Original Article: Synthesis of Pyrazolo[1,2-*b*]phthalazine-5,10-dione Derivatives: A New Class of α –Glucosidase Inhibitors

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ABSTRACT

Background: Hyperglycemia is a metabolic disorder that refers to an increase in blood sugar in diabetic patients. α-Glucosidase has been introduced as a membrane-bound enzyme, and it is the main enzyme for carbohydrate digestion in some parts of the intestine. Inhibition of α -glucosidase enzyme activity is a reliable approach to control post-prandial hyperglycemia condition.

Objectives: In this study, a series of Pyrazolo[1,2-b]phthalazine-5,10-dione derivatives 5a–t were synthesized via a multicomponent reaction and evaluated as new inhibitors for α-glucosidase.

Methods: The biological activity of the synthesized compounds was studied using a source of the α-glucosidase enzyme (EC3.2.1.20, *Saccharomyces cerevisiae*) at 20 U/mg concentration.

Results: Four compounds showed higher α-glucosidase inhibitory activity in comparison to a standard, i.e., Acarbose. Compound 5q displays the most potent α-glucosidase inhibitory activity (IC_{50} = 155.4 ± 6.0 µM).

Conclusion: In conclusion, some of the synthesized compounds, including heterocyclic core molecules, have shown remarkable activity that could be considered as subjects for the development of new, more efficient inhibitors of the α-glucosidase enzyme.

1. Introduction

For the treatment of diabetes mellitus, five different chemical categories have been recommended by Food and Drug Administration (FDA, USA): Biguanides, thiazolidinediones, sulfonylureas, meglitinides, and α-glucosidase inhibitors. However, some of these drugs show undesired side effects in diabetic patients or lose their activity over some time. Therefore, many researcher teams try to develop new antidiabetic agents. For this purpose, the natural products, as well as the chemical compounds, have been studied for antidiabetic activity [1-3].

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One of the best ways for controlling and preventing diabetes diseases is the inhibition of α-glucosidase that can help reduce carbohydrate digestion and consequently decreases D-glucose absorption [4, 5]. One of the most critical parameters in diabetes mellitus management is controlling blood glucose levels within a range of 70-100 mg/dL [6, 7]. Also, for diabetes prevention, avoiding hyperlipidemia, hyperlipoproteinemia, and obesity have been exclusively emphasized by physicians [8]. Because of the ability of α-glucosidase in monosaccharide removal from viral glycoproteins, inhibition of this enzyme could alter cell-to-cell signaling and virus recognition by the cell. Hence, α-glucosidase inhibition may be used in treating viral diseases, cancers, and immune dysregulations, too [9-13].

In recent years, heterocyclic rings have received extensive interest. The Original Articles have introduced a broad range of applications for heterocyclic compounds in pharmacological and biological properties. In this regard, one of the heterocyclic rings, phthazine core, has been attracted due to a broad wide range of biological activities, including anti-inflammatory, anti-microbial, anti-fungal, and cytotoxic activity [14-22].

Some phthalazinone derivatives (Figure 1A) were studied as an antidiabetic agent in vivo model. Some of them showed a remarkable potential for decreasing the level of plasma glucose. Furthermore, 2-amino-4-(aryl)-4H-naphtho[1,2-b]pyran-3-carbonitrile derivatives prevent the glucose-induced increase in blood flow and permeability in rat granulation tissue and corresponding vascular changes in the retina, sciatic nerves, and aorta of diabetic rats [14, 15].

In the current study, the synthesis of pyrazolo[1,2-*b*] phthalazine-5,10-diones (Figure 1C) as an important fused bicyclic nitrogen-containing heterocycles derivatives with inhibitory properties against α-glucosidase (α-Gls) was targeted in which hybridization of functional groups of A and B was considered.

Recently, multicomponent reactions have been emphasized as a powerful tool in the area of synthetic chemistry. Several advantages have been mentioned for this type of chemical reaction, including the intrinsic atom economy, more straightforward procedures, intensive diversity of structure, and reducing waste by-products [17, 22, 23].

In the current report, the synthesis of 1H-pyrazolo[1,2-b]phthalazine-5,10-dione derivatives was reported from the condensation of phthalimide, with different aryl-aldehydes using malononitrile and hydrazine hydrate via one-pot four-component reactions. The chemical reaction was carried out in the presence of $NiCl_2$-$6H_2O$ as Lewis-acid. The synthesized compounds were evaluated for their α-glucosidase inhibitory activities.

2. Materials and Methods

General

Melting points were taken on a Kofler hot stage apparatus and uncorrected. 1H spectra were recorded on Bruker FT-500, using TMS as an internal standard. A Nicolet Magna FT-IR spectrophotometer was used for acquiring IR spectrum using KBr disk. The solvents and reagents were purchased from Sigma-Aldrich or Merck and were used directly without any further purification. For column chromatography, silica gel 60 (0.040–0.063 mm) and for Tthin-Layer Chromatography (TLC) silica gel 60/Kieselguhr F254 percolated on Aluminum sheets (thickness 0.2 mm) were used commercially available from Merck. Visualization of spots on the TLC plate was accomplished with UV light.

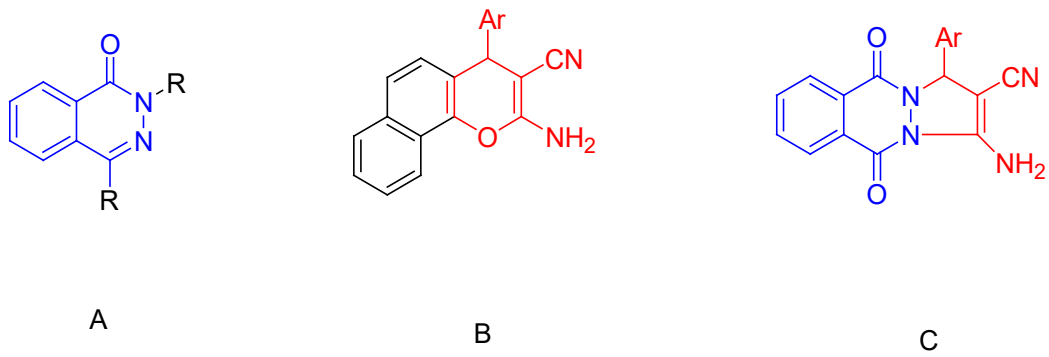

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Figure 1. A: General structures of phthalazinone; B: Pyran-3-carbonitrile derivatives; and C: Pyrazolo[1,2-b]phthalazine-5,10-diones.

α-Glucosidase inhibition assay

The enzyme solution at a concentration of 50 mM and pH 6.8 was prepared to solve the protein in potassium phosphate buffer from the following source: (EC3.2.1.20, *Saccharomyces cerevisiae*, 20 U/mg). *p*-Nitrophenyl-α-D-glucopyranoside (pNPG) was purchased from Sigma-Aldrich and used as the main substrate of the enzyme. Dimethyl sulfoxide (DMSO) was used as a solvent for the preparation of the test compounds solution at 10% concentration. The 96-well plates were used for the assessment of α-glucosidase inhibition activity. To each well, 20 µL of enzyme solution, 135 µL of phosphate buffer solution, and 20 µL of various concentrations of 5a-t were added. The plate was incubated at 37oC for 10 min. Then, 25 µL of the substrate, pNPG, previously dissolved in the 0.05 M phosphate buffer (pH = 6.8), was added and incubated at 37oC for 20 min. Finally, the reaction was quenched by adding 50 µL of sodium carbonate solution (0.2 M). The absorption of each well was recorded at 405 nm with a UV spectrophotometer (Gen5, Power wave xs2, BioTek, America). The blank solution was DMSO at a concentration similar to those used for the sample solution. Acarbose was used as a reference drug (positive control) [24]. The following formula was used for the determination of α-glucosidase inhibition property for the synthesized compounds and was expressed as the percentage of enzyme inhibition [25, 26]:

$$\%\ Inhibition = \frac{(Control\ Absorption - Sample\ Absorption)}{Control\ Absorption} * 100$$

IC_{50} was defined to the needed concentration of each of the synthesized compounds required to inhibit 50% of the enzyme activity from the non-linear regression curve using the Logarithmic method.

Enzyme kinetic study

Compound 5q as the most active compound was selected to investigate the inhibition mode of the action against α-glucosidase enzyme using different concentrations of substrate, p-nitrophenyl α -D-glucopyranoside (1-10 mM) in two different experiments. In the first experiment, the 5q compound was substituted by a blank buffer solution with the same volume and amount of other material, and in the second, compound 5q was used as an inhibitor at three different concentrations (95, 135 and 155 µM). The inhibition type and K_m (the Michaelis–Menten constant) were studied using a Lineweaver-Burk plot experiment involved with the plotting of 1/V versus1/[S], in which 1/[S] is the reciprocal of the concentration of substrate and 1/V presented the reciprocal of enzyme rate. The secondary plot resulted from the plotting of Km versus the concentration of inhibitor.

Molecular docking

We used molecular docking study for developing and more understanding the binding mode interaction of the synthesized compounds and α-glucosidase enzyme. For this propose, AutoDock version 4.2.6, AutoDock Tools (ADT) version 1.5.6, and Discovery studio v16.1.0.15350 were used to perform the docking studies. Regarding the biological data, the most potent compounds, 5h, 5n, 5s, 5q, and the reference drug (acarbose), were selected for docking study, and their 3D structure was generated using ChemDraw Ultra 12.0.2 version of Cambridge University. The crystal structure of α-glucosidase from *Saccharomyces cerevisiae* is not available in Protein Data Bank (www.rcsb.org/pdb/), and also, there is no report about its crystal structure. Therefore, the crystal structure of isomaltase from *Saccharomyces cerevisiae* (PDB ID: 3A4A; Resolution 1.6 Å) was retrieved from Protein Data Bank (www.rcsb.org/pdb/). Then, the water molecules were removed from the protein structure and original inhibitor for starting the docking study. The PDBQT files for the ligand and the enzyme were prepared, and AutoGrid Tools software was used for carrying out grid box formation. A set of grid size with the dimensions of 60 × 60 × 60 and 0.375 Å of grid space was established for the docking study. The center of grid box was designated in coordinates of x= 21.277, y= -0.773, and z= 18.648. The Lamarckian genetic algorithm was used via 50 runs of the AutoDock search for each docked system separately. Analysis of the interactions between the inhibitor and the targeted enzyme was carried out using Discovery Studio Visualizer v16.1.0.15350 (Accelrys, San Diego, USA) with considering the best pose of each ligand.

Synthesis of 3-amino-5,10-dioxo-1-phenyl-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5a-t)

General procedure: a mixture of phthalic anhydride (1 mmol), hydrazine hydrate (1 mmol), malononitrile (1.2 mmol), various aryl aldehydes (1 mmol), and a catalytic amount of $NiCl_2$-$6H_2O$ (10 mol %) in EtOH (10 mL) were refluxed for 2-3 h. After completing the reaction as monitored by TLC using petroleum ether:ethyl acetate 2:1 as mobile phase, the residue was filtered and washed with boiling ethanol to obtain the pure products 5a-t. Their properties are as follows:

3-Amino-5,10-dioxo-1-phenyl-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5a)

Yield: 91%; m.p.: 274-276, IR (KBr): 3383, 3014, 2895, 2226, 1657, 1603, 1492 cm^{-1}. 1H NMR (500 MHz, DMSO-d_6) δ ppm: 6.24 (s, 1H, -CH), 7.32-7.461 (m, 5H, H-Ar), 7.78-7.82 (m, 2H, H-Ar), 8.13 (s, 2H, NH_2), 8.21 (dd, *J*=5.5, 3 Hz, 1H, H-Ar), 8.33 (dd, *J*=5.5, 3 Hz, 1H, H-Ar) [13]. CNMR (125 MHz, $CDCl_3$, ppm): δ 159.3 156.7, 155.3, 155.0, 154.5, 136.7, 134.6, 134.4, 133.8, 129.9, 129.5, 129.2, 128.7, 128.1, 127.5, 125.4, 123.3, 99.5; MS (m/z, %): 418 (M+, 100), 337 (10), 318 (7), 157 (7), 132 (26), 101 (16), 89 (16), 77 (10).

3-Amino-1-(4-methoxyphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5b)

Yield: 94%; m.p.: 259-261, IR (KBr): 3383, 3180, 2890, 2220, 1654, 1615, 1482 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 3.75 (s, 3H, OCH_3), 6.22 (s, 1H, -CH), 6.68-7.09 (m, 2H, H-Ar), 7.30-7.57 (m, 2H, H-Ar), 7.59-8.41 (m, 6H, H-Ar, NH_2).

3-Amino-1-(3-methoxyphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2- carbonitrile (5c)

Yield: 91%; m.p.: 249-250, IR (KBr): 3363, 3259, 3056, 2221, 1654, 1566 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 3.74 (s, 3H, OCH_3), 6.09 (s, 1H, -CH), 6.89 (dd, *J*=8, 1.5 Hz, 1H, H-Ar), 6.97-7.30 (m, 2H, H-Ar), 7.28 (t, 1H, *J* = 8, H-Ar), 7.93-7.99 (m, 2H, H-Ar), 8.03-8.21 (m, 3H, H-Ar, NH_2), 8.22-8.29 (m, 1H, H-Ar).

3-Amino-1-(2-methoxyphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5d)

Yield: 93%; m.p.: 259-261, IR (KBr): 3380, 3250, 3156, 2224, 1657, 1564 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 3.74 (s, 3H, OCH_3), 6.34 (s, 1H, -CH), 6.91 (t, *J*=7.5 Hz, 1H, H-Ar), 7.04 (d, *J*=8 Hz, 1H, H-Ar), 7.24-7.33 (m, 2H, H-Ar), 7.93-8 (m, 2H, H-Ar), 8.1 (s, 2H, NH_2), 8.10 (dd, *J*=5.5, 2.5 Hz, 1H, H-Ar), 8.29 (dd, *J*=7, 3.5 Hz, 1H, H-Ar).

3-Amino-1-(2,3-dimethoxyphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5e)

Yield: 92%; m.p.: 255-257, IR (KBr): 3346, 3250, 3056, 2225, 1657, 1554 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 3.75 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 6.30 (s, 1H, -CH), 6.92 (d, *J*=7 Hz, 1H, H-Ar), 6.97-7.1(m, 2H, H-Ar), 7.97 (dd, *J*=5.5, 3.5 Hz, 2H, H-Ar), 8.05-8.13 (m, 3H, H-Ar, NH_2), 8.29 (dd, *J*=6.5, 3.5 Hz, 1H, H-Ar).

3-Amino-1-(3,4-dimethoxyphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5f)

Yield: 91%; m.p.: 235-237, IR (KBr): 3330, 3225, 3056, 2225, 1657, 1561 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 3.72 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 6.12 (s, 1H, -CH), 6.90-7.12 (m, 3H, H-Ar), 7.90-8.3 (m, 6H, H-Ar, NH_2).

3-Amino-1-(Trimethoxyphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5g)

Yield: 94%; m.p.: 253-255, IR (KBr): 3350, 3145, 3026, 2226, 1657, 1561 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 3.66 (s, 3H, OCH_3), 3.76 (s, 6H, OCH_3), 6.07 (s, 1H, -CH), 6.85 (t, *J*=7.5 hz, 1H, H-Ar), 7.05 (d, *J*=8 Hz, 1H, H-Ar), 7.24-7.33 (m, 2H, H-Ar), 6.78 (s, 2H, H-Ar), 7.89-8.23 (m, 6H, H-Ar, NH_2).

3-Amino-5,10-dioxo-1-p-tolyl-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5h)

Yield: 82%; m.p.: 253-255, IR (KBr): 3370, 3197, 3056, 2225, 1653, 1562 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 2.29 (s, 3H, CH_3), 6.08 (s, 1H, -CH), 7.11 (d, *J*=8 Hz, 2H, H-Ar), 7.30 (d, *J*=8 Hz, 2H, H-Ar), 7.91-7.99 (m, 2H, H-Ar), 8.1 (s, 2H, NH_2), 8.04-8.12 (m, 1H, H-Ar), 8.22-8.29 (m, 1H, H-Ar).

3-Amino-1-(3-methylphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5i)

Yield: 81%; m.p.: 250-252, IR (KBr): 3356, 3184, 3065, 2223, 1653, 1542 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 2.29 (s, 3H, CH_3), 6.07 (s, 1H, -CH), 7.12 (d, *J*=7 Hz, 1H, H-Ar), 7.19-7.29 (m, 3H, H-Ar), 7.91-7.99 (m, 2H, H-Ar), 8.02 (s, 2H, NH_2), 8.07-8.13 (m, 1H, H-Ar), 8.24-8.29 (m, 1H, H-Ar).

3-Amino-1-(2-methylphenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5j)

Yield: 81%; m.p.: 248-250, IR (KBr): 3366, 3123, 3045, 2225, 1645, 1564 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 2.50 (s, 3H, CH_3), 6.44 (s, 1H, -CH), 7.1-7.34 (m, 4H, H-Ar), 7.95-8.20 (m, 5H, H-Ar, NH_2), 8.26-8.29 (m, 1H, H-Ar).

3-Amino-1-(methyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5k)

Yield: 87%; m.p.: 251-253, IR (KBr): 3346, 3143, 3025, 2219, 1645, 1564 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 1.53 (d, *J*=5.9 Hz, 3H, -CH_3). 5.24 (q, *J*=5.9 Hz, 1H,-CH), 7.94-8.06 (m, 4H, H-Ar and NH_2), 8.16-8.26(m, 2H).

3-Amino-1-(4-fluorophenyl)-5,10-dioxo-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5l)

Yield: 86%; m.p.: 265-266, IR (KBr): 3373, 3260, 2182, 1683, 1663.cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.15 (s, 1H, -CH), 7.01-7.2 (m, 2H, H-Ar), 7.49-7.69 (m, 2H, H-Ar), 7.89-8.18 (m, 5H, H-Ar, NH_2), 8.22-8.29 (m,1, H-Ar).

3-Amino-1-(2-fluorophenyl)-5,10-dioxo-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5m)

Yield: 84%; m.p.: 265-267, IR (KBr): 3378, 3184, 3065, 2223, 1653, 1542.cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.35 (s, 1H, -CH), 7.15-7.24 (m, 2H, H-Ar), 7.31-7.43 (m, 1H, H-Ar), 7.51-7.58 (m, 1H, H-Ar), 7.94-8.01 (m, 2H, H-Ar), 8.04-8.15 (m, 2H, H-Ar, NH_2), 8.25-8.32 (m, 1H, H-Ar).

3-Amino-1-(4-bromophenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5n)

Yield: 91%; m.p.: 266-268, IR (KBr): 3420, 3110, 2887, 2202, 1693, 1660cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.15 (s, 1H, -CH), 7.48 (d, *J*=7.5 Hz, 2H, H-Ar), 7.59 (d, *J*=7.5 Hz, 2H, H-Ar), 7.91-7.99 (m, 2H, H-Ar), 8.02 (s, 2H, NH_2), 8.04-8.13 (m, 1H, H-Ar), 8.32-8.39 (m, 1H, H-Ar).

3-Amino-1-(3-bromophenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5o)

Yield: 83%; m.p.: 269-271, IR (KBr): 3363, 3248, 2187, 1683, 1663cm−1. 1H NMR (500 MHz, DMSO-d_6) δ ppm: 6.15 (s, 1H, -CH), 7.31 (t, *J*=7.5 Hz, 1H, H-Ar), 7.44-7.55 (m, 2H, H-Ar), 7.73 (bs, 1H, H-Ar), 7.91-8.01 (m, 2H, H-Ar), 8.04-8.13 (m, 3H, H-Ar, NH_2), 8.23-8.31(m, 1H, H-Ar).

3-Amino-1-(2-bromophenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5p)

Yield: 84%; mp.:. 272-274, IR (KBr): 3356, 3184, 3065, 2223, 1653, 1542 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.44 (s, 1H, -CH), 7.25 (t, *J*=7.5 Hz, 1H, H-Ar), 7.35 (t, *J*=7.5 Hz, 1H, H-Ar), 7.55 (d, *J*=7.5vHz, 1H, H-Ar), 7.62 (d, *J*=7.5 Hz, 1H, H-Ar), 7.91-8.05 (m, 2H, H-Ar), 8.09-8.12 (m, 3H, H-Ar, NH_2), 8.25-8.29 (m, 1H, H-Ar).

3-Amino-1-(4-chlorophenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5q)

Yield: 85%; m.p.: 272-274, IR (KBr): 3350, 3110, 2887, 2222, 1660, 1463 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.15 (s, 1H, -CH), 7.36-7.43 (d, *J*=7.5 Hz, 2H, H-Ar), 7.54-7.62 (d, *J*=7.5 Hz, 2H, H-Ar), 7.85-7.92 (m, 2H, H-Ar), 8.02-8-15 (m, 3H, H-Ar, NH_2), 8.2-8.29 (m, 1H, H-Ar).

3-Amino-1-(2-chlorophenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5r)

Yield: 83%; m.p.: 259-261, IR (KBr): 3367, 3232, 3171, 2206, 1655, 1568, 1379 cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.15 (s, 1H, -CH), 7.31-7.38 (m, 2H, H-Ar), 7.46-7.48 (m, 1H, H-Ar), 7.65 (dd, *J*=7, 2 Hz, 1H, H-Ar), 7.96-8.01 (m, 2H, H-Ar), 8.07-8.11 (m,1H, H-Ar), 8.14 (bs, 2H, NH_2), 8.27-8.29 (m, 1H, H-Ar) .

3-Amino-1-(4-nitrophenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5s)

Yield: 96%; m.p.: 228-229, IR (KBr): 3433, 3321, 3076, 2160, 1658, 1558, 1515cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.30 (s, 1H, -CH), 7.81 (d,

J=8.6 Hz, 2H, H-Ar), 7.97-7.99 (m, 2H, H-Ar), 8.08-8.10 (m, 1H, H-Ar), 8.20 (bs, 2H, NH2), 8.22 (d, *J*=8.6 Hz, 2H, H-Ar), 8.27- 8.29 (m, 1H, H-Ar).

3-Amino-1-(3-nitrophenyl)-5,10-dioxo-5,10-dihydro-1*H*-pyrazolo[1,2-*b*]phthalazine-2-carbonitrile (5t)

Yield: 93%; m.p.: 228-229, IR (KBr): 3321, 3076, 2216, 1657, 1565, 1485cm^{-1} [1]. H NMR (500 MHz, DMSO-d_6) δ ppm: 6.34 (s, 1H, -CH), 7.68 (t, *J*=8 Hz, 1H, H-Ar), 7.93-8.05 (m, 3H, H-Ar), 8.06-8.11 (m, 1H, H-Ar), 8.16-8.23 (m, 3H, H-Ar, NH_2), 8.25-8.31(m, 1H, H-Ar), 8.428 (bs, 1H, H-Ar).

3. Results

Chemistry

Compounds 5a-t were prepared by the reaction of phthalic anhydride and different aldehyde derivatives in the presence of hydrazine hydrate, malononitrile, and the catalytic amount of nickel chloride via a one-pot reaction (Figure 2). The yield is presented in Table 1.

Biological activity

The α-glucosidase inhibition activity of the synthesized compounds was studied using *p*-nitrophenyl-α-D-glucopyranoside (pNPG), known as a substrate and a source of the α-glucosidase enzyme (EC3.2.1.20, *Saccharomyces cerevisiae*, 20 U/mg) in potassium phosphate buffer (pH= 6.8, 50 mM). The UV absorption of control, samples, and the reference compound (acarbose) were recorded at 405 nm per the reported method. The percentage inhibition and determination of the concentration of the sample compounds required to inhibit 50% of the enzyme activity (IC_{50}) were determined using the logit method.

According to Table 2, some of the tested compounds showed significant inhibition of the enzyme. The IC_{50} values for the synthesized compounds were less than the reference standard, acarbose (IC_{50}=750 µM). The most active compounds were 5h, 5n, 5q, and 5s with the Mean ±SD IC_{50} values of 159.3±7.7, 231.0±6.6, 155.4±6.0, and 230.5±7.0 µM, respectively.

Enzyme kinetic study

According to Figure 3A, the Lineweaver-Burk plot shows a competitive inhibition process with a gradually increasing Km and a constant value for Vmax followed by the increasing inhibitor (5a-t) concentration. The results of the kinetic study showed a binding to the enzyme active site for 5q after competing with the substrate. Furthermore, Figure 3b demonstrates an estimated inhibition constant for Ki (152 µM) when the Km value was plotted versus different concentrations of inhibitor 5q.

Docking study

For the evaluation of the possible mode interaction between potent compounds 5h, 5n, 5q, 5s, and the enzyme active site, a molecular docking study was carried out. For compound 5h, VAL308, PRO312, and VAL319 residues showed pi-alkyl interaction with the compound. ASP307 and THR310 residues showed hydrogen bond interaction, and ALA329 residues showed pi-sigma interaction with 5h. There are also van der Waals interactions between 5h and some residues of the active site. The result of the docking study for 5n showed hydrogen bond interaction between TYR158, HIS280, and ARG315 residues and a pi-anion interaction between ASP307 and the compound. Other interactions between 5n and the active site residues are mostly van der Waals type. In the case of 5q, there is a hydrogen bond interaction between TYR158, HIS280, and ARG315 residues and the compound. Furthermore, there are several interactions between 5q and the active site, including a pi-anion interaction with ASP307, a pi-alkyl interaction with PHE303, and several van der Waals type interactions. Finally, for 5s, there is a pi-pi interaction with HIS280 and some hydrogen bond interaction with ASN302, ASP307, GLY309, and THR310 residues. On the other hand, there is pi-alkyl interaction between PRO312 and 5s.

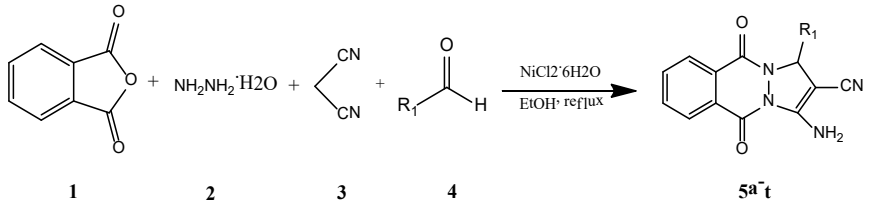
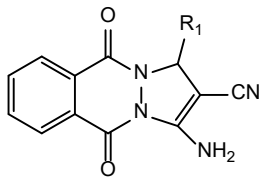


Figure 2. Synthesis of pyrazolo[1,2-*b*]phthalazinone derivatives

Table 1. The yield of the reaction for preparation of pyrazolo[1,2-b]phthalazinone derivatives



Entry	R_1	Product	Time/h	Yield(%)
1	C_6H_5	5a	2	91
2	4-OMe-C_6H_4	5b	2	94
3	3-OMe-C_6H_4	5c	2	91
4	2-OMe-C_6H_4	5d	2	93
5	2,3-diOMe-C_6H_3	5e	2	92
6	3,4-diOMe-C_6H_3	5f	2	91
7	3,4,5- (OMe)–C_6H_2	5g	2	94
8	4-Me-C_6H_4	5h	4	82
9	3-Me-C_6H_4	5i	4	81
10	2-Me-C_6H_4	5j	4	81
11	CH_3	5k	2	87
12	4-F-C_6H_4	5l	2	86
13	2-F-C_6H_4	5m	2	84
14	4-Br-C_6H_4	5n	2	91
15	3-Br-C_6H_4	5o	2	83
16	2-Br-C_6H_4	5p	2	84
17	4-Cl-C_6H_4	5q	2	85
18	2-Cl-C_6H_4	5r	2	83
19	4-NO2-C_6H_4	5s	2	96
20	3-NO2-C_6H_4	5t	2	93

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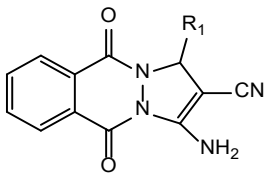
4. Discussion

Chemistry

The reaction of phthalic anhydride (1) with hydrazine hydrate gave the related phthalhydrazide (6) [27]. In another pathway, the intermediate (7) was prepared from the reaction of malononitrile (3) and appropriate benzaldehyde via the Knoevenagel condensation reaction. Finally, a cyclization reaction between intermediate (7) and phthalhydrazide (6) was carried out using the Michael-type addition reaction to afford the target products 5a-t in good yield (80%-95%) (Figure 3) [27]. In the presence of a catalyst, phthalic anhydride 1 reacts with hydrazine hydrate 2 to generate phthalhydrazide 6. Meanwhile, the Knoevenagel condensation of malononitrile 3 with aldehyde 4 produces intermediate 7. Subsequently, Michael-type addition of phthalhydrazide 6 and intermediate 7 followed by cyclization afforded the corresponding product 5a-t in good yields.

Table 2. Inhibitory effect (IC_{50} = µM) of selected compounds on α-glucosidase



Compound	R_1	Inhibition% (750 µM)	IC_{50} (µM) Mean±SD
5a	C_6H_5	8.0	_
5b	4-OMe-C_6H_4	7.0	_
5c	3-OMe-C_6H_4	3.0	_
5d	2-OMe-C_6H_4	4.0	_
5e	2,3-diOMe-C_6H_3	8.0	_
5f	3,4-diOMe-C_6H_3	17.0	_
5g	3,4,5- (OMe)–C_6H_2	3.0	_
5h	4-Me-C_6H_4	94.0	159.3±7.7
5i	3-Me-C_6H_4	27.0	_
5j	2-Me-C_6H_4	13.2	_
5k	CH_3	17.0	_
5l	4-F-C_6H_4	34.0	_
5m	2-F-C_6H_4	25.0	_
5n	4-Br-C6H4	88.0	231.0±6.6
5o	3-Br-C_6H_4	21.0	_
5p	2-Br-C_6H_4	8.0	_
5q	4-Cl-C_6H_4	95.0	155.4±6.0
5r	2-Cl-C_6H_4	35.0	_
5s	4-NO2-C_6H_4	89.0	230.5±7.0
5t	3-NO2-C_6H_4	19.0	_
Acarbose	-	50	750±8.6

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After synthesis steps and structure elucidation, the target compounds were evaluated regarding their activity as an α-glucosidase inhibitor. At first, the percentage of inhibition was determined for all the synthesis compounds. Among them, 5h, 5n, 5q, and 5s showed good activity with a percentage of inhibition of more than 85% at a concentration of 750 µM. Therefore, four compounds were selected for the determination of IC_{50}. Two compounds (5h and 5q) inhibited α-glucosidase with IC_{50} values of 159 µM and 155 µM, respectively. The structure-activity relationship study demonstrated that compounds with chlorine or methyl group in the para position of the phenyl ring (R1) showed the best activity. On the other hand, compounds containing methoxy groups in the same position did not show any inhibitory activity (Table 2).

In the docking study, the binding mode of the most active compounds was performed using AutoDock tools (version 1.5.6). According to experimental results, it

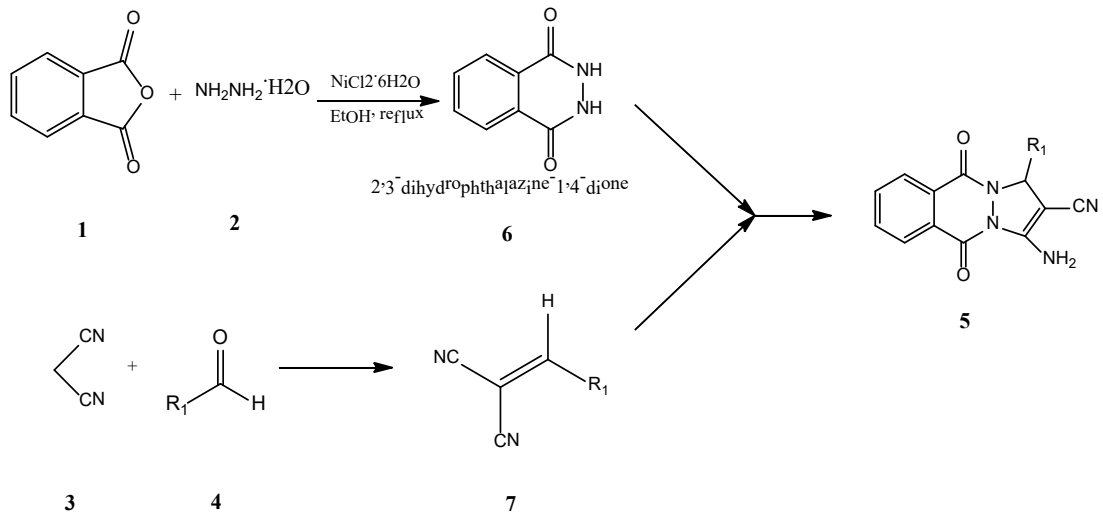

Figure 3. The synthesis pathway for the formation of pyrazolo[1,2-*b*]phthalazinone derivatives

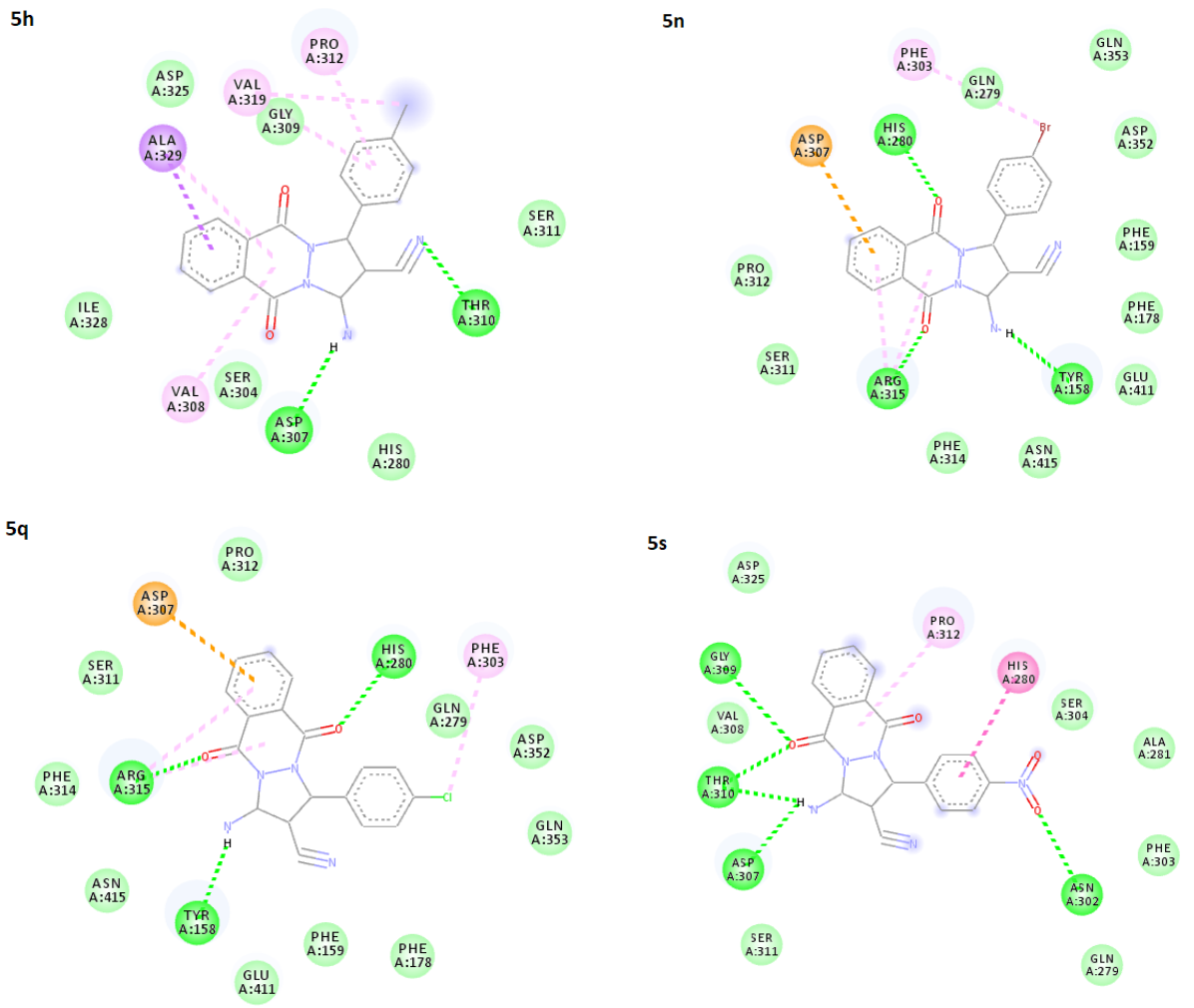

Figure 4. Possible mode of interactions between active compounds and enzyme active site

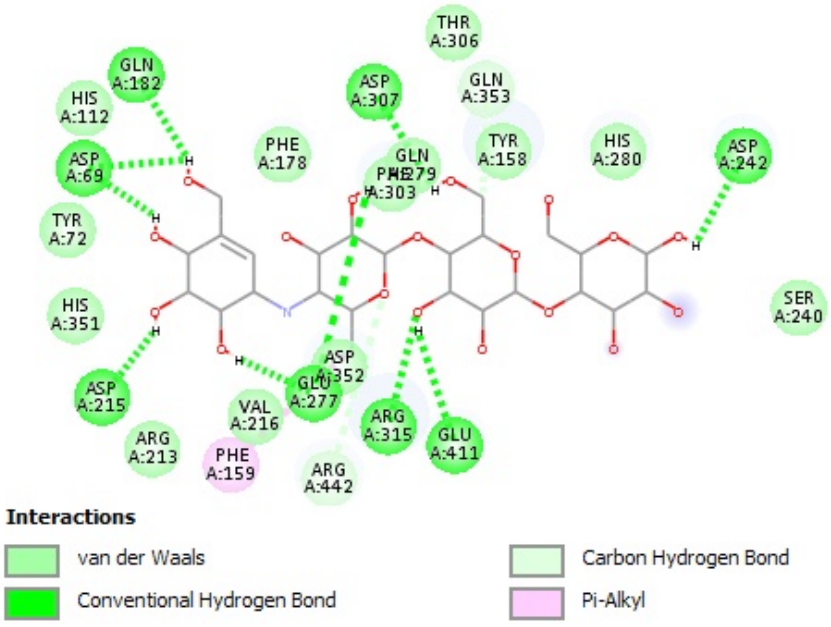

Figure 5. The predicted binding mode of Acarbose in the active site pocket

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seems that interactions between 5h and 5q with the active site of the enzyme are stronger than the interactions between 5n and 5s (Figure 4). These results are parallel with their biological activities. The binding mode of acarbose (Figure 5) demonstrates that it interacts with the active site of isomaltase via ten hydrogen bonds, two carbon-hydrogen bond, and one hydrophobic interaction with the residues Gln189, Asp242, Asp215, Phe159, Asp215, Glu277, Asp69, Arg315, Asp307, Gln353, Arg442, and Glu411.

5. Conclusion

A series of pyrazolo[1,2-b]phthalazine-5,10-dione derivatives 5a–t were synthesized using a new heterogeneous catalyst, $NiCl_2$-$6H_2O$ in EtOH at a concentration of 10 mol% and evaluated as new inhibitors for α-glucosidase. Among the tested compounds with α-glucosidase inhibitory activity, 5q and 5h compounds were the most active compounds with an IC_{50} Mean±SD value of 155.4±6.0 and 159.3±7.7 µM, respectively. They showed a significant activity far better than the reference drug, acarbose. A competitive inhibition mode on α-glucosidase of *Saccharomyces cerevisiae* was distinguished from the kinetic study of compound 5q with Ki of 152 µM. The molecular docking study also revealed that the four compounds (5h, 5n, 5q, and 5s) have significant binding interactions with the enzyme active site. The molecular docking studies results of compounds 5e and 5a were supported by the results of the in vitro assay test (Figure 6).

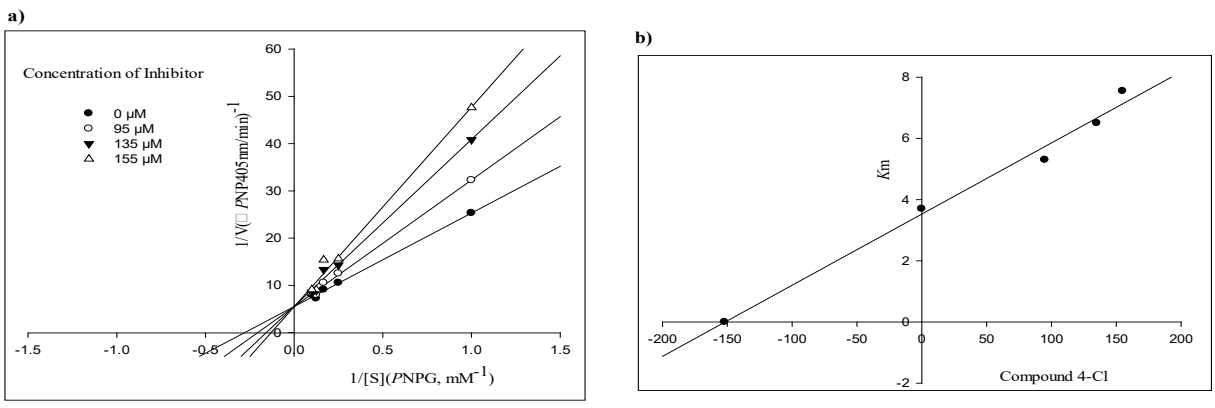

Figure 6. Kinetics of α-glucosidase inhibition by 5q

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A: The Lineweaver-Burk plot in the absence and presence of different concentrations of 5q; B: The secondary plot between Km and various concentrations of 5q.

The current study results showed that the pyrazolo[1,2-b]phthalazine-5,10-dione skeleton has potential for α-glucosidase inhibitory activity and could be considered for designing new chemical agents with higher potencies. In conclusion, the synthesized compounds of this class of heterocyclic themes have shown promising results for further development of potent, selective, and efficacious inhibitors against the α-glucosidase enzyme that could be a potential candidate for the treatment of diabetes.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization and supervision: Ahmad Mirshokrayi and Mohsen Amini; Methodology: Mehdi Khoobi; Data collection and data analysis: Maryam Hosseinpoor Tehrani.

Conflict of interest

The authors declared no conflict of interest.

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