SYNTHESIS OF MULTIPLE BIOLOGICALLY ACTIVE 1, 2-DIHYDRO-PYRIMIDO [1, 2-A]-BENZIMIDAZOLE-3- CARBONITRILE

Ravindra M. Kumbhare,* Umesh Kosurkar, K. Vijay Kumar, Tulshiram Dadmal, A. K. Tiwari

*Division of Flouroorganics, Indian Institute of Chemical Technology, Tarnaka, Hyderabad-500067, INDIA

ABSTRACT: A variety of 1,2-dihydropyrimido-[1,2-a]-benzimidazole-3-carbonitrile derivatives were synthesized under microwave irradiation using water and acetonitril as solvent system. All the compounds were tested in vitro for α-glucosidase inhibitory and DPPH free radical scavenging activity. 4-Amino-2-(4-flourophenyl)-1,2-dihydropyrimido [1,2-a]-benzimidazole-3-carbonitrile (4**c**) was found to be a potent intestinal α-glucosidase inhibi tory activity (IC_{50}; 91μM) along with moderate DPPH scavenging property. This compound was further evaluated for cytotoxicity activity against HT-29 colon cancer cell line. The IC_{50} value for its cytotoxicity activity was found to be 662 μM.

Keywords: Benzimidazole carbonitriles, α-glucosidase, free radical scavenger, cytotoxic.

INTRODUCTION:

A protracted increase in blood glucose levels beyond the usual fasting and or postprandial ranges is termed hyperglycemia and is frequently used as a synonym for diabetes.[1] The diabetes is associated with an increased risk for cardiovascular disease (CVD).[2] Post prandial hyperglycemia (PPHG) in particular, has emerged as an important indepen dent risk factor for CVD. Pathological PPHG is characterized by hyperglycemic spikes that induce endothelial dysfunction, inflammatory reactions and oxidative stress, which may lead to progression of atherosclerosis and occurrence of cardiovascular events. Control of PPHG therefore, is becoming the focus of future clinical investi gations[4] and emerging as a legitimate therapeutic target to minimize CVD risk.[3] Diabetes is also emerging as major risk factor for cancer, specifically colorectal cancer and associated with increased cancer mortality.[5-7] Therefore, to combat the epidemic of this cardiometabolic disorder, development of agents that possess multiple biological activities may present better therapeutic opportunities.

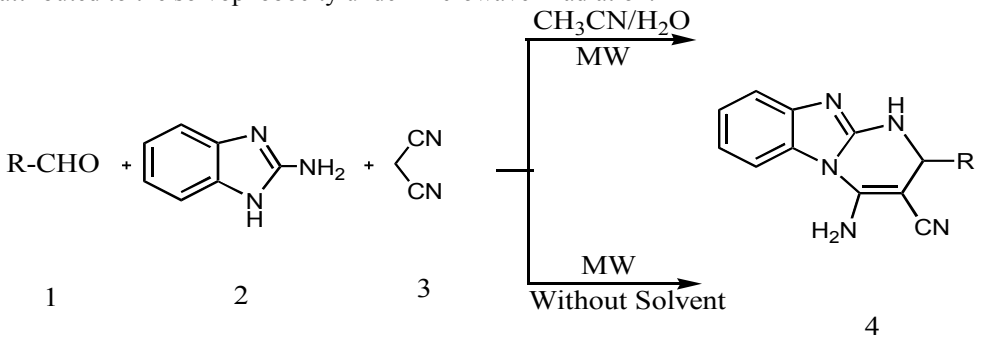
Intestinal α-glucosidase inhibitors that delay the absorption of carbohydrates from the small intestine have been found to reduce PPHG[8] and have been suggested as a promising metabolic modifier that could reduce the risk of CVD in patients with the metabolic syndrome[2]. Recently, inhibitors of glycosidases have become important not only in the control of PPHG but also in development of newer therapeutics targeted at, for example, cancer, viral infections, and lysosomal storage disease, with a number of drugs in current clinical use[9]. As part of our on going program of identifying molecules with multiple biological activities like, α-glucosidase inhibitory, cytotoxic particularly against colon cancer cell lines, free radical scavenging (antioxidants) and antihyperglycemic from natural products[10,11] and synthetic routs.[12] In this communication, we wish to report, a simple, novel and cost effective synthesis of the biological active new 1,2-dihydro-pyri mido-[1, 2-a]-benzimidazole-3-carbonitriles.

RESULTS AND DISCUSSION

Chemistry

Multi-component reactions (MCRs) have become preferred area of research in chemistry that yields novel chemical scaffolds for design and synthesis of a diversity oriented molecules. It requires simple efforts with minimal environment loads suitable from Green-Chemistry view point.[13,14] Though the synthesis of 1,2-dihydropyrimido [1,2-a] benzimidazole-3-carbonitriles derivatives in presence of ethanol and Me_2NH/Methanol as a catalyst has been reported, it has resulted poor yields.[15] In order to make this synthesis efficient applying green chemistry protocols that results in higher yields, we designed a multicomponent condensation reaction of 1, 2 and 3 to result product 4 (scheme I). In order to optimize the best possible conditions, equimolar amounts of chlorobenzaldehyde, malononitrile and 2-aminobenzimidazole were incubated in a microwave irradiation with water and acetonitril (1:1) as solvent. The optimum conditions were set as 180 W and 80 ^{0}C. Progress of the reactions was monitored by TLC and crude products were purified by column chromatography.

Secondly, the same reaction was attempted in the absence of solvents and procedure was followed until pure products were obtained. Surprisingly, good yield was obtained in this condition also, though longer reaction time was required. Both the procedures were adopted for synthesis of aromatic aldehydes like fluoro, methoxy, methyl etc Table I. All the compounds were analyzed by ^{1}HNMR, mass spectrometry and elemental analysis. The marginal lower yields (Table I) in this reaction condition, in the absence of solvent may be attributed to the solvophobocity under microwave irradiation.



Scheme 1

This is the first report synthesizing1,2-dihydrobenzo-(4,5-imidazole-1,2-a)-pyrimidine-3- carbonitrile derivatives under microwave irradiation condition in presence of water and acetonitrile. Both the methods resulted in high yield of the products.

Biological Activity:

For *in vitro* α- glucosidase inhibitory activities, we screened compounds using two sources of α-glucosidase; one from yeast that represents α- glucosidase type-I [16] and has also been extensively used as a model for screening potential inhibitors against cancer and viral diseases[17] and another from rat intestine that serves as potential target for screening of antihyperglycemic agents active against carbohydrate – induced postprandial hyperglycemia[12]. It is evident from Table II that compounds displayed variable activity levels in inhibiting glucosidase of different origin. Difference in α-glucosidase inhibitory activity of compounds may be explained on the basis of molecular anatomy of the enzymes and source of its origin.

Table 1: Synthesis of 4 under Microwave irradiation condition:

Compd	R	Microwave Irradiation [a]		MP(˚C)
		With solvent Yield (%), Reaction Time (min)	Without solvent Yield (%), Reaction Time (min)	
4a	4-ClC_6H_4	93 (5)	85 (5)	240
4b	4-BrC_6H_4	90 (5)	82 (5)	228
4c	4-FC_6H_4	90 (6)	80 (5)	244
4d	4-C_6H_5	92 (5)	85 (5)	232
4e	4-$CH_3C_6H_4$	93 (5)	82 (5)	238
4f	4-$OCH_3C_6H_4$	90 (5)	82 (5)	230

[a]Pulsed irradiated at 180 W.

Table II: Percent α-glucosidase inhibitory & DPPH scavenging activities of compounds:

Compd	Yeast α-glucosidase inhibition at 0.3 mM concentration	Intestinal α-glucosidase inhibition at 0.3mM concentration,	DDPH scavenging at 75µM concentration
4a	25.8±7.53	NA	50.34±0.04
4b	7.26±0.44	6.98±2.8	19.19±0.07
4c	11.77±2.34	62.45±1.18	42.2±1.59
4d	19.8±6.13	17.45±3.3	48.26±1.14
4e	39.32±2.56	34.43±0.01	49.62±2.54
4f	13.44±7.14	31.64±2.1	38.47±0.27

Values represent mean±SD, n=3, NA; not active.

It has been observed that though the amino acid sequences in the catalytic site of the α-glucosidase family may be highly conserved, the aglycon specificity may not be the same.[18] It suggested therefore, using enzymes of target tissues or the organs for screening and identification of agent against diabetes, cancer and viral diseases etc.[19] Compound **4c** displayed maximum intestinal α-glucosidase inhibitory activity and therefore was selected for cytotoxicity and relevant dose response studies (Figure I, II).

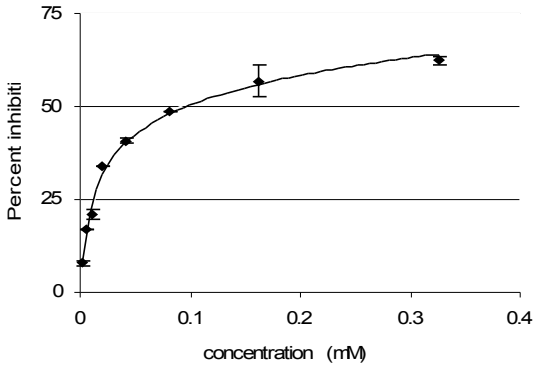


Figure I - Dose response curve of compound **4c** for intestinal α-glucosidase inhibitory activity. Curve represents logarithmic regression line. Values represent mean ±SD, n=3

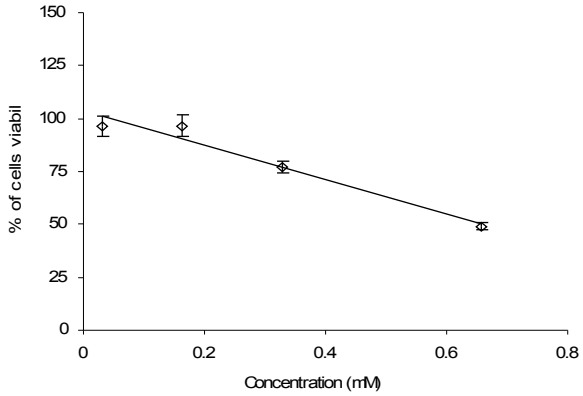


Figure II- Dose response curve of compound **4c** for cytotoxicity study activity against HT-29 (Colon cancer) cell line. Curve represents linear regression line. Values represent mean ±SD, n=3.

It displayed IC_{50} value 91µM for intestinal α-glucosidase inhibition and 662 µM for cytotoxicity activity. Though 4c displayed potent intestinal α-glucosidase inhibitory activity, the activity was comparatively less than the standard intestinal α-glucosidase inhibitor drug molecule acarbose (IC_{50}; 27 µM) in our experiment. All the compounds were tested for DPPH free radical scavenging activity also and were found to display varying degrees of free radical scavenging activity (Table-2). This is the first report assigning *in vitro* α-glucosidase inhibitory, cytotoxicity against colon cancer cell line HT-29 and free radical (DPPH) scavenging activity for 1, 2-dihydro-pyrimido [1, 2-a] benzi midazole-3-carbonitriles. Less number of compounds limits structure activity relationship discussion. However, efforts are on in our laboratory to synthesize more number of compounds with various substitutions for this purpose and development of this novel group of multiple active 1, 2-dihydro-pyrimido [1,2-a] benzimidazole-3-carbonitril α-glucosidase inhibitors as antihyperglycemic agents.

Experimental Section:

General: IR Spectra were recorded on a Perkin-Elmer FT-IR 240-c spectrophotometer using KBr optics. ^1H NMR spectra were recorded on Bruker-300 MHz, spectrometer in $CDCl_3$ using TMS as internal standard. Mass spectra were recorded on a Finnigan MAT 1020 mass spectrometer operating at 70 eV. Elemental analysis (C, H and N) were performed on Vario EL (Germany) data were with in ±0.4 of the theoretical values.

Method A. With solvent:

A mixture of malononitrile (1 mmol), 2-aminobenzthiazole (1 mmol), benzaldehyde (1 mmol), and solvent mixture (1.5 mL, acetonitrile/water) were placed in a 10 mL pressure tube. The mixture was subjected to microwave irradiation (CEM Discover, 180 W, 250 psi, 80 ^{0}C) for 5 min and then diluted with dichloromethane (5 ml) and filtered. The solid was rinsed with dichloromethane (2x 5 mL) and the combined extracts were concentrated and purified by column chromatography to afford the corresponding pure product.

Method B. Without solvent:

A mixture of malononitrile (1 mmol), 2-aminobenzthiazole (1 mmol), benzaldehyde (1 mmol), in a 10 mL pressure tube was subjected to microwave irradiation (CEM Discover, 180 W, 250 psi, 80 ˚C) for 5 min and then diluted with dichloromethane (5 mL) and filtered. The solid was rinsed with ethyl acetate (2x5 mL) and the combined extracts were concentrated and purified by column chromatography to afford the corresponding pure products.

Biological assays:

Chemicals

Yeast α-glucosidase, rat intestinal acetone powder as a source of intestinal α-glycosidase, *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG) and 1,1-Diphenyl-2-picryhydrazyl (DPPH) free radical, Dulbecco's modified Eagle's medium (DMEM), 3-(4,5- dimethylthiazole-2-yl)-2,5- diphenyl tetrazolium bromide (MTT), and Trypsin were purchased from Sigma Chemical Co., St Louis, MO, USA. Other chemicals of Analytical grade were procured from indigenous manufacturers. HT-29 (colon cancer) cell line was obtained from National Centre for Cell Science (NCCS) Pune (India).

4-Amino-2-(4-chlorophenyl)-1,2-dihydropyrimido[1,2-a]-benzimidazole-3-carbo nitrile (4a): IR (KBr): υ 3422, 3327, 3214, 3052, 2189, 1598, 1463 cm.$^{-1}$; ^{1}H NMR ($CDCl_3$+DMSO): δ 5.24 (s, 1H), 6.87 (s, 2H), 7.05 (t, 1H), 7.14 (t, 1H), 7.24 (d, 1H), 7.32 (d, 2H), 7.45 (d, 2H), 7.62 (d,1H), 8.61 (s, 1H).; Anal. Calcd. For $C_{17}H_{12}ClN_5$: C, 63.46; H, 3.76; N, 24.77. Found: C, 63.40; H, 3.72; N, 21.65.

4-Amino-2-(4-bromophenyl)-1,2-dihydropyrimido [1,2-a]-benzimidazole-3-carbo nitrile (4b): IR (KBr): υ 3420, 3327, 3215, 3052, 2201, 1610, 1463 cm.$^{-1}$; ^{1}H NMR ($CDCl_3$+DMSO): δ 5.20 (s, 1H), 6.80 (s, 2H), 7.05 (t, 1H), 7.10 (t, 1H), 7.22-7.29 (m, 3H), 7.55-7.65 (m, 3H), 8.62 (s, 1H).; Anal. Calcd for $C_{17}H_{12}BrN_5$: C, 55.75; H, 3.30; N, 1915. Found: C, 55.87; H, 3.32; N, 19.23.

4-Amino-2-(4-flourophenyl)-1,2-dihydropyrimido[1,2-a]-benzimidazole-3-carbo nitrile (4c): IR (KBr): υ 3422, 3317, 3214, 3052, 3892, 2189, 1673, 1598 cm.$^{-1}$; ^{1}H NMR ($CDCl_3$ +DMSO): δ 5.25 (s, 1H), 6.84 (s, 2H), 7.01 (t, 1H) 7.12 (t, 1H), 7.18-7.25 (m, 3H), 7.31-7.36 (m, 2H), 7.69 (d, 1H), 8.58 (s, 1H).; Anal. Calcd for $C_{17}H_{12}FN_5$: C, 66.88; H, 3.96; N, 22.94. Found: C, 66.85; H, 3.95; N, 22.84.

4-Amino-2-phenyl-1,2-dihydropyrimido [1,2-a]-benzimidazole-3-carbonitrile (4d): IR (KBr): υ 3422, 3327, 3214, 3052, 2189, 1598, 1463 cm.$^{-1}$; ^{1}H NMR ($CDCl_3$+DMSO): δ 5.21 (s, 1H), 6.84 (s, 2H), 7.01 (t, 1H), 7.13 (t, 1H), 7.24-7.35 (m, 6H), 7.61 (d, 1H), 8.60 (s, 1H).; Anal. Calcd for $C_{17}H_{13}N_5$:c, 71.06; H, 4.56; N, 24.37. Found: C, 71.12; H, 4.54; N, 24.44.

4-Amino-2-*p*-totyl-1,2-dihydropyrimido [1,2-a]-benzimidazole-3-carbonitrile (4e): IR (KBr): υ 3482, 3323, 3218, 3055, 2184, 1687, 1598, 1403 cm.$^{-1}$; ^{1}H NMR ($CDCl_3$+ DMSO): δ 2.26 (s, 3H), 5.15 (s, 1H), 6.80 (s, 2H), 7.04 (t, 1H), 7.09-7.23 (m, 6H), 7.64 (d, 1H), 8.58 (s, 1H).; Anal. Calcd for $C_{18}H_{15}N_5$: C, 71.74; H, 5.02; N, 23.24. Found: C, 71.82; H, 5.04; N, 23.16.

4-Amino-2-(4-methoxyphenyl)-1,2-dihydropyrimido[1,2-a]-benzimidazole-3-carbo nitrile (4f): IR (KBr): υ 3423, 3327, 3216, 3152, 3006, 2909, 1598, 1423 cm.$^{-1}$; ^{1}H NMR ($CDCl_3$+ DMSO): δ 3.72 (s, 3H), 5.22 (s, 1H), 6.80 (s, 2H), 6.84 (d, 2H), 7.04 (t, 1H), 7.12 (t, 1H), 7.19-7.24 (m, 3H), 7.64 (d, 1H), 8.51 (s, 1H).; Anal. Calcd for $C_{18}H_{15}N_5O$: C, 68.13; H, 4.76; N, 22.07.Found: C, 68.23; H, 4.72; N, 22.02.

In vitro α-Glucosidase inhibitory assay:

α-Glucosidase inhibitory activities were determined according to earlier reported methods.[20,12] Rat intestinal acetone powder in normal saline (100: 1; w/v) was sonicated properly and the supernatant was used as a source of crude intestinal α-glucosidase after centrifugation. In brief, 10 µL of test samples dissolved in Dimethyl sulfoxide (DMSO) were reconstituted in 100 µL of 100 mM-phosphate buffer (p^H 6.8) in 96-well microplate and incubated with 50 µL of crude intestinal α-glucosidase or yeast α-glucosidase (0.76 Units/mL) for 5 minutes before 50 µL substrate (5 mM, *p*-nitrophenyl- α-D-gluco pyranoside prepared in same buffer) was added. Release of *p*-nitrophenol was measured at 405 nm spectrophotometrically (Spectra $_{MAX}$ Plus,[384] Molecular Devices Corporation, Sunnyvale, CA, USA) 5 min after incubation with substrate. Individual blanks for test samples were prepared to correct background absorbance where substrate was replaced with 50 µL of buffer. Control sample contained 10 µL DMSO in place of test samples. Percentage of enzyme inhibition was calculated as (1-B/A) x 100 where [A] represents absorbance of control without test samples, and [B] represents absorbance in presence of test samples. The IC_{50} values were determined by applying logarithmic regression analysis to the data of at least five concentration dilutions of the compound.

In vitro DPPH free radical scavenging activity:

Assay for the scavenging of stable free radical, DPPH was done as reported earlier [20]. Briefly, in a 96-well micro plate, 25 µL of test sample dissolved in DMSO, 100 µL of 0.1M tris-HCl buffer (pH 7.4) and 125 µL of 0.5mM DPPH solution dissolved in absolute ethyl alcohol were added. The reaction mixture was shaken well and incubated in dark for 30 min and read at 517nm spectrophotometrically (Spectra $_{MAX}$ plus ^{384}, Molecular Devices Corporation, Sunnyvale, CA, USA). Percentage of DPPH scavenging was calculated as (1-B/A) x 100 where A represents absorbance of control without test samples, and B represents absorbance in presence of test samples.

In vitro Cytotoxity Assay:

HT-29 (Colon cancer) cell were seeded at a density of $1X10^3$ cells per well in 100µl of DMEM supplemented with 10% FBS medium in 96 well plate and grown for 24 hours [12]. The cells were then exposed to a series of concentrations of test compound **4c** for 24 hours and the viability of cells was measured with MTT method. Briefly, the above media was replaced with 90µl of fresh serum free DMEM and 10 µl of MTT reagent (5mg/ml) and plates were incubated at 37^0C for 4h, there after the above media was replaced with 200µl of DMSO and incubated for 15min.The absorbance at 570nm was measured on a spectrophotometer (Spectra $_{MAX}$ plus ^{384}, Molecular Devices Corporation, Sunnyvale, CA, USA) The values for each point were calculated from triplicate wells. IC_{50} values were determined from plot: % inhibition (from control) versus concentration.

Acknowledgments:

Authors thank Dr. J. S. Yadav, Director IICT for his constant encouragement. This work was supported in part from the Grant No. SR/FTP/CS-93/2006 to RMK from Department of Science and Technology, Government of India under the Fast Track Scheme for Young Scientists.

REFERENCES

[1] NA. Calcutt, ME. Cooper, TS. Kern, AM. Schmidt. Nature Reviews: Drug Discov. 8, 417 (2009).
[2] SI. Yamagishi, T. Matsui, S. Ueda, K. Fukami, S. Okuda. Curr. Drug Metab. 10, 159 (2009).
[3] R. Peter, OE. Okoseime, A. Rees, DR. Owens. Curr. Vascular Pharmacol. 7, 68 (2009).
[4] K. Node, T. Inoue. Cardiovoscu Diabetol. 8, 23 (2009).
[5] CJ. Smith, GA. McKay, M. Fisher. Int. J. Clin. Pract. 62, 810 (2008).
[6] NH. Othman, AAM. Zin. Asian Pacific. J. Cancer Prev. 9, 747 (2008).
[7] JM. Berester, B. Goke. Arch. Physiol. Biochem.114, 84 (2008).
[8] AJ. Scheen. Lancet. 373, 1579 (2009).
[9] TM. Gloster, P. Meloncelli, RV. Stick, D. Zechel, A. Vasella, GJ. Davies. J. Am Chem. Soc. 129, 2345 (2007).
[10] PP. Reddy, AK. Tiwari, RR. Rao, K. Madhusudana, VRS. Rao, AZ. Ali, KS.Babu, JM. Rao. Bioorg. Med. Chem. Lett. 19, 2562 (2009).
[11] PP. Reddy, RR. Rao, KS. Babu, J. Shashidhar, G. Bhashikiran, VV. Lakashim, JM. Rao. Bioorg. Med. Chem. Lett. 19, 192 (2009).
[12] JA. Kumar, AK.Tiwari, AZ. Ali, MK. Reddy, BS. Ramakrishana, BC. Raju. J. Enzyme Inhib. Med. Chem. DOI: 10.1080/14756360903017122.
[13] JC. Menendez. Synthesis. 15, 2624 (2006).
[14] AT. Khan, T. Parvin, LH. Choudhury. Tetrahedron. 63, 5593 (2007).
[15] B. Insuasty, A. Salcedo, R. Abonia, J. Quiroga, A. Sanchez. Heterocycl. Commun. 8, 287 (2002).
[16] S. Chiba. Biosc Biotechnol Biochem. 61, 1233 (1997).
[17] N. Asano. Glycobiol. 13, 934 (2003).
[18] A. Kimura. Trends Glycosci Glycotechnol. 12, 373 (2000).
[19] W. Hakamata, M. Muroi, K. Kadokura, T. Nishio, T. Oku, A. Kimura. Bioorg. Med. Chem. Lett. 15, 1489 (2005).
[20] P. Aparna, AK. Tiwari, PV. Srinivas, AZ. Ali, V. Anuradha, JM. Rao. Phytother Res. 23, 591 (2009).

****