatives

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ABSTRACT

A series of novel substituted benzimidazole derivatives by the condensation of different diamines with anthranilicacid were synthesized. The subsequent reactions of the benzimidazole derivatives were reacted with different aromatic acid chlorides to get tetrazole moieties. These compounds were screened for their potential anti-cancer, anti-diabetic, anti-tumor and anti- asthmatic properties, which exhibited some authentic results towards testing organism invitro and invivo studies.

Key words: anthranilic acid, benzimidazole derivatives, benzoyl chloride, anti-cancer, anti-diabetic and anti-asthmatic

INTRODUCTION

Life threatening infections caused by pathogenic fungi are becoming increasingly common, especially in individuals with suppressed immune systems such as cancer chemotheraphy and AIDS patients. The compounds bearing benzimidazole moiety are reported to possess a number of interesting biological activities and the widespread importance of benzimidazole structure has extensive studies for practical synthetic method of heterocycles[1-5]. Benzimidazole derivatives have found the appreciation in diverse therapeutic areas including antimicrobial activity[6-10], the activity against several viruses such as HIV[11-13], antiallergic[14,15], antioxidant[16-18], antihistaminic[19], antitubercular[20,21], antiasthmatic[22], anti-diabetic[23,23a], anticancer[24-28], antitumor[29-30], antiulcer[31,32], antihelmentic[33], HIV-1 reverse transcriptase inhibitors[34,34a], antiviral[35], anticoagulant[36], anti inflammatory[37], antibacterial[38,39], the series of biologically active benzimidazoles[40].

Owing to the immense biological importance of benzimidazole derivatives, on the basis of these reports and as a continuation of our research program on benzimidazole derivatives.

We report were the synthesis of novel benimidazole derivatives to evaluate their anticancer, anti-diabetic, anti tumor and anti asthmatic properties. In addition benzimidazole is very important intermediates in organic synthesis.

EXPERIMENTAL SECTION

General procedures: Melting points are uncorrected and were recorded on a REMI series, lab India instrument. TLC analysis was done using pre-coated silica gel plates and visualization was done using iodine. IR spectra were recorded in KBr on schimadzu FT-IR Spectrometer. 1H & 13C-NMR spectra were recorded on a Bruker (AC 400MHz) using TMS as an internal standard. Elemental analysis was carried out on a Perkin-Elmer series –II CHNS/O Analyzer 2400. All the chemicals were obtained from Aldrich; all the solvents used were of commercial grade only.

Synthesis of 4(1H-benzo[d]imidazol-2yl) aniline (1a, 2b,)
A mixture of p-Phenylendiamine / m- Phenylendiamine (0.1mol) and 2-aminobenzoic acid (0.1 mol) was heated on a water bath for 2 1/2 hours. It was cooled and add 10% NaOH was added slowly with constant stirring until just alkaline. The crude product was filtered, washed with cold ice water, decolorized and washed repeatedly and dried well. The product was recrystalized from ethanol.

Compound- 1a (Found C, 74.6; H, 5.26; N, 20.0 $C_{13}H_{11}N_3$) IR(KBr): 3420 (N-H stretching for 1^0 amine), 3300 (N-H stretching for 2^0 amine), 3012 (aromatic C-H stretching), 1620 (C=N stretching), 1379 (C-N stretching). MASS ES: 210 (M+)
Compound- 2b (Found C, 74.5; H, 5.20; N, 20.01 $C_{13}H_{11}N_3$) IR (KBr): 3400 (N-H stretching for 1^0 amine), 3280 (N-H stretching for 2^0 amine), 3030 (aromatic C-H stretching), 1634 (C=N stretching), 1325 (C-N stretching). MASS ES: 210 (M+)

Synthesis of 3-(1H methyl-1H-benzo[d]imidazol-2yl) aniline (3c)
To a mixture of compound (1a) (2mmole) in dimethylformide (10 mL) was added sodium hydride (55%, 2 mmole) lot wise at 0 ºC. After completion of addition the temperature for 1.5 h. The reaction mixture was again cooled to 0 ºC and the respective alkyl halid(2.4 mmole) was added at 0 ºC . The temperature of the reaction mixture was then allowed to warm to room temperature and stirred for 3h. After completion of the reaction, water (50 mL) was slowly added to reaction mixture and extracted with ethyl acetate (2×25 mL). The organic layer was washed with water (2×25 mL), brine and dried over anhydrous magnesium sulfate and concentrated under vacuum to yield the corresponding N-substituted different derivatives. The crude compounds were recrystallized from hot aq. Ethanol to obtained pure products.
Compound- 3c (Found C, 75.3; H, 5.8; N, 18.8 $C_{14}H_{13}N_3$) IR (KBr): 3379 (N-H stretching for 1^0 amine), 3272 (N-H stretching for 2^0 amine), 3047 (aromatic C-H stretching), 1640 (C=N stretching),1390 (CH_3), 1329 (C-N stretching). MASS ES: 223 (M+)

Synthesis of N-(4-(1Hmethyl-1H-benzo[d]imidazol-2yl) phenyl) benzamide (1ai, 2bi, 3ci)

A mixture of compound (0.001 moles) of (1a) and equivalent amount of benzoyl chloride (0.001 moles) was refluxed with pyridine (40 ml) for 8hours. The reaction mixture was cooled, treated with cold ice and neutralized with conc. HCl. The separating solid was filtered and washed with ice cold water. The product was recrystalized from ethanol.

Compound- 1ai (Found C, 76.6 ; H,4.7 ; N, 13.4 ; O, 5.0 $C_{21}H_{18}N_3O$) IR (KBr): 3291 (N-H stretching), 3068 (aromatic C-H stretching), 16564 (C=O stretching), 1604 (C=N stretching), 1320 (C-N stretching), ^{1}H NMR: δ 7.2-7.8 (13H, m, Ar-H), 8.1 (1H, s, CO-NH), 11.4 (1H, s, imidazole ring NH), ^{13}C NMR: δ 115-128 (18C, Ar-C), 169 (1C, C=O), 150 (1C, C=N). MASS ES: 314 (M+)

Compound - 2bi (Found C, 76.5 ; H,4.6 ; N, 13.4 ; O, 5.0 $C_{21}H_{18}N_3O$) IR (KBr): 3270 (N-H stretching), 3060 (aromatic C-H stretching), 1650 (C=O stretching), 1609 (C=N stretching), 1312 (C-N stretching), ^{1}H NMR: δ 7.26-7.75 (13H, m, Ar-H), 8.2 (1H, s, CO-NH), 11.7 (1H, s, imidazole ring NH), ^{13}C NMR: δ 114-129 (18C, Ar-C), 169 (1C, C=O), 150 (1C, C=N). MASS ES: 314(M+)

Compound – 3ci (Found C, 77 ; H,5 ; N, 12.8 ; O, 4.8 $C_{21}H_{17}N_3O$) IR (KBr): 3281 (N-H stretching), 3078 (aromatic C-H stretching), 1644 (C=O stretching), 1630 (C=N stretching), 1352 (C-N stretching), ^{1}H NMR: δ 7.22-7.79 (13H, m, Ar-H), 8.1 (1H, s, CO-NH), 11.9 (1H, s, imidazole ring NH), ^{13}C NMR: δ 115-128 (18C, Ar-C), 168 (1C, C=O), 152 (1C, C=N). MASS ES: 327 (M+)

Table 1 - Physical and Analytical Data of Compounds

Compounds	**position**	**Time in Hours**	**m.p (ºC)**	**Yield %**	**Molecular Formula**	**Analysis % calcd.(Found)**			
						C	**H**	**N**	**O**
1a	Para	2.5	108	70	$C_{13}H_{11}N_3$	74.6	5.26	20.0	-
2b	Meta	3.5	110	74	$C_{13}H_{11}N_3$	74.5	5.20	20.01	-
3c	Methyl	6	119-121	78	$C_{14}H_{13}N_3$	75.3	5.8	18.8	-
1-ai	Para	8	190	60	$C_{20}H_{15}ON$	76.6	4.7	13.4	5
2-bi	Meta	9	194	61	$C_{20}H_{15}ON$	76.5	4.6	13.4	5
3-ci	Methyl	10	180	65	$C_{21}H_{17}N_3O$	77	5	12.8	4.8
1-at	Para	20	241	50	$C_{20}H_{14}N_6$	71.0	4.1	24.8	-
2-bt	Meta	21	245	52	$C_{20}H_{14}N_6$	71.3	4.5	24.6	-
3-ct	Methyl	22	248	51	$C_{21}H_{16}N_6$	71.5	4.5	23.86	-

Synthesis of 1-methyl-2-(4-(5-phenyl-1H-tetrazol-1-yl)phenyl)-1H-benzo[d]imidazole (1at, 2bt, 3ct)

Compound - 1at (Found C, 71 ; H, 4.1; N, 24.8; $C_{20}H_{14}N_6$) IR (KBr): 3297 (N-H stretching), 3050 (aromatic C-H stretching), 1665 (C=N stretching), 1030 (tetrazole), ^{1}H NMR: δ 7.0-7.94

(13H, m, Ar-H), 11.8 (1H, s, imidazole ring NH), ^{13}C NMR: δ 114-132 (18C, Ar-C), 160 (1C, C=N in tetrazole ring), 151 (1C, C=N). MASS ES: 338 (M+)
Compound - 2bt (Found C, 71.3 ; H, 4.5; N, 24.6; $C_{20}H_{14}N_6$) IR (KBr): 3289 (N-H stretching), 3060 (aromatic C-H stretching), 1660 (C=N stretching), 1020 (tetrazoles), ^{1}H NMR: δ 7.0-7.94 (13H, m, Ar-H), 11.8 (1H, s, imidazole ring NH), ^{13}C NMR: δ 114-131 (18C, Ar-C), 159 (1C, C=N in tetrazole ring), 150 (1C, C=N). MASS ES: 338 (M+)
Compound - 3ct (Found C, 71.5 ; H, 4.5; N, 23.8; $C_{21}H_{16}N_6$) IR (KBr): 3294 (N-H stretching), 3088 (aromatic C-H stretching), 1669 (C=N stretching), 1027 (tetrazole), ^{1}H NMR: δ 7.0-7.94 (13H, m, Ar-H), 11.8 (1H, s, imidazole ring NH), ^{13}C NMR: δ 114-131 (18C, Ar-C), 159 (1C, C=N in tetrazole ring), 150 (1C, C=N). MASS ES: 353 (M+)

BIOLOGICAL ACTIVITY

Antimicrobial Screening

The antimicrobial activity for the given samples was carried out by Disc Diffusion Technique (Indian Pharmacopoeia 1996, Vol II A-105). The test micro organisms of Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli* and fungus *Candida albicans*, *Aspergillus Niger* were obtained from National Chemical Laboratory (NCL) Pune and maintained by periodical sub culturing on Nutrient agar and Sabourad dextrose medium both bacteria and fungus respectively. The effect produced by the sample was compared with the effect produced by the positive control (Reference standard Ciprofloxacin 5µg/discfor bacteria and Fluconazole 10 µg/disc for fungi). The result indicated that compounds were more active against all four organisms with reference to standard.

The results are shown in the Table 2

Table 2 – Antimicrobial screening results of the compounds

S.No.	Micro Organisms	Diameter zone of inhibition in mm							
		2b	1ai	1bi	1ci	1at	1bt	1ct	Std
1.	*Staphylococcus Aureus* (NCIM2O79)	23	02	39	20	32	35	30	40
2.	*Escherichia Coli* (NCIM 2O65)	33	27	25	34	30	38	39	40
3.	*Candida Albicans* (NCIM 3102)	20	17	20	19	16	18	19	20
4.	*Aspergillus Niger* (NCIM 105)	10	05	15	18	19	01	17	20

In vitro anti tumor screening[42]

Daltons Lymphoma Ascites (DLA) cell were collected, counted and adjusted to 1×10^6 cells/m L. the drug dilutions were made with phosphate buffer saline and the drug dilutions were further adjusted to required concentrations. The drug dilutions were then added to the DLA cell and incubated at 37 °C for 3 h. At the end of 3 h, tryphan blue dye exclusion test was performed and percentage viability was calculated.

Dalton's Lymphoma Ascites Tumor Model

The anti tumor activity of the test compounds was determined by an ascites cells were propagated in Swiss albino mice by Kuttan *et al*[45,46]. Dalton's Lymphoma Ascites cells were propagated in Swiss albino mice by injecting 1×10^6 cells intraperitoneally. The cells were aspirated aseptically from the developed tumor during the log phase of the 11^{th} day of tumor transplantation by with drawing the fluid from intraperitoneal cavity.

The ascetic fluid was washed 3 times with phosphate buffer saline by centrifugation at 300-400 rpm. The supernatant liquid was discarded and cells were diluted with normal saline and the tumor cell count was done using tryphan blue dye exclusion method using a haemocytometer. The cell suspension was diluted to get 1×10^6 cells in 0.1 m L of phosphate buffer saline. The tumor cells were injected in to the peritoneal cavity of all the animals and treatment was started 24 h after the tumor inoculation (once daily) for 10 d as described below.

The mice were divided into VI groups With 5 animals in each group as follows: Group-I: Solvent control and received 0.3 % CMC suspension. Group-II: Positive control and treated with cyclophophamide[43] (27.3 mg/kg body wt.). Group-III-VI: Test groups and were treated with test compounds as a single dose 100 mg/kg body weight by oral route, once daily for 10 d.

During the course of anticancer study, the animals were subjected to the following screening methods: Determination of body weight analysis[44], mean survival time (MST) reported on Table-3.

Table 3 - Effect of Test Compounds on Mean Survival Time Inoculated with DLA Cells (1×10^6)

Group	Dose(mg/kg)	Mean survival time(d)
Carboxy methyl cellulose	10 mL / kg	20.0 ± 0.70
Cyclophosphamide	27.3	27.2 ± 0.73
Compound-1ai	50	26.5 ± 0.39
Compound-2bi	50	19.7 ± 0.40
Compound-1at	50	23.3 ± 0.31
Compound-2bt	50	25.7 ± 0.37

The Anti-Asthmatic Screening

All the compounds prepared herein were screened for their potential anti-asthmatic activities such as, they were tested against PDE-IV for potential anti-asthmatic effect, and against DPP-IV and PTP1B for potential anti-diabetic effects. Moderate activity was found. The anti-asthmatic activity was carried out using *phosphodiesterase* IV enzyme (PDE-IV)[22](Table-4) and the primary screening of the compounds was done at 1 u M concentration using human PDE-IV enzyme, where Rolipram
& Ariflo were used as standard compounds.

The Anti-Diabetic Screening

The anti-diabetic activity was carried out with dipeptidyl peptidase (DPP-IV)[23] enzyme (Table-5) and the primary screening of the compounds was carried at 300 nM concentration using recombination human DPP-IV enzyme by the use of 1-(2-amino-3,3-dimethylbutanoyl pyrrolidine-2-carbonitrile as the standard compound at 100 nM. Similarly, the PTP1B[23a] (in house compound, also for anti-diabetic) activity (Table-4) was done using the test compounds at 30 μ M with the standard compound N-[5-[N-Acetyl-4-[N-(2-carboxyphenyl)-N-(2-

hydroxyoxalyl)amino]-3-ethy-DL-phenylalanyl-amino]-pentanoyl]-L-methionine at a concentration of 0.3 µM.

Protocol for PDE-IV-inhibition assay
Phosphodiesterase IV enzyme converts [^{3}H] cAMP to the corresponding [^{3}H] 5'-AMP in proportion to the amount of *Phosphodiesterase* IV present. The [^{3}H] 5'-AMP then was quantitatively converted to free [^{3}H] adenosine and phosphate by the action of snake venom 5'-nucleotidase hence the amount of [^{3}H] adenosine librated is proportional to *Phosphodiesterase* IV activity.

The assay was performed at 34 °C in a 200 mL total reaction mixture. The reaction mixture contained 25 mM of tris buffer, 10 mM $MgCl_2$, 1µM cAMP (cold) and [^{3}H] cAMP (0.1µCi) stock solutions of the compounds to be investigated were prepared in dimethyl sulfoxide in concentrations such that the dimethyl sulfoxide content in the test samples did not exceed 0.05% by volume to avoid affecting the *Phosphodiesterase* IV activity. Compounds were then added in the reaction mixture (25µL/tube). The assay was initiated by addition of enzyme mix (75µL) and the mixture was incubated for 20 minutes at 34 °C. The reaction was stopped by boiling the tubes for 2 min at 100 °C in a water bath. After cooling on ice for 5 minutes and addition of 50 µg 5'-nucleotidase snake venom from *Crotalus atrox* incubation was carried out again for 20 min at 34 °C. The un-reacted substrate was separated from [^{3}H] adenosine by addition of Dowex AG IX-8 (400 µL), which was pre equilibrated in (1:1) water:ethanol. Reaction mixture was then thoroughly mixed, placed on ice for 15 minutes, vortexed and centrifuged at 14,000 rpm for 2 min. after centrifugation, a sample of the supernatant (150 µL) was taken and added in 24 well optiplates contaning scinillant (1 mL) and mixed well. The samples in the plates were then determined for radioactivity in a Top Counter and the *Phosphodiesterase* IV activity was calculated. *Phosphodiesterase* IV enzyme was present in quantities that yield < 30% total hydrolysis of substrate (linear assay conditions). Rolipram and Cilomilast were used as a standard in all assays.

Protocol for the DPP-IV assay
DPPIV inhibition measurement in vitro
DPPIV activity was determined by the cleavage rate of 7-amino-4-methyl-coumarin (AMC) from synthetic substrate Glycyl-Prolyl-AMC. In brief, the assay was conducted by adding 10 mg of human recombinant Dipeptidyl peptidase IV enzyme (DPPIV, available commercially from R & D Systems) in 50 µL of the assay buffer (25 mM Tris, p^H 7.4, 140 mM NaCl, 10 mM KCl, 1% BSA) to 96 well black flat bottom micro-titer plates. The reaction was initiated by adding 50 µL of 100 µM substrate Gly-Pro-AMC. The incubation was carried out in the kinetic mode at 30 °C for 30 min. (Fluorescence was measured using Fluorostar at excitation filter of 380 mM and emission filter of 460 mM) test compounds and solvent controls were added as 1 µL additions. Test compounds were dissolved in DMSO and tested at 300 mM concentration. Percent inhibition was calculated with respect to the solvent control sample (no test compound added). Dipeptidyl peptidase (i.e., anti-diabetic).

Table-4: Anti-asthmatic and Anti-diabetic Screening of compounds

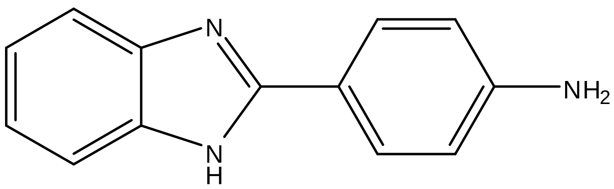
Compound No	PDE-IV (1 µM) % Inhibition	DPP-IV (0.3 µM) % Inhibition
1a	32.45	15
2b	27.72	13
3c	29.07	14
1-ai	33.01	11
2-bi	39.27	09
3-ci	37.02	12
1-at	35.43	07
2-bt	41.12	17
3-ct	40.21	19

Standard compound assay

1. PDE-IV: Rolipram and Cilomilast were used as a standard in all assays. Rolipram shows percentage inhibition 67.41% at a concentration of 2 µM. Cilomilast shows percentage inhibition 45.28 % at a concentration of 0.075 µM.
2. DPP-IV: 1-(2-amino-3, 3-dimethylbutyryl) pyrrolidine-2-carbonitrile is used as A standard in all assays and shows percentage inhibition of 96 % at a concentration of 0.1 µM.

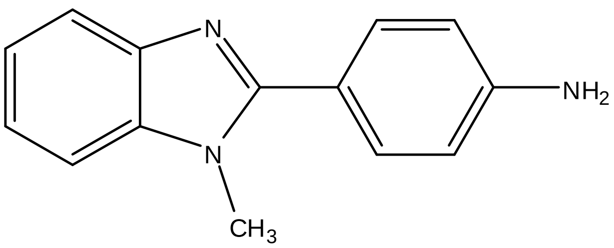
RESULTS AND DISCUSSION

The condensation of p-phenylene diamine with anthanilic acid was fused at 100° C for 2.5h to obtain the compound [41].

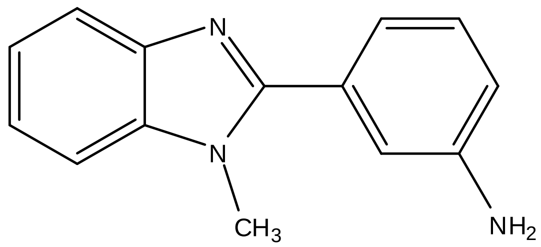


4-(1*H*-benzo[*d*]imidazol-2-yl)aniline

Further we treated with alkylating agent in presence of NaH / DMF to get the compound below.



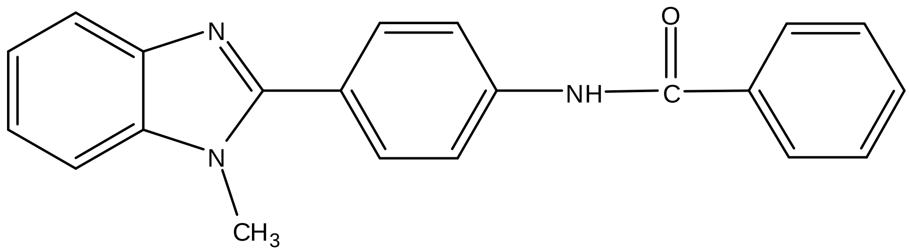
4-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)aniline



3-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)aniline

SCHEME-1

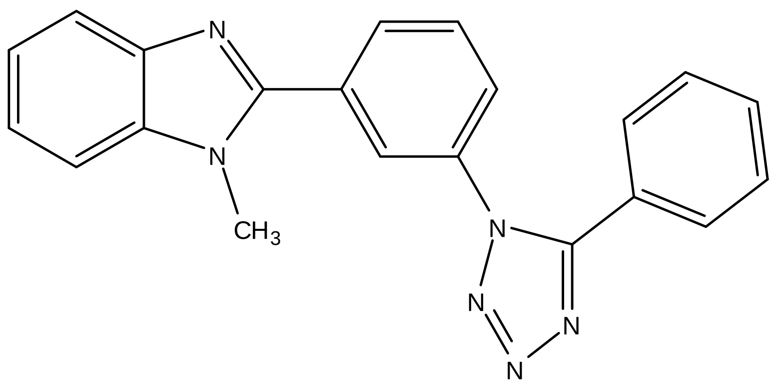
Compound-II was treated with different aromatic acid chlorides in presence of pyridine as base to obtain the corresponding aromatic acid chloride derivatives.



N-(4-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)phenyl)benzamide

SCHEME-2

Compound-III has also been treated with PCl_5 to yield an intermediate compound, further more; we treated with NaN_3 the compound obtained as a tetrazole moiety.



1-methyl-2-(3-(5-phenyl-1*H*-tetrazol-1-yl)phenyl)-1*H*-benzo[*d*]imidazole

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