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SYNTHESIS, ANTICANCER, ANTI-TUMOR, ANTI-DIABETIC AND ANTI-ASTHMATIC SCREENING OF SOME NOVEL CLUBBED TETRAZOLE MOIETY COMPOUNDS

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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 anticancer, anti-diabetic, anti tumor and anti asthmatic properties, which exhibited some authentic results towards testing organism invitro and invivo studies.

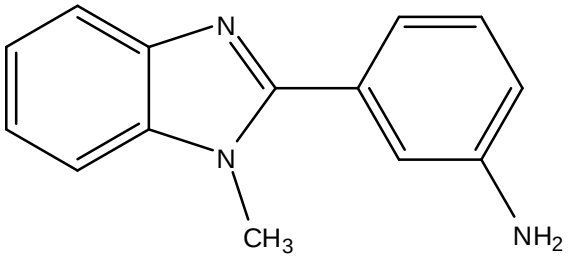
Key Words: Anthranilicacid, Benzoyl Chloride, Anticancer, Anti-Diabetic, PCl_5 & NaN_3

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 are very important intermediates in organic synthesis, vitamineB12 constitutes a milestone in the chemistry of benzimadazole.

Result and Discussion:



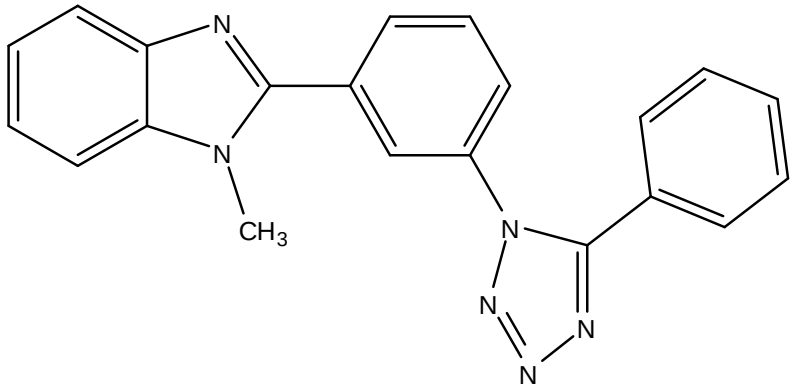
3-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)aniline

The condensation of m-phenylenediamine with anthanilicacid was fused at 100° C for 3.5h to obtain the compound [41], Further we treated with alkylating agent in presence of NaH/ DMF to get the compounds below.

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.

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

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 a 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

A mixture of m- Phenylendiamine (0.1mol) and 2-aminobenzoic acid (0.1 mol) was heated on a water bath for 3 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 methanol.

Compound- I (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 (I)

To a mixture of compound (I) (2mmole) in dimethylformide (10 mL) was added sodium hydride (55%, 2 mmole) lot wise at 0 ºC. After completion of addition the temperature for 2.5 h. The reaction mixture was again cooled to 0 ºC and the respective alkyl halide (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 -4-fluoro-N-(4-(1-Hmethyl-1H-benzoimidazol-2yl)phenyl) benzamide (II)

A mixture of compound (0.001 moles) of (I) and equivalent amount of benzoyl fluoride (0.001 moles) was refluxed with pyridine (40 ml) for 8 hours. 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 - II (Found C, 72.50; H, 4.26; F, 5.73; N, 12.68; O, 4.83: $C_{20}H_{14}FN_3O$: M.Wt: 331) 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: 332 (M+)

Synthesis of 2-(3-(5-(4-fluro-phenyl)-tetrazol-1-yl)phenyl)-1-methyl-1H-benzoimidazole (III)

A mixture of compound (**2;** 0.01 mole) was which taken in a beaker and added a known amount of PCl_5 (0.01 mole) and heated at 100 ^{0}C. The resulting mixture was treated with ice cold solution of known weight of NaN_3 (0.02 mole), a known volume (40 mL) of acetone, known volume of sodium acetate was added. The reaction mixture was long time stirred. The product filtered and washed with ice cold water. The residue was recrystalized from benzene–pet-ether mixture and purified by column chromatography to give **III.**

Compound - III (Found C, 68.0; H, 4.18; F, 5.1; N, 22.82 $C_{21}H_{15}FN_6$ M.Wt: 370) 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: 371 (M+)

Table.1 Physical and Analytical Data of Compounds

Com pounds	R	Time in Hours	m.p (ºC)	Yield %	Molecular Formula	Analysis % calcd.(Found)			
						C	H	N	O
I	Meta	3.5	110	74	$C_{13}H_{11}N_3$	74.5	5.20	20.01	-
II	Meta	9	194	61	$C_{20}H_{15}ON$	76.5	4.6	13.4	5
III	Meta	21	245	52	$C_{20}H_{14}N_6$	71.3	4.5	24.6	-

Biological Screening:

***In vivo* anticancer screening** [42]

A healthy adult swiss mouse (20-30 g) was well ventilated and animals had +12h day and night schedule with temperature between 11-20° C. The animals were housed in large spacious hygienic cages during the course of experimental period. The animals were fed with rat pellet. The experiments were performed as per the recommendations of CPCSEA, Chennai, Tamilnadu.

Table-2: Effect of Test Compounds on Body Weight of Mice, Inoculated With DLA Cells 1×10⁶

Group	Treatment	Dose (mg/kg)	Body weight 0th d	11th d	20th d	Decearse in body wt from 11th day to 20th day
I	Carboxy methyl cellulose	10 m L/ kg	26.60	33.80	37.66	-
II	Cyclophosphamide	27.3	21.40	36.20	33.60	5.29
III	Compound-I	50	19.00	33.20	30.30	2.72
IV	Compound-II	50	18.50	32.00	30.50	1.50
V	Compound-III	50	27.57	35.72	32.10	3.62

Data expressed as mean ± SEM of four animals.

Drugs treated with 100 mg/kg were compared with control.

***In vitro* anti tumor screening** [43]:

Daltons Lymphoma Ascites(DLA) cell were collected, counted and adjusted to 1×10⁶ 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*[46,47]. Dalton's Lymphoma Ascites cells were propagated in Swiss albino mice by injecting 1×10⁶ cells intraperitoneally. The cells were aspirated aseptically from the developed tumor during the log phase of the 11th 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 cell 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 1×10⁶ 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 [44] (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 [45], mean survival time (MST) and percentage increase in life span (% ILS) [46].

All the mice were weighed daily, after tumor inoculation. Average gain in body weight was determined and recorded in Table-3 and % decrease in body weight was calculated. The surviving time of DLA tumor –bearing mice was noted and mean survival time (MST) was calculating. Using mean survival time percentage increase in life span was calculated and recorded in 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-II	50	19.7 ± 0.40
Compound- III	50	25.7 ± 0.37

Data expressed as mean ± SEM of four animals.
Drugs treated with 100 mg/kg were compared with control

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-5) and the primary screening of the compounds was done at 1 u M concentration using human PDIV 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 n M 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 n M. Similarly, the PTP1B[23a] (in house compound, also for anti-diabetic) activity (Table-5) 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 µ M.

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 m L total reaction mixture. The reaction mixture contained 25 m M of tris buffer, 10 m M MgCl2, 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 (400µL), 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 determine 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 ng of human recombinant Dipeptidyl peptidase IV enzyme (DPPIV, available commercially from R & D Systems) in 50 µL of the assay buffer (25 m M Tris, p^H 7.4, 140 m M NaCl, 10 m M 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 nM and emission filter of 460 nM) test compounds and solvent controls were added as 1 µL additions. Test compounds were dissolved in DMSO and tested at 300 nM 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 activity of compounds

Compound No	**PDE-IV** (1 µM) % Inhibition	**DPP-IV** (0.3 µM) % Inhibition
I	27.72	13
II	39.27	09
III	41.12	17

Standard Compound Assay:

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.

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

Conclusion:

The present research work demonstrates an unforced and convenient method for synthesizing compounds vice versa I, II and III. The solvent using method for the condensation step proved to be more efficient and eco friendlier to the environment than the standard procedures. The condensation reaction takes place at relatively moderate temperatures. This method also simplifies the handling of the reactions and yields tetrazole derivative. This procedure is simple, non-toxic and low cost. The reactions scheme exhibited good activity and valuable contribution to the existing methodologies. All the biological screening has good inhibition property.

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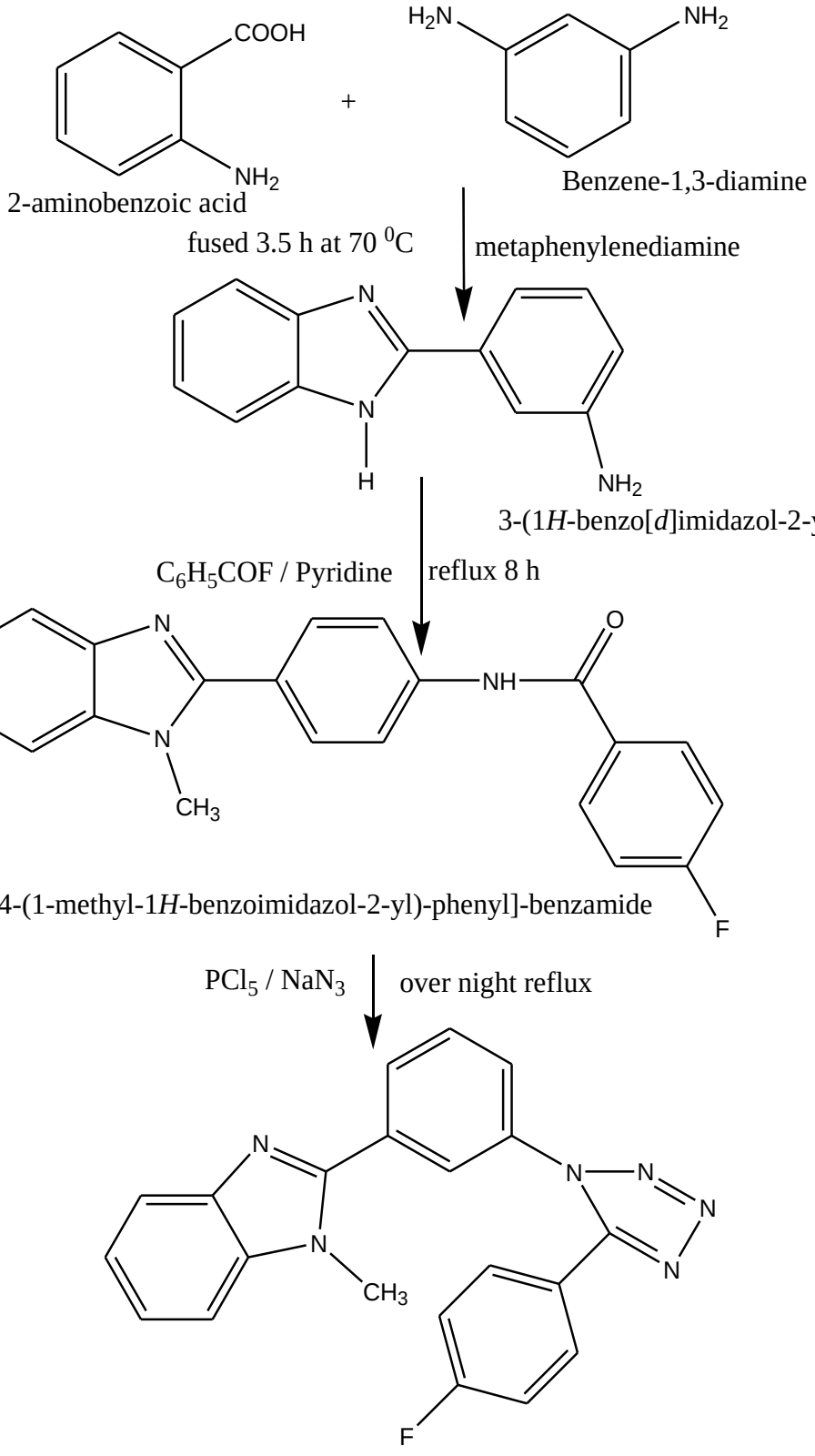
Reaction Scheme



2-{3-[5-(4-Fluoro-phenyl)-tetrazol-1-yl]-phenyl}-1-methyl-1*H*-benzoimidazole