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Novel pyridine-2,4,6-tricarbohydrazide derivatives: Design, synthesis, characterization and *in vitro* biological evaluation as α- and β-glucosidase inhibitors

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ABSTRACT

A range of novel pyridine 2,4,6-tricarbohydrazide derivatives **(4a–4h)** were synthesized and its biological inhibition towards α- and β-glucosidases was studied. Most of the compounds demonstrate to be active against α-glucosidase, and quite inactive/completely inactive against β-glucosidase. A number of compounds were found to be more active against α-glucosidase than the reference compound acarbose (IC_{50} 38.25 ± 0.12 μM); being compound **4d** with the *p*-hydroxy phenyl motive the most active (IC_{50} 20.24 ± 0.72 μM). Molecular modeling studies show the interactions of compound **4d** with the active site of target α-glucosidase kinase.

1. Introduction

Diabetes is one of those chronic diseases, which are considered among the main death causing factors in the world. WHO proposed that diabetes will be the 7th major disease responsible for most of the deaths by 2030. In fact, the number of diabetes patients has become more than double since 1980–2008 [1]. To cure diabetes, we need to control the postprandial glucose levels with the inhibition of α-glucosidase [2,3]. α-Glucosidase is located on the brush border surface of the small intestine, which reversibly inhibit digestive α-glucosidase and decreases glucose levels produced from dietary complex carbohydrate and starch. Hence, it decreases absorption of glucose into the blood stream and reduces plasma glucose levels.

In addition, α-glucosidase inhibitors are also used for the treatment of carbohydrate initiated diseases such as hepatitis, cancer and viral infections [4–6]. α-Glucosidase inhibitors are also known for their antitumor, anti-viral and immunoregulatory activities [7–10]. From α-glucosidase inhibitors, deoxynojirimycin (DNJ), *N*-butyl-deoxynojirimycin (NB-DNJ) and castanospermine are known to be potent inhibitors of both HIV replication and HIV mediated syncytium development [9]. Acarbose [10], miglitol [11], voglibose [12] and nojirimycin [13] are well known drugs, which are prescribed to control blood glucose levels (Fig. 1). These drugs are effective, but show a number of side effects, like abdominal distension, flatulence, meteorism and diarrhea [14]. Potent and safer α-glucosidase inhibitors are highly desired. A major synthetic focus has been put on non-glucosidic based inhibitors [15,16]. Our group has recently discovered pyrimidine derivatives as significant α-glucosidase inhibitors, 4-methylphenyl bearing pyrimidine (ethyl 6-methyl-4-(4-methylphenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate) was identified as most active of them all (IC_{50} 112.21 ± 0.97 μM) [17].

As part of our ongoing medicinal chemistry research interests [39–43] and vast medicinal potential of pyridine hydrazides have convinced us to synthesize novel pyridine hydrazide derivatives and evaluate their α-glucosidase inhibition potential.

Pyridine derivatives display a broad range of biological activities such as antimicrobial, antioxidant, anti-inflammatory [18–20], anti-cancer [21], analgesic, agonistic, hypotensive, hallucinogenic and anti-metastatic [22–24]. In particular it has been fused as a core skeleton in drug candidates involved in enzyme inhibitions [25]. Pyridine bearing hydrazide moieties such as isonicotinic acid

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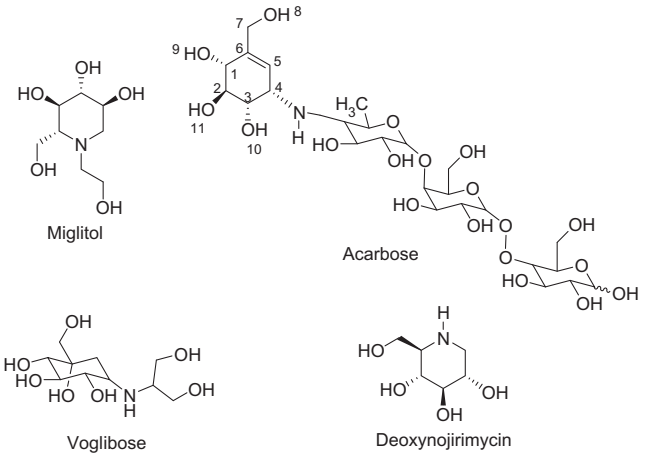


Fig. 1. Acarbose, voglibose, deoxynojirimycin and miglitol structures.

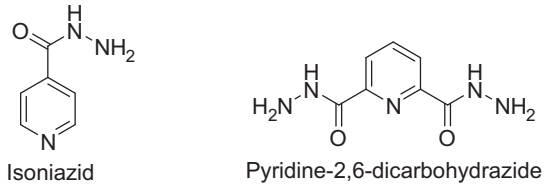


Fig. 2. The molecular structures of biologically well-known pyridine hydrazides.

hydrazide (INH, isoniazid) [26] and pyridine-2,6-dicarbohydrazide are pharmacologically useful targets as anticonvulsants [27], antidepressants [28], anti-inflammatory [29], antimalarials [30], antimycobacterials [31], anticancer [32], and antimicrobials [33–36] (Fig. 2).

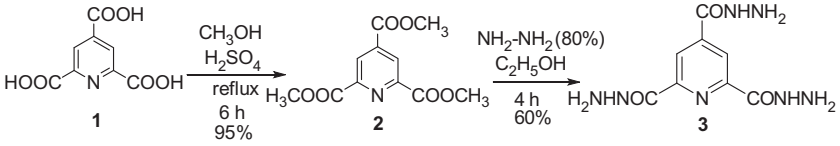
The docking method is used to estimate the conformation of the compounds and its orientation within the binding site of the enzyme. Docking studies aims: (1) specific structural modeling, (2) the accurate prediction of the activity [37]. MOE-Dock [38] was selected because allow ligands to be flexible during docking so that they can adjust their conformations in the binding pocket of the receptor.

In this paper, we report a facile synthesis and characterization of novel pyridine derivatives, their *in vitro* α-glucosidase inhibition activity and molecular modeling to demonstrate the pattern of binding interactions of our synthesized compounds into the targeted α-glucosidase.

2. Results and discussion

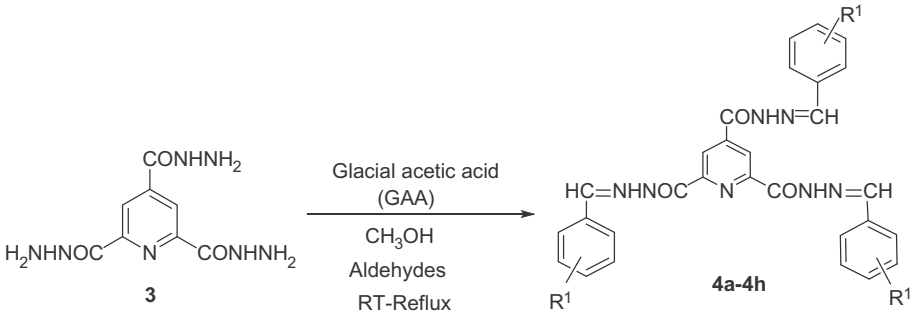
2.1. Chemistry

Compounds **3** and **4a–4f** were synthesized in good yields and purity from commercially available pyridine tricarboxylic acid (Scheme 1). Pyridine tricarboxylic acid was heated under reflux in the presence of methanol and sulfuric acid which resulted in trimethyl pyridine-2,4,6-tricarboxylate **2** in excellent yield (95%) [44,45]. Treatment of ester **2** with hydrazine in the presence of methanol (Scheme 1) afforded novel pyridine-2,4,6-tricarbohydrazide **3** in 60% yield.



Scheme 1. Synthesis of pyridine-2,4,6-tricarbohydrazide.

Table 1
Reaction conditions and yields of products **4a–h**.



Entry	Sample code	Time (h)	R^1	Yield (%)
1	**4a**	3	H	82
2	**4b**	4	*o*-OH	75
3	**4c**	3	*m*-OH	95
4	**4d**	4	*p*-OH	85
5	**4e**	5	*p*-*N*-$(CH_3)_2$	86
6	**4f**	6	*m*-NH_2	70
7	**4g**	5	-cinnamyl	60
8	**4h**	3	*p*-OCH_3	69

Table 2
α- and β-Glucosidase inhibition activity, and IC_{50} values (mean ± SEM, $n = 3$) of pyridine-2,4,6-tricarbohydrazide **3** and its derivatives **4a–4h**.

Entry	Sample code	α-Glucosidase from baker's yeast		β-Glucosidase from almonds	
		%Inhibition (0.5 mM)	IC_{50} μM ± SEM[a]	%Inhibition (0.5 mM)	IC_{50} μM ± SEM[a]
1	**3**	14.3 ± 0.9	–	2.83 ± 0.95	–
2	**4a**	98.1 ± 0.5	28.1 ± 0.4	NI[b]	–
3	**4b**	98.6 ± 0.4	26.8 ± 0.3	4.33 ± 1.92	–
4	**4c**	95.7 ± 0.8	22.5 ± 0.7	6.39 ± 1.64	–
5	**4d**	93.8 ± 0.7	20.4 ± 0.3	NI	–
6	**4e**	44.9 ± 0.9	>500	NI	–
7	**4f**	40.1 ± 1.2	>500	NI	–
8	**4g**	99.1 ± 0.6	23.3 ± 0.4	NI	–
9	**4h**	98.1 ± 0.8	28.1 ± 0.4	NI	–

[a] All reactions were performed in triplicates and averaged, and SEM is standard mean error of the experiments.
[b] No inhibition detected at 0.5 mM.

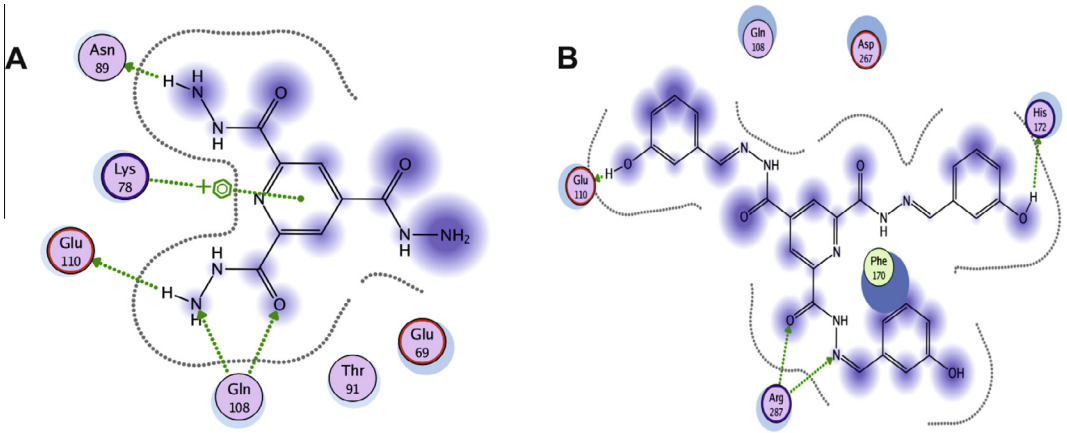


Fig. 3. 2D Interaction analysis of compounds in the active site of the enzyme: (A) **3** and (B) **4c**.

Pyridine-2,4,6-tricarbohydrazide **3** was treated with a wide range of selected aldehydes in the presence of methanol and few drops of glacial acetic acid. Good to excellent yields of the corresponding Schiff bases **4a–4h** (60–95%) were obtained as shown in Table 1.

2.2. Biological

2.2.1. α-Glucosidase activity from baker's yeast

The synthesized compounds **3** and **4a–4h** were screened for their α-glucosidase inhibitory activity and acarbose was used as a reference compound. The IC_{50} values of these compounds are listed in Table 2.

Pyridine 2,4,6-tricarbohydrazide **3** (Table 2, Entry 1) was found to be very weak inhibitor (% inhibition of 14.29 ± 1.21). In the Schiff bases series **4a–4h,** the un-substituted compounds (**4a** and **4g**) or and those containing a hydroxyl group (**4b–d**) were the most active, and with comparable inhibition. Compounds bearing amino substituents (**4e** and **4f**) showed to be inactive. In the phenol derivatives cases (**4b–4d**) the higher activity observed could be due to their proton donating ability, possibly related to reversible protonation [46]. Among these phenol compounds, **4d** with *p*-hydroxyl

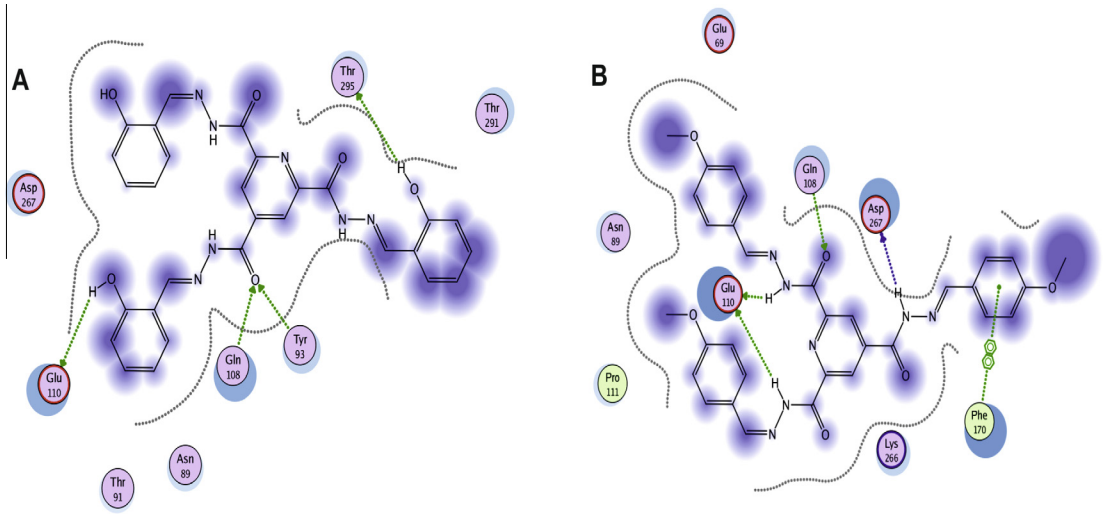


Fig. 4. 2D Interaction analysis of compounds in the active site of the enzyme: (A) **4b** and (B) **4h**.

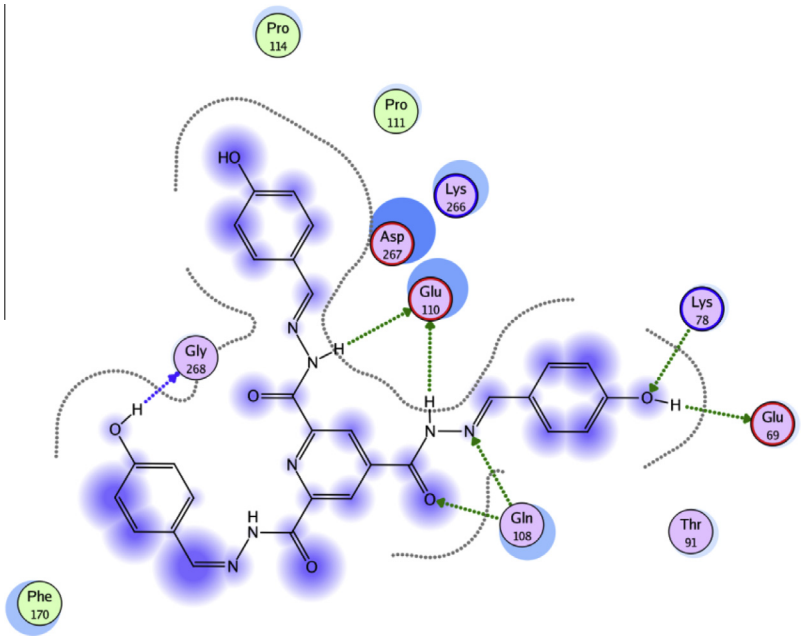


Fig. 5. 2D interaction analysis of compound **4d** in the active site of the enzyme.

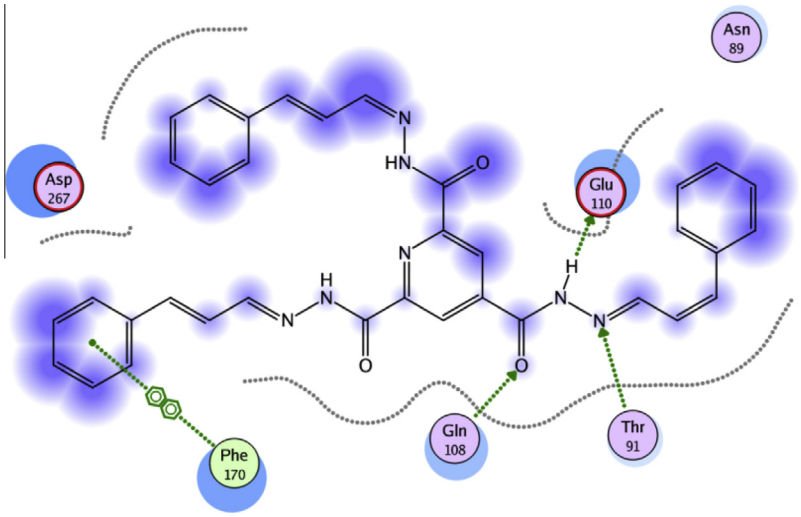


Fig. 6. 2D interaction analysis of compound **4g** in the active site of the enzyme.

group is the most active. This is may be due to the higher acidity of compound **4d** compared to the other phenol derivatives. On the other hand the lack of inhibition activity of compounds bearing amino groups (**4e** and **4f**), may be connected to its protonation inability. The presence of *p*-methoxy in the phenyl group seems to have no biological effect, since compound **4a** (R^1 = H) and **4h** (R^1 = *p*-OMe) gave the same IC_{50} value (28.12 ± 0.44 μM).

The overall order of synthesized pyridine derivatives based on IC_{50} value was as follows:

4d > **4c** > **4g** > **4b** > **4a** = **4h** > **4e** = **4f**

2.2.2. Inhibition towards β-glucosidase from almonds

All the synthesized pyridine derivatives (**3**, **4a–4h**) were investigated for their β-glucosidase inhibitory potential. The three compounds **3, 4b** and **4c** were found to be very weak inhibitors, whereas remaining compounds were completely inactive against β-glucosidase (Table 2).

2.2.3. Molecular modeling studies for α-glucosidase

*2.2.3.1. Interaction analysis of compound **3** and **4c**.* In case of compound **3**, a total of five interactions with the residues of the binding site were observed. Asn89, Gln108 and Glu110 interacts with formohydrazide moieties attached to pyridine ring. Whereas, Lys78 make arene-hydrogen bonding with pyridine ring as shown in Fig. 3A. The interaction analysis of compound **4c** (Fig. 3B) revealed hydroxyl benzylidene formohydrazide as an important interacting moiety, as three amino acid residues Glu110, His172 and Arg287 interact with the very same chemical moiety.

*2.2.3.2. Interaction analysis of compound **4b** and **4h**.* The molecular structure of compound **4b** is very much similar to compound **4c**. As observed in compound **4c**, the hydroxyl benzylidene formohydrazide moieties were found as the interacting feature of the compound. The active site residues Tyr93, Gln108, Glu110 and Thr295 interact with compound as shown in Fig. 4 A. In case of compound **4h**, five interactions were observed. The active site

Table 3
Molecular docking results showing binding energies and docking score.

Entry	Sample code	Docking score	Binding energy (GBVI/WSA)
1	**3**	−6.3050	−12.6011
2	**4a**	−12.0689	−23.9313
3	**4b**	−11.9923	−23.4106
4	**4c**	−12.6625	−24.1839
5	**4d**	12.7878	−26.2320
6	**4e**	−7.8998	−18.1921
7	**4f**	−7.6350	−17.2923
8	**4g**	−11.8713	−21.8861
9	**4h**	−10.9936	−20.7101
10	Acarbose	−11.5348	−22.3214

residues Gln108, Glu110, Phe170 and Asp267 interact with methoxy benzylidene formohydrazide moieties of the compound. Phe170 make arene-arene bonding with anisole ring of methoxy benzylidene formohydrazide moiety (Fig. 4B).

*2.2.3.3. Interaction analysis of compound **4d**.* The molecular structure of compound **4d** is closely related to compound **4b**. A total of seven interactions were observed in case of compound **4d**: Glu69, Lys78, and Gly268, Gln108, and Glu110 as shown in Fig. 5.

*2.2.3.4. Interaction analysis of compound **4g**.* In case of compound **4g**, a total of four interactions were observed: Thr91, Gln108 and Glu110 interact with formo hydrazide group of phenylallylidene formohydrazide moiety, whereas, Phe170 interacts through arene-arene bonding with terminal benzene ring (Fig. 6).

In order to rationalize the obtained IC_{50} values for the synthesized compounds against α-glucosidase, docking score and binding energy for all compounds have been calculated (Table 3). The Generalized-Born Volume Integral/Weighted Surface Area (GBVI/WSA) is a scoring function which estimates the free energy of binding of the ligand from a given pose. For all scoring functions, lower scores indicate more favorable poses [50]. In molecular docking studies, binding energies and docking score are the primary bases for the selection of active and non-active compounds.

3. Conclusion

In summary, a facile synthesis of a range of pyridine 2,4,6-tricarbohydrazide derivatives bearing different level of substituents was achieved in good to excellent yields. The α-glucosidase inhibitory activity of these pyridine derivatives was evaluated; acarbose was used as the reference compound. Structure activity relationship demonstrated that the inhibition potential of the synthesized compounds is greatly dependent upon the nature of R^1; compounds bearing hydroxyl-phenyl groups were comparatively more active, while amino compounds were inactive. Compound **4d** with *p*-hydroxyl-phenyl motif was the most active among this series (IC_{50} 20.24 ± 0.72 μM). Molecular modeling was performed on the inactive parent trihydrazide compound **3** together with the most active compounds (**4b–d**, **4g**, **4h**) for comparison purposes and understanding of the interaction patterns responsible for the inhibition activity.

4. Experimental

All chemicals and solvents used are of analytical grade and were purchased from Sigma Aldrich and Merck Chemical Company and used without further purification. TLC was run on the silica-coated aluminum sheets (silica gel 60 F254, E Merck, Germany) and visualized in low UV light. IR spectra in KBr pellets were recorded on the FT-IR Perkin Elmer spectrum BX spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz on JEOL-Lambda NMR instrument. Chemical shifts are quoted as δ ppm and the coupling constants J in Hz. Signals are described as s (singlet), d (doublet), m (multiplet) and br (broad). Melting points were measured on a Buchi 434 melting point apparatus and are uncorrected. Combustion analysis was performed on a Elementar, variomicrocube, Germany.

4.1. Synthesis of trimethyl pyridine-2,4,6-tricarboxylate 2

Trimethyl pyridine-2,4,6-tricarboxylate **2** was prepared using reported method [44]. Pyridine tricarboxylic acid **1** (10 g, 47 mmol) in methanol (70 mL) with concentrated sulfuric acid, 98% (5 mL) was heated under reflux for six hours until complete disappearance of compound **1**. The solution was neutralized with $CaCO_3$, filtered and solvent was evaporated under reduced pressure. The crude product was recrystallised from toluene to give a white crystalline product; Yield: 95%; m.p.: 161 °C IR (νmax, KBr, cm^{-1}): 3462, 3304 (NH stretch), 2976, 1737 (C=O), 1644 (C=N), 1488 (C—N); ^{1}H NMR (DMSO-d_6) δ_H: 8.54 (2H, apparent s), 3.95 (9H, apparent s).

4.2. Synthesis of pyridine 2,4,6-tricarbohydrazide 3

Hydrazine hydrate (80%, 5 eq.) was added to a stirred solution of trimethyl pyridine-2,4,6-tricarboxylate **2** (5 g, 24 mmol) in ethanol (50 mL) [47]. The reaction mixture was heated at reflux for 4 h and allowed to cool to RT, filtered, dried and recrystallized from ethanol to obtain pure product **3** (3 g, 70%); m.p.: 295 °C; IR (ν_{max}, KBr pellets, cm^{-1}): 3317 (NH stretch), 1649 (C=O), 1623 (C=N), 1235 (C—N), 1063(N—N); ^{1}H NMR (DMSO-d_6) δ_H: 10.66 (2H, apparent s), 10.42 (1H, s), 8.478 (2H, s), 4.66 (4H, s), 3.94 (2H, s).

4.3. General procedure for the synthesis of compounds (**4a–4h**)

4.3.1. General method A

Compounds **4a–4h** were prepared by following the method reported by Hearn et al. [48] with modifications. Pyridine 2,4,6-tricarbohydrazide **3** (1 equiv.) was dissolved into absolute ethanol (50 mL) in the presence of glacial acetic acid (2–3 mL). The reaction mixture was stirred at RT and then treated with selected aldehyde (3.3 equiv.). After being refluxed for 3–5 h, the reaction mixture was cooled, and placed in refrigerator overnight. The resulting solid was filtered, washed with water (200 mL), and *n*-hexane (30 mL).

4.3.2. N′-2,N′-4-dibenzylidene-6-((2benzylidenehydrazinyloxy)methyl) pyridine-2,4-dicarbohydrazide (**4a**)

Compound **4a** was prepared by following general method A: pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2.6 mmol, 1 equiv.), benzaldehyde (0.29 mL, 2.9 mmol), 3 h reflux. White amorphous solid; Yield: 82%; m.p. 310–312 °C; Rf (*n*-hexane: EtOAc 1:1) 0.5; IR (ν_{max}, KBr pellets, cm^{-1}): 1660 (C=O), 1551 (C=N), 1199 (C—N), 3412 (NH); ^{1}H NMR (DMSO-d_6) δ_H: 12.38 (3H, s), 8.84 (2H, s), 8.25 (2H, s), 8.12(1H, s), 7.84 (6H, s), 7.50 (6H, apparent s) 7.48 (3H, s); Anal. Calcd. for $C_{29}H_{23}N_7O_3$: C, 67.30; H, 4.48; N, 18.94. Found: C, 66.30; H, 4.34; N, 18.17.

4.3.3. N′-2, N′-4, N′-6-tris(2-hydroxybenzylidene)pyridine-2,4,6-tricarbohydrazide (**4b**)

The compound **4b** was prepared according to general method A: pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2.6 mmol), salicylaldehyde (0.27 mL, 2.9 mmol), 4 h reflux. White amorphous solid (735 mg); Yield: 75%; m.p.: 324–325 °C; R_f (*n*-hexane: EtOAc, 1:1) 0.42; IR (ν_{max}, KBr pellets, cm^{-1}): 1665 (C=O), 1609 (C=N), 1254 (C—N), 3375 (NH); ^{1}H NMR (DMSO-d_6) δ_H: 12.22 (3H, s), 11.28 (3H, s), 9.70 (2H, s), 8.69 (3H, d, J = 9.9 Hz), 8.64 (3H, d,

J = 9.3 Hz), 8.52 (3H, s), 7.33(3H, d, J = 9.2 Hz), 6.88 (3H, t, J = 6.3 Hz); Anal. Calcd for $C_{29}H_{23}N_7O_6$: C, 61.59; H, 4.10; N, 17.34. Found: C, 60.30; H, 4.34; N, 16.17.

4.3.4. N'-2, N'-4, N'-6-tris(3-hydroxybenzylidene)pyridine-2,4,6-tricarbohydrazide (**4c**)

Compound **4c** was prepared by following general A: pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2 mmol, 1 equiv.), 3-hydroxy benzaldehyde (0.8 g, 6.5 mmol), 3 h reflux. White amorphous solid; Yield: 95%, m.p.: 280°C; R_f (n-hexane: EtOAc, 1:1) 0.4; IR (ν_{max}, KBr pellets, cm^{-1}): 1660 (C=O), 1607 (C=N), 1254 (C—N), 3355(NH).^{1}H NMR (DMSO-d_6) δ_H: 12.32 (3H, s), 9.69 (3H, s), 9.44 (2H, s), 8.66 (3H, d, J = 9.9 Hz), 8.60 (3H, d, J = 9.3 Hz), 8.53 (3H, s), 7.23 (3H, t, J = 9.2 Hz), 6.83 (3H, t, J = 6.2 Hz); Anal. Calcd for $C_{29}H_{23}N_7O_6$: C, 61.59; H, 4.10; N, 17.34. Found: C, 60.20; H, 4.32; N, 16.06.

4.3.5. N'-2,N'-4,N'-6-tris(4-hydroxybenzylidene)pyridine-2,4,6-tricarbohydrazide (**4d**)

The compound **4d** was prepared by following general method A: pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2 mmol, 1 equiv.), 4-hydroxy benzaldehyde (0.8 g, 6.5 mmol), 4 h reflux. White amorphous solid. Yield: 85%, m.p.: 324 °C; R_f (n-hexane: EtOAc, 1:1) 0.4; IR (ν_{max}, KBr, cm^{-1}): 1660 (C=O), 1607 (C=N), 1254 (C—N), 3355(NH).^{1}H NMR (DMSO-d_6) δ_H: 12.19 (3H, s), 10.00 (3H, s), 8.88 (2H, s), 8.66 (3H, d, J = 9.9 Hz), 7.65 (6H, s), 6.86 (6H, s); Anal. Calcd for $C_{29}H_{23}N_7O_6$: C, 61.59; H, 4.10; N, 17.34. Found: C, 60.10; H, 4.22; N, 16.04.

4.3.6. N'-2,N'-4,N'-6-tris(4-(dimethylamino)benzylidene)pyridine-2,4,6-tricarbohydrazide (**4e**)

Compound **4d** was prepared by general method A: pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2 mmol, 1 equiv.), 4-(dimethylamino)benzaldehyde (0.97 g, 6.5 mmol), 5 h reflux. White amorphous solid; Yield: 86%; m.p.: 240–242 °C; R_f (n-hexane: EtOAc, 1:1) 0.6; IR (ν_{max}, KBr, cm^{-1}): 1660 (C=O), 742 (C—Cl), 1557 (C=N), 1200 (C—N), 3400 (NH); ^{1}H NMR (DMSO-d_6) δ_H: 12.12 (3H, s), 8.60 (2H, s), 8.51(2H, s), 7.65–7.68 (6H, m), 7.57 (1H, s), 6.7 (6H, d, J = 9 Hz), 3.00 (18H, s); Anal. Calcd for $C_{35}H_{38}N_{10}O_3$: C, 65.00; H, 5.92; N, 21.66. Found: C, 64.10; H, 4.92; N, 20.01.

4.3.7. N'2, N'4, N'6-tris(3-aminobenzylidene)pyridine-2,4,6-tricarbohydrazide (**4f**)

The compound **4f** was prepared by general method A: Pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2 mmol, 1 equiv.), 3-aminobenzaldehyde (0.78 g, 6.5 mmol), 6 h reflux. Yellow amorphous solid. Yield: 70%; m.p.: 320–321 °C; R_f (n-hexane: EtOAc, 1:1) 0.5; IR (ν_{max}, KBr, cm^{-1}): 1724 (C=O), 1557 (C=N), 1277 (C—N), 3430 (NH); ^{1}H NMR (DMSO-d_6) δ_H: 12.20(3H, s), 10.02 (2H, s), 9.97 (1H, s), 8.63 (3H, d, J = 6.9 Hz), 8.59 (2H, s), 8.53 (3H, d, J = 5.1 Hz), 7.66 (2H, d, J = 8.1 Hz), 7.58 (1H, d, J = 8.1 Hz), 6.88 (3H, d, J = 6.3 Hz), 3.97 (6H, s); Anal. Calcd for $C_{29}H_{26}N_{10}O_3$: C, 61.91; H, 4.66; N, 24.90. Found: C, 60.10; H, 3.92; N, 23.60.

4.3.8. N'-2, N'-4, N'-6-tris(3-phenylallylidene)pyridine-2,4,6-tricarbohydrazide (**4g**)

The compound **4g** was prepared by general method A: pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2 mmol, 1 equiv.), cinnamaldehyde (0.86 g, 6.5 mmol), 5 h reflux. Yellow amorphous solid; Yield: 60%; m.p.: 276 °C; R_f (n-hexane: EtOAc, 1:1) 0.6; IR (ν_{max}, KBr, cm^{-1}): 1724 (C=O), 1557 (C=N), 1277 (C—N), 3430 (NH); ^{1}H NMR (DMSO-d_6) δ_H: 12.24 (3H, s), 8.79 (3H, s), 8.62 (2H, s), 8.50(6H, d, J = 6.0 Hz), 7.67 (3H, d, J = 7.5 Hz), 7.46–7.35 (6H, s), 7.18 (3H, d, J = 8.7 Hz), 3.97 (3H, s); Anal. Calcd for $C_{35}H_{29}N_7O_3$ (595): C, 70.57; H, 4.91; N, 16.46. Found: C, 69.10; H, 3.91; N, 15.82.

4.3.9. N'-2,N'-4,N'-6-tris(4-methoxybenzylidene)pyridine-2,4,6-tricarbohydrazide (**4h**)

The Compound **4h** was prepared according to general method A: pyridine 2,4,6-tricarbohydrazide **3** (500 mg, 2 mmol, 1 equiv.), 4-methoxybenzaldehyde (0.88 g, 6.5 mmol), 3 h reflux. White amorphous solid; Yield: 69%; m.p.: 248–250 °C; R_f (n-hexane: EtOAc, 1:1) 0.4; IR (ν_{max}, KBr, cm^{-1}): 1724 (C=O), 1557 (C=N), 1277 (C—N), 3430 (NH); ^{1}H NMR (DMSO-d_6) δ_H: 12.31 (3H, s), 9.85(2H, s), 8.70 (2H, s), 8.68 (1H, s), 7.75 (4H, apparent d, J = 8.1Hz), 7.70 (2H, d, J = 8.4 Hz), 7.06–7.01 (6H, s), 3.81(9H, s); Anal. Calcd for $C_{32}H_{29}N_7O_6$: C, 63.25; H, 4.81; N, 16.14. Found: C, 61.25; H, 4.21; N, 16.04.

4.4. α-Glucosidase assay procedure

The α-glucosidase inhibition activity was performed with slight modifications as given by Pierre et al. [49]. Total volume of 100 μL reaction mixture contained, 70 μL 50 mM phosphate buffer, pH 6.8, 10 μL (0.5 mM) test compound, followed by the addition of 10 μL (0.0234 units, Sigma Inc.) α-glucosidase enzyme. The contents were mixed, preincubated for 10 min at 37 °C and pre-read at 400 nm. The reaction was initiated by the addition of 10 μL of 0.5 mM substrate (p-nitrophenyl glucopyranoside, Sigma Inc.). After 30 min of incubation at 37 °C, absorbance of the yellow color produced due to the formation of p-nitrophenol was measured at 400 nm using Synergy HT (BioTek, USA) 96-well microplate reader. Acarbose was used as positive control. All experiments were carried out in triplicates. The percent inhibition was calculated by the following equation;

$$\text{Inhibition}(\%) = (\text{abs of control} - \text{abs of test}/\text{abs of control}) \times 100$$

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IC_{50} values (concentration at which there is 50% in enzyme catalyzed reaction) were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

4.5. β-Glucosidase assay procedure

Total volume of 100 μL reaction mixture contained, 70 μL 50 mM phosphate buffer, pH 6.8, 10 μL (0.5 mM) test compound, followed by the addition of 10 μL (1.2 units/mL; G0395; Sigma) of β-glucosidase from almonds. The contents were mixed, pre-incubated for 10 min at 37 °C and pre-read at 400 nm. The reaction was initiated by the addition of 10 μL of 0.5 mM substrate (p-nitrophenyl β-glucopyranoside, Sigma Inc.). After 30 min of incubation at 37 °C, absorbance of the yellow color produced due to the formation of p-nitrophenol was measured at 400 nm using 96-well microplate reader. All experiments were carried out in triplicates. The percent inhibition was calculated by the following equation;

$$\text{Inhibition}(\%) = (\text{abs of control} - \text{abs of test}/\text{abs of control}) \times 100.$$

4.6. Protein preparation

The protein molecule included in our study, α-glucosidase kinase was obtained from Protein Data Bank. Water molecules were removed and the 3D protonation of the protein molecule was carried out. The energy of the protein molecule was minimized using the Energy minimization algorithm of MOE tool. The following parameters were used for energy minimization; gradient: 0.05, Force Field: MMFF94X + Solvation, Chiral Constraint: Current Geometry. Energy minimization was terminated when

the root mean square gradient falls below the 0.05. The minimized structure was used as the template for docking [46].

4.7. Molecular docking

The binding of the ligand molecule with the protein molecule was analyzed using MOE docking program to find the correct conformation of the ligand, so as to obtain minimum energy structure. After the completion of docking we analyze the best poses for hydrogen bonding/π–π interactions by using MOE applications.

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