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Design, Synthesis and Glucose-6-Phosphatase Inhibitory Activity of Diaminoguanidine Analogues of 3-Guanidinopropionic Acid and Amino Substituted (Pyridin-2-Yl)thiourea Derivatives

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Abstract

A series of diaminoguanidine analogues of 3-guanidinopropionic acid was designed by substitution at alpha position. Another series of amino substituted (pyridin-2-yl)thiourea derivatives was designed. Both groups of compounds were evaluated for in vivo glucose-6-Phosphatase inhibitory activity. Glucose-6-phosphatase activity was analyzed by release of phosphate during incubation at 37°C of liver homogenate containing enzyme with glucose-6-Phosphate as substrate. The amount of phosphate liberated was analyzed spectrophotometrically.

Keywords: Glucose-6-phosphatase, diaminoguanidine, (pyridine-2-yl)thiourea

Introduction

The glucose-6-phosphatase (G-6-Pase) system catalyzes the terminal enzymatic step of both gluconeogenesis and glycogenolysis by converting glucose-6-phosphate (G-6-P) to glucose and inorganic phosphate. Inhibition of the G-6-Pase system in the liver is expected to result in a reduction of hepatic glucose production irrespective to hepatic glucose output.[1] Very few compounds with activity on the G-6-Pase enzyme complex are known and most of them have been found to inhibit the T1 or T2 translocase, such as 2-hydroxy-5-nitrobenzaldehyde and chlorogenic acid derivatives. Recently, it was reported a series of 4,5,6,7-tetrahydrothienopyridines, which are potent competitive inhibitors of the G-6-Pase catalytic enzyme, the first report on non-carbohydrate compounds with potent activity at the catalytic site only. This suggests that specific inhibitors of the G-6-Pase catalytic protein could be an alternative to the T1-translocase inhibitors as potential antidiabetic drugs targeting G-6-Pase.[1] Though numerous compounds known to control type 2 diabetes with different mechanism but inhibitors of glucose-6-phosphatase as antihyperglycemic agents are not exploited extensively.[2,3] The significance of this enzyme was realized in controlling blood glucose level with the discovery of vanadium and pyridine derivatives as inhibitors of glucose-6-phosphatase.[4]

To evaluate the long-held concept that acidic guanidines lack glycemic effects, guanidinoalkanoic acids and the biguanide metformin (positive control) were administered to KKAy mice, a model of noninsulin-dependent diabetes. Two acidic guanidines, 3-guanidinopropionic acid (3-GPA) and guanidinoacetic acid, decreased the plasma glucose level; other compounds were ineffective. 3-GPA was more potent than even metformin.[5,6]

Recently, it has been reported that 3-guanidinopropionic acid **(I)**, 3-(pyridin-2-yl-amino)-propionic acid **(II)**, and 3-carbamimidoylsulfanyl-propionic acid **(III)** possess both antihyperglycemic and antiobesity activities in KKAy mouse.[4,7]

The high intolerance of these compounds necessitated to modify the structures to obtain efficacious compounds. The structural modification of **I–III** led to design and synthesize (3-pyridin-2-yl-thiouriedo)alkanoic acid ester **IV** (Figure 1), which were equipotent and less toxic.[4]

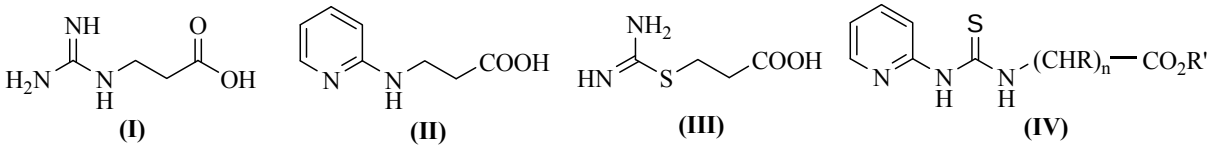
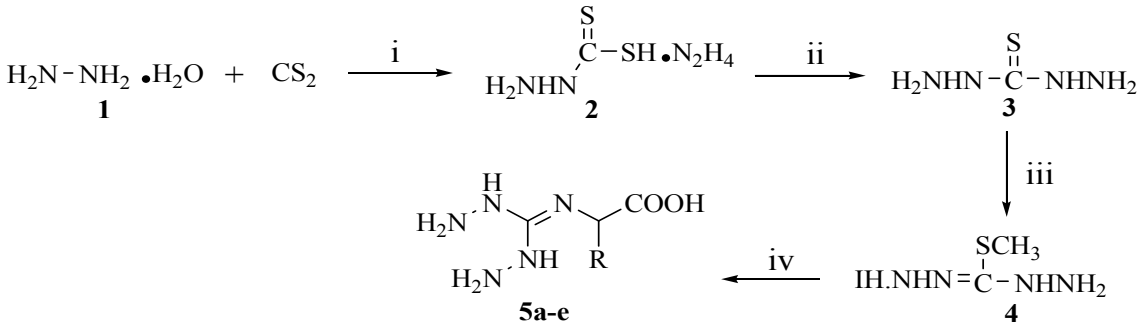


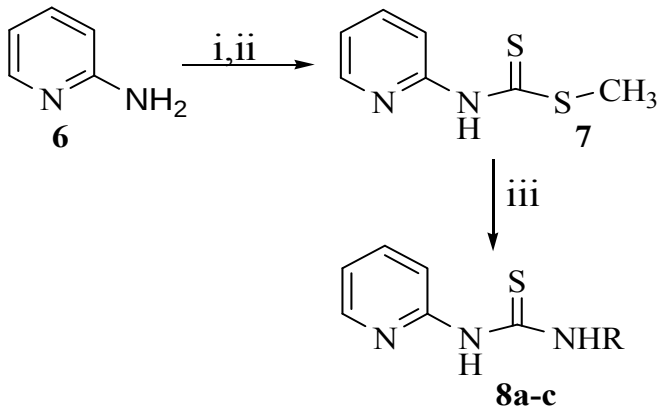
Figure 1. 3-guanidinopropionic acid **(I)**, 3-(pyridin-2-yl-amino)-propionic acid **(II)**, 3-carbamimidoylsulfanyl-propionic acid **(III)**, (3-pyridin-2-yl-thiouriedo)alkanoic acid ester **(IV)**

In search of more effective and least toxic antihyperglycemic agents, compounds within built amidine, thiourea, and amino acid ester pharmacophores were synthesized as potential inhibitors of glucose-6-phosphatase (G-6-pase), an enzyme responsible for maintaining normal blood glucose level.[8]

A new series of diaminoguanidine analogues of 3-guanidinopropionic acid was designed by substitution at alpha position (Scheme 1). Another series of amino substituted (pyridin-2-yl) thiourea derivatives was designed (Scheme 2) for having giucose-6-phosphatase inhibitory activity. The present study aims to create novel synthetic lead compounds for the future development of glucose-6-phoshatase inhibitory activity.



Scheme 1. Synthesis of diaminoguanidine analogues. Reagents and conditions: (i) 1 hr. stirring/reflux 1 hr. at 90°C; (ii) hydrazine hydrate/reflux 1.5 hr. at water bath; (iii) Methyl iodide; (iv) $NH_2CH(R)COOH/NaOH/EtOH/H_2O$/reflux 6 hr.



Scheme 2. Synthesis of (Pyridin-2-yl)thiourea derivatives. Reagents and conditions: (i) DMSO/KOH; (ii) CS_2/MeI/20°C; (iii) RNH_2/C_2H_5OH/85°C

Experimental Section

Melting Point was determined using capillary method on electric melting point apparatus and is uncorrected. ^{1}H NMR spectra were recorded on Bruker DRX-300 at 300 MHz using D_2O and $CDCl_3$ as solvent. The chemical shifts were measured at δ units (reported at ppm) relative to TMS. The IR spectra of the synthesized compounds were taken on Shimadzu IR Prestige-21. Elemental analysis was done with vario EL CHNS, Germany (Serial no.1106047). The progress of the reaction was checked by TLC in a solvent vapour saturated chamber on glass plates coated with Silica Gel G. Absorbance was taken on UV-1700, UV-Visible spectrophotometer, Shimadzu.

Synthesis of diaminoguanidine derivatives:

Hydrazinium dithiocarbazinate (2). Carbon disulfide (9.88 g, 0.13 mol) was added drop wise, with stirring, over a period of 1 hr to a warm (50°C) solution of hydrazine hydrate (13 g, 0.26 mol) and water (25ml). The mixture was then refluxed at 90°C for 1 hr. further and cooled; the colorless crystals which separated out were recrystallized from water. Yield: 52.1%; mp 119-120°C (lit.[21] 121°C); Anal. Calcd for $CH_8N_4S_2$: C, 8.56, H, 5.75, N, 39.95, S 45.73. Found: C, 8.61, H, 5.6, N, 39.81, S 46.04.

Thiocarbohydrazide (3). Hydrazinium dithiocarbazinate **(2)** (10 g, 0.071 mol) was dissolved in 50 ml of aqueous solution containing (3.55 g, 0.071 mol) hydrazine hydrate. The resulting solution was refluxed on steam bath for 90 min. Then it was cooled in ice bath and the resulting precipitate was washed with ethanol. It was recrystallized with water with few drops of conc. HCl. Yield: 51.7%; mp 169-171°C (lit.[21] 171°C); Anal. Calcd for CH_6N_4S: C, 11.31, H, 5.70, N, 52.78, S 30.21. Found: C, 11.10, H, 5.6, N, 53.21, S 30.47.

S-Methyl isothiocarbohydrazide (4). (2 g, 0.018 mol) of thiocarbohydrazide **(3)** in 40ml absolute ethanol was taken and heated under reflux on water bath. Thereafter (3.97 g,0.028 mol) of methyl iodide was added drop wise over a period of 30 min. Heating was continued until all the thiocarbohydrazide had undergone reaction. The resulting solution was filtered hot and the desired product crystallized on cooling. The product was obtained in the form of hydroiodide salt. Yield: 56.30%; mp 143°C; Anal. Calcd for $C_2H_9IN_4S$: C, 9.6; H, 3.6; N, 22.5; S, 12.9. Found: C, 9.7; H, 3.7; N, 22.5; S, 15.0

2-{[bis(hydrazine)methylene]amino}-4-methylpentanoic acid (5a). To (260 mg, 2.02 mmol) of L-leucine suspended in 20ml absolute ethanol, in a oil bath was added in order, triethylamine (4.04 mmol) and water 10 ml. The mixture was stirred until the majority of the amino acid has dissolved, then S-methyl isothiocarbohydrazide (1 g, 4.04 mmol) was added in six approximately equal portions over 1 hr. The mixture was allowed to stir for 8 hr at 60°C, then cooled to room temperature. The solid was isolated by filtration, washed with ethanol and dried. Yield: 49.9%; mp 193-195°C; IR (KBr): 3315 cm^{-1} (broad N—H stretch), 2990 cm^{-1} (C—H stretch), 1664 cm^{-1} (C═O stretch), 1294 cm^{-1} (C—N stretch); Anal Calcd. for $C_7H_{17}N_5O_2$: C, 41.3; H, 8.4; N, 34.4; O, 15.7. Found: C, 42.5; H, 7.5; N, 35.6.

2-{[bis(hydrazino)methylene]amino}-3-methylpentanoic acid (5b). 260 mg, 2.02 mmol) of L-isoleucine was taken and the same procedure was followed as that of **(5a)**. Yield: 0.35 g (42.6%); mp 196-200°C; IR (KBr): 3265 cm^{-1} (N—H stretch), 2989 cm^{-1} (C—H stretch), 1654 cm^{-1} (C═O stretch), 1498 cm^{-1} (C—H bending); ^{1}H NMR D_2O (300 MHz) δ 8.58 (s, 1H, COOH), 3.82 (m, 2H, NH), 3.262-3.333 (m, 4H, NH_2), 2.62 (d, 1H, CH), 1.81 (m, 1H, CH), 1.35-1.4 (m, 2H, CH_2), 1.04 (d, 3H, CH_3), 1 (t, 3H, CH_3); Calcd. for $C_7H_{17}N_5O_2$: C, 41.3; H, 8.4; N, 34.4; O, 15.7. Found: C, 42.2; H, 7.8; N, 33.9

2-{[bis(hydrazino)methylene]amino}-3-hydroxypropanoic acid (5c). (210 mg, 2.02mmol) of L-serine was taken and the same procedure was followed for the synthesis as that of **(5a)**. Yield: 0.37 g, (51.7%); mp 184-188°C; IR (KBr): 3448 cm^{-1} (broad N—H stretch), 3010 cm^{-1} (O—H stretching), 1600 cm^{-1} (C═O stretch), 1469 cm^{-1} (C—H deforming);

Calcd. for $C_4H_{11}N_5O_3$: C, 27.3; H, 6.2; N, 39.5; O, 27.0. Found: C, 26.5; H, 7.5; N, 40.7.

2-{[bis(hydrazino)methylene]amino}-3-(4-hydroxyphenyl)propanoic acid (5d). (360 mg, 2.02 mmol) of L-tyrosine was taken and the same procedure was followed as that of **(5a)**. Yield: 0.4 g, (39.2%); mp 210-215°C; IR (KBr): 3265 cm^{-1} (N—H stretch), 3138 (aromatic C—H stretching), 1674 cm^{-1} (C═O stretch); Calcd. for $C_{10}H_{15}N_5O_3$: C, 47.4; H, 5.9; N, 27.6; O, 18.9. Found: C, 46.9; H, 6.1; N, 26.8.

2-{[bis(hydrazino)methylene]amino}-3-hydroxybutanoic acid (5e). (240 mg, 2.02mmol) of L-threonine was taken and the same procedure was followed as that of **(5a)**. Yield: 0.35 g (45.3%); mp decomposes at 205°C; IR (KBr): 3300 cm^{-1} (N—H stretch), 3205 (O—H stretching), 2964 cm^{-1} (C—H stretch), 1641 cm^{-1} (C═O stretch); Calcd. for $C_5H_{13}N_5O_3$: C, 31.4; H, 6.8; N, 36.6; O, 25.1. Found: C, 30.6; H, 7.5; N, 35.5.

(Pyridin-2-yl)thiourea derivatives:

pyridine-2-yl-dithiocarbamic acid methyl ester (7). (1 g, 0.062 mol) of conc. Ammonia solution was slowly added with stirring to a solution of (3 g, 0.031 mol) of 2-aminopyridine, (4.7 g, 0.062 mol) of carbon disulfide and 20 ml of methanol at 10-15°C. The ammonium salt formed was added to 20 ml of methanol and (4.3 g, 0.062mol) of methyl iodide was added drop wise while stirring at 10-15°C. Yield: 2.3 g (40.3%); mp 115-118°C; IR (KBr): 3450 cm^{-1} (NH stretch), 3050 cm^{-1} (C—H stretch), 1450 cm^{-1} (C—C stretch), 1272 cm^{-1} (C═S stretch)

N-(pyridine-2-yl)morpholine-4-carbothioamide (8a). A mixture of pyridine-2-yl-dithiocarbamic acid methyl ester (0.7 g, 3.8 mmol) and morpholine (0.34 g, 3.8 mmol) was refluxed in 20 ml methanol for 5 hr. The reaction mixture was left overnight at room temperature. The crystal thus formed were filtered and washed with chilled alcohol. Yield: 0.4 g (45.4%); mp 153-157°C**;** IR (KBr): 3472 cm^{-1} (NH stretch), 3030 cm^{-1} (C—H stretch), 1492 cm^{-1} (C—C stretch), 1288 cm^{-1} (C═S stretch), 1180 cm^{-1} (C—O stretch); ^1H NMR $CDCl_3$ (300 MHz) δ 7.32-7.37 (t, 3H, CH), 7.26 (s, 1H, NH), 7.11-7.19 (t, 3H, CH), 3.8-3.83 (t, 4H, CH_2), 3.71-3.75 (t, 4H, CH_2); Calcd. for $C_{10}H_{13}N_3OS$: C, 53.7; H, 5.8; N, 18.8; O, 7.1; S, 14.3. Found: C, 52.3; H, 6.9; N, 19.2; S, 15.1.

N-(pyridine-2-yl)piperidine-4-carbothioamide (8b). The same procedure as of **(8a)** was used by taking (0.32 g, 3.8 mmol) of piperidine. Yield: 0.35 g (42.1%); mp 164-166°C; IR (KBr): 3261 cm^{-1} (NH stretch), 3043 cm^{-1} (C—H stretch), 1410 cm^{-1} (C—C stretch), 12281288 cm^{-1} (C═S stretch); ^1H NMR $CDCl_3$ (300 MHz) 7.3-7.35 (t, 1H, CH), 7.26 (s, 1H, NH), 7.08-7.15 (t, 3H, 3H), 3.77-3.78 (d, 4H, CH_2), 1.56-1.66 (m, 6H, CH_2); Calcd. for $C_{11}H_{15}N_3S$: C, 59.6; H, 6.8; N, 18.9; S, 14.4. Found: C, 58.5; H, 7.7; N, 19.3; S, 15.2.

1-propyl-3-(pyridyn-2-yl)thiourea (8c). The same procedure as of **(8a)** was used by taking (0.22 g, 3.8 mmol) of piperidine. Yield: 0.3 g (40.5%); mp 151-153°C; IR (KBr): 3473 cm^{-1} (NH stretch), 3035 cm^{-1} (C—H stretch), 1492 cm^{-1} (C—H deforming), 1442 cm^{-1} (C—C stretch), 1288 cm^{-1} (C═S stretch); Calcd. for $C_9H_{13}N_3S$: C, 55.3; H, 6.7; N, 21.5; S, 16.4. Found: C, 54.5; H, 7.8; N, 21.3; S, 17.2.

The synthesized compounds were evaluated for in vitro glucose-6-phosphatase inhibitory activity. Among the 8 screened compounds, three compounds demonstrated good inhibitory activity ranging from 35% to 40% tested at 100µM concentration.

Partial purification of G-6-Pase (D-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9) from goat liver

The liver of goat (~10 g) was taken and homogenate was prepared in a 40 ml solution containing 250 Mm sucrose, 4 mM EDTA and 1 mM sodium fluoride. Following homogenization the total volume of tissue suspension was brought to 60 ml with buffer solution. The homogenate was centrifuged at 10000 rpm for 20 minutes. The supernatant was decanted and used as enzyme source.

Glucose-6-phosphatase enzyme assay

Glucose-6-phosphatase activity can be analyzed by either release of phosphate or

glucose during incubation at 37°C of liver homogenate with glucose-6-phosphate as substrate.[9,10] Phosphate is estimated spectrophotometrically.[11-14] The assay of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase; *EC* 3.1.3.9) is complicated by the fact that glucose-6-phoshate may be hydrolysed by more than one enzyme. So, a specific assay system for glucose-6-phosphatase should, therefore, include EDTA and sodium fluoride to inhibit alkaline phosphatase (orthophosphoric monoester phosphohydrolase; *EC* 3.1.3.1) and acid phosphatase (orthophosphoric momoester phosphohydrolase; *EC* 3.1.3.2) respectively and thus allow the determination of the true glucose-6-phosphatase activity.[15,16]

The reagents used in the assay were prepared as following:

- Standard phosphate solution: 0.438 g of potassium dihydrogen phosphate was dissolved in 100 ml water, 1ml conc. H_2SO_4 was added as preservative. 5 ml of it was diluted to 100 ml water to give 50µg/ml concentration.
- 10% ammonium molybdate solution: 25 ml of it was prepared in 5000 mM of sulphuric acid.
- Taussky-Shorr color reagent (TSCR): It was prepared by adding 10 ml of ammonium molybdate solution to 70 ml of water. Then 5 g ferrous sulphate was dissolved. Final volume was adjusted to 100 ml.
- Glucose-6-phosphate solution (G-6-P): 0.140 g was dissolved in 100 ml water to give 5mM concentration.
- 50 ml of 5% (w/v) sodium dodecyl sulphate (SDS) solution.
- Maleate buffer (pH 6.0): It was prepared by adding 0.2 N NaOH to 25 ml of 0.2 M maleic acid and pH was adjusted to 6.0. Then volume was adjusted to 100 ml by water.

The effect of test compounds was studied by incubating 100 µM of the compound in 2.5 ml reaction system containing 1 ml enzyme buffer soln., 0.5 ml test compound and 1 ml G-6-P. The reaction was terminated after 15 minutes by adding 0.5 ml SDS soln. Then 2 ml of TSCR was added for development of color. The amount of phosphate liberated was analyzed spectrophotometrically. The amount of inorganic phosphate liberated in the absence of substrate and enzyme respectively should be subtracted from that obtained with the complete system.

Results and Discussion

Determination of λ_{max} of phosphate at a concentration of (5µg/ml):

The λ_{max} of standard inorganic phosphate was determined at a concentration of 5µg/ml. The spectrum was recorded in a Shimadzu Model UV-1700 UV/VIS spectrophotometer over the wavelength span of 600 to 780 nm. The absorbance measurement was carried out at the end of 15 minutes. The λ_{max} was found to be 701.7 nm. (Figure. 2)

Standard curve of phosphate:

Standard curve for inorganic phosphate was determined at λ_{max} 701.7 nm. In a 5.0 ml assay system the concentration of Pi was varied from 1 to 7 µg. The absorbance data is depicted in Table 1. The standard curve is shown in (Figure. 3)

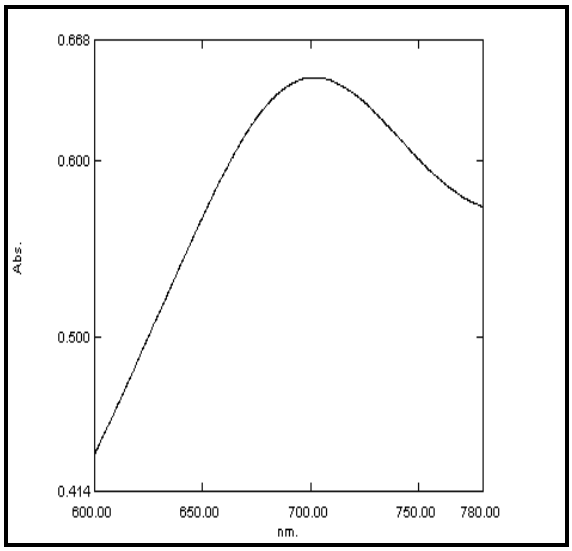


Figure. 2: Spectra of molybdenum blue

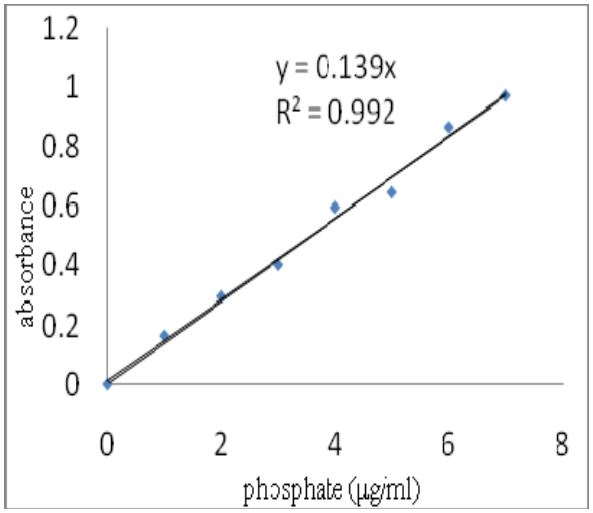


Figure.3: Standard curve for inorganic phosphate

Glucose-6-phosphatase assay and testing of synthesized compounds:

4.9714 µg/ml of phosphate was liberated when 1 ml of enzyme buffer solution was incubated at 37°C with G-6-P in 5 ml reaction system for 15 minutes. (Table 1)

Sl. No.	*Contents*	*Avg. Pi(µg/ml) ± SD (n=3)*
1	G-6-Pase + G-6-P	5.002 ± 0.0357
2	G-6-Pase	0.0293 ± 0.0025
3	G-6-P	0.0013 ± 0.0005

Table 1: Glucose-6-phosphatase assay: Amount of phosphate liberated after incubation at 37°C for 15 minutes

Test compounds (100 µM) were added to reaction mixture containing 1 ml G-6-Pase in maleate buffer and 1 ml G-6-P. Sodium dodecyl sulphate was added to terminate reaction and color was developed by adding Taussky-Shorr color reagent (TSCR). Out of the 8 compounds evaluated for their glucose-6-phosphatase, only three compounds demonstrated significant activity (Table 2). Compound **8c** was found to be most active among them most active (40.71% inhibition) followed by 8b and 8a having 38.88 % and 35.32 % inhibitory activity respectively.

Sl. No.	*Compound*	*Avg. Pi(µg/ml) ± SD(n=3)*	*% inhibition*[a]
1	5a	4.872 ± 0.0181	2.61
2	5b	4.834 ± 0.0220	3.37
3	5c	4.477 ± 0.0311	10.56
4	5d	4.673 ± 0.0149	6.61
5	5e	4.421 ± 0.0181	11.68
6	8a	3.069 ± 0.0250	35.32
7	8b	2.978 ± 0.0190	38.88
8	8c	3.246 ± 0.0181	40.71

[a] Values are mean of three experiments

Table 2: Testing of synthesized compounds % inhibition of the test compounds

Conclusion

The diaminoguanidine analogues of 3-GPA 5a-e were found to have relatively insignificant as compared to the (pyridine-2-yl)thiourea derivatives 8a-c. This may be due to their non cyclic structure and presence of carboxyl group. From the result it can be concluded that pyridine nucleus may have a significant role in antidiabetic activity. The lipophilicity and the long chain structure of diaminoguanidines might be causing decrease in activity due to steric effect.

Acknowledgements

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