RESEARCH ARTICLE

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# Synthesis And Evaluation of Novel 1-(2-(5-Aryl-1,3,4-Thiadiazol-2-Ylamino)Acetyl)Pyrrolidine-2-Carbonitrile Derivatives for their DPP-4 Inhibiting Activity

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**Abstract:** The current therapeutic agents for type 2 diabetes (like Insulin, Sulphonylureas, Biguanides, α-Glucosidase inhibitors, PPAR agonist and GLP-1 agonist), although effective in increasing insulin secretion, are associated with some safety issue and undesirable side effects, including hypoglycemia, abnormalities in cardiovascular responses and β-cell apoptosis. DPP-4 inhibitors offer several potential advantages over existing therapies including decreased risk of hypoglycemia, potential for weight loss and the potential for regeneration and differentiation of pancreatic β-cells. Moreover, DPP-4 inhibitors can also be administered orally. Among all DPP-4 inhibitor derivatives, 2-Cyano pyrrolidine-based inhibitors have been studied most extensively. Apart from behaving as a proline mimic, the presence of the nitrile on the five-membered ring was shown to provide (i) nanomolar inhibition of DPP-4 and (ii) chemical stability adequate for oral administration. These intermediate was fused with 2-amino-5-aryl-1, 3, 4-thiadiazole derivative to get series of novel 1-(2-(5-aryl-1,3,4-thiadiazol-2-ylamino)acetyl)pyrrolidine-2-carbonitrile derivatives. The synthesized DPP-4 inhibitor derivatives were evaluated by fluorescence assay using Gly-Pro-AMC as a DPP-4-specific fluorescent substrate.

## INTRODUCTION

Overall, DPP 4 inhibitors are promising new class of antidiabetics and intense research in this area has resulted in the launch of sitagliptin, vildagliptin, saxagliptin (Onglyza™), alogliptin and linagliptin as new weapon in the arsenal of oral antihyperglycemic agents. [1, 2] In clinical studies, DPP 4 inhibitors have generally been well tolerated. Clinical trials have indicated that DPP 4 inhibitors generally do not cause severe hypoglycemia or weight gain. The weight neutrality of this class distinguishes these agents from other commonly used antidiabetic medications, including insulin, sulfonylureas and thiazolidinediones. Nowadays, many newer anti-diabetic therapies like 11β-hydroxysteroid dehydrogenase 1 inhibitors, sodium-glucose co-transporter 2 inhibitors, glucagon-receptor antagonists, dipeptidyl peptidase-4 inhibitors metabolic inhibitors of hepatic glucose, pancreatic-G-protein-coupled fatty-acid-receptor agonists, insulin-releasing glucokinase activators have been emerge to overcome such side effects. [3] Among these, DPP 4 inhibitors have proven their potential for long term glycemic control globally.

DPP 4 is a serine exopeptidase enzyme which selectively binds to substrates containing proline or alanine at the penultimate P1- position. Hence, those DPP-4 inhibitors which contain proline mimic 5-membered heterocyclic rings like pyrrolidine, thiazolidine, cyanopyrrolidine or cyanothiazolidine at P1 site are classified as peptidomimetics. These DPP-4 inhibitors usually contain electrophile which forms covalent bond to the catalytic residue $Ser_{630}$ and shows a time-dependent kinetic profile.

As mention earlier, DPP 4 enzyme selectively cleaves the N-terminal dipeptides (X-Ala or X-Pro) from target polypeptides, such as GLP-1 and GIP. [4] Also, structure of DPP-IV enzyme resembles with several other protease enzymes and it exhibit broad substrate specificity. Thus, in order to develop selective DPP-IV inhibitor, we decided to design dipeptide based DPP-IV inhibitors, based upon the sequence homology of first two amino acids of GLP-1 peptide (His-Ala) and SAR study of DPP-IV inhibitors, which are in clinic or in clinical development. [5, 6]

However, reported events are often mild and include nasopharyngitis, upper respiratory tract infection, headache and cough. The currently available DPP 4 inhibitors, vildagliptin and sitagliptin leads to the increased risk of infection (e.g., nasopharyngitis and urinary tract infection) and headache due to the potential involvement of DPP 4 in immune functions as indicated by clinical studies. Short half life; inefficient inhibition of gastrointestinal functions and diabetic complications with the administration of vildagliptin has always been a matter of concern. Short half life is attributed to cyanopyrrolidine structural class of DPP 4 inhibitor which has suffered from varying degrees of chemical instability which have hampered formulation efforts.

## MATERIALS AND METHODS

All chemicals like L-proline, dicyclohexylcarbodiimide, ammonium bicarbonate, trifluoroacetic anhydride, thiosemicarbazide, Benzoic acid, 4-chloro benzoic acid, 4-methyl benzoic acid, 4-methoxy benzoic acid, 4-fluoro benzoic acid, 4-hydroxy benzoic acid and 4-bromo benzoic acid were purchased from S.D Fine Chemicals, Baroda and solvents like chloroacetyl chloride, dichloromethane, Tetrahydrofuran, 1, 4 – dioxane, Concentrated sulphuric acid, were purchased from Loba Chemie, Mumbai. When needed, the solvents were dried by distillation. Reaction progress was monitored on Merck precoated silica gel thin-layer chromatography (TLC) plates (without fluorescent indicator) the spots were visualized in UV and/or in iodine vapours. The chromatographic purification was performed on silica gel (200-400 mesh). Melting points were determined on a SMP 203 digital melting point apparatus

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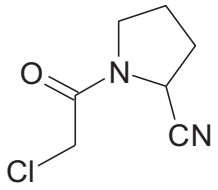



1-(2-chloroacetyl)pyrrolidine-2-carbonitrile (1)

**Figure 1:** Chemical structure of 1-(2-chloroacetyl) pyrrolidine-2-carbonitrile(1)

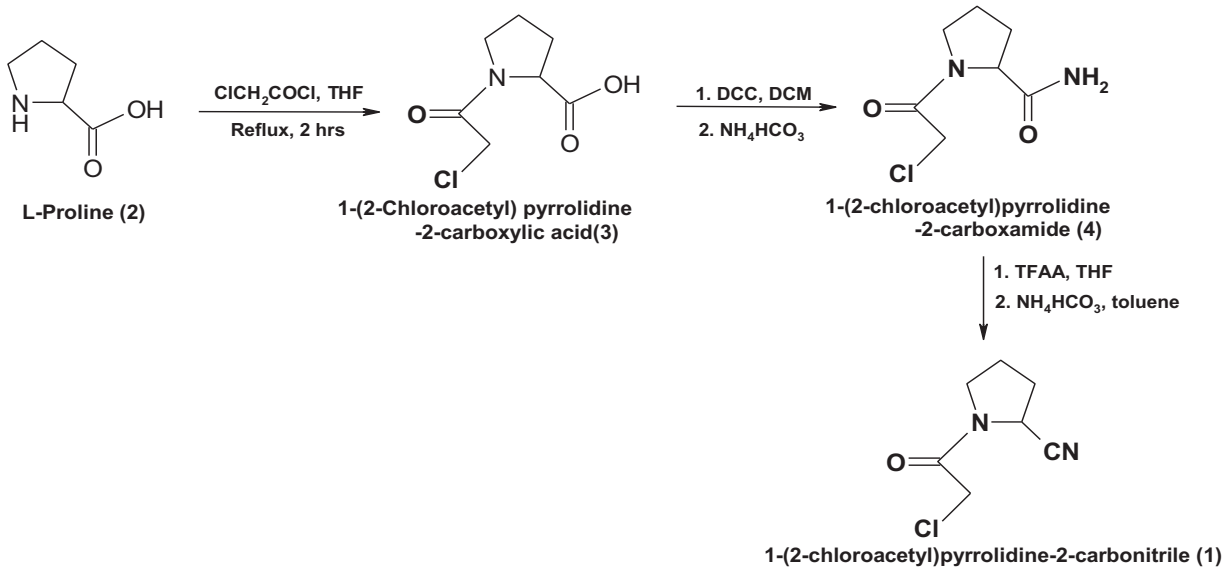



**Figure 2:** Synthesis of 1-(2-chloroacetyl)pyrrolidine-2-carbonitrile(1)

from Lab Intelligence Appliances. IR spectra of the synthesized compounds were recorded on Shimadzu FT-IR 8400 model using KBr method. Various functional groups present were identified by characteristic frequency obtained for them. Mass spectra of the synthesized compounds were recorded on Shimadzu GCMS QP 2010 model using direct injection probe technique. The molecular ion peak was found in agreement with molecular weight of the respective compound. Fragmentation pattern can be observed to be particular for these compounds and the characteristic peaks obtained for each compound. $^1$H NMR spectra of the synthesized compounds were recorded on Bruker Avance II 400 spectrometer by making a solution of samples in DMSO-$d_6$ solvent. Chemical shifts were measured in parts per million (ppm) downfield from an internal tetramethylsilane (TMS) standard. Elemental analysis was performed with Elementar Vario EL III analyzer for C, H, N and the results were found within ±0.4% of the calculated value.

Incorporation of a 2-cyanopyrrolidine moiety into a molecule can be carried out by using 1-(2-chloroacetyl) pyrrolidine-2-carbonitrile(1) as a reactant. Hence, this reactant has become a key intermediate for the synthesis of many DPP 4 inhibitors like vildagliptin.

By alternative synthetic method, 1-(2-chloroacetyl) pyrrolidine-2-carbonitrile(1), synthesized from L-proline(2) which is less expensive and readily available. [7] This synthetic route neither involves *N*-protection-/ deprotection strategy nor a complicated isolation method as it was in earlier routes.

In this method, chloroacetyl group play the role of a protecting group so that the use of an additional protecting group and its removal (i.e. deprotection) can be avoided. Moreover, chloroacetyl group is also a part of the key intermediate. Therefore, L-proline(2) was *N*-acylated with chloroacetyl chloride to prepare 1-(2-chloroacetyl) pyrrolidine-2-carboxylic acid(3) in good yield. We also observed that compound(3) can be prepared with 78 % yield by *N*-acylation of L-proline which proceeds faster in THF at an elevated temperature within 2 hours.

The compound(3) was treated with dicyclohexylcarbodiimide (DCC) at room temperature in dichloromethane followed by ammonium bicarbonate (Figure 2) to convert the carboxylic acid moiety of compound(3) to the amide moiety of compound(4) in good yield.

Further, 1-(2-chloroacetyl) pyrrolidine- 2-carbonitrile, a key intermediate for the synthesis of DPP 4 inhibitors was also found to be soluble in water. Thus it can be prepared by treatment of a solution of amide(4) in THF with trifluoroacetic anhydride. The side product trifluoroacetic acid was neutralized by ammonium bicarbonate after completion of the reaction and the desired product(1) was isolated (HPLC purity 99.25%) from the toluene extract.

**Synthesis of 1-(2-chloroacetyl)pyrrolidine-2-carbonitrile (1)**

A trifluoroacetic anhydride (4.4 ml, 0.0315 mol) was added at 0-5°C to a suspension of amide(4) (4.0 g, 0.0209 mol) in THF (40 ml) and the reaction mixture was then stirred at

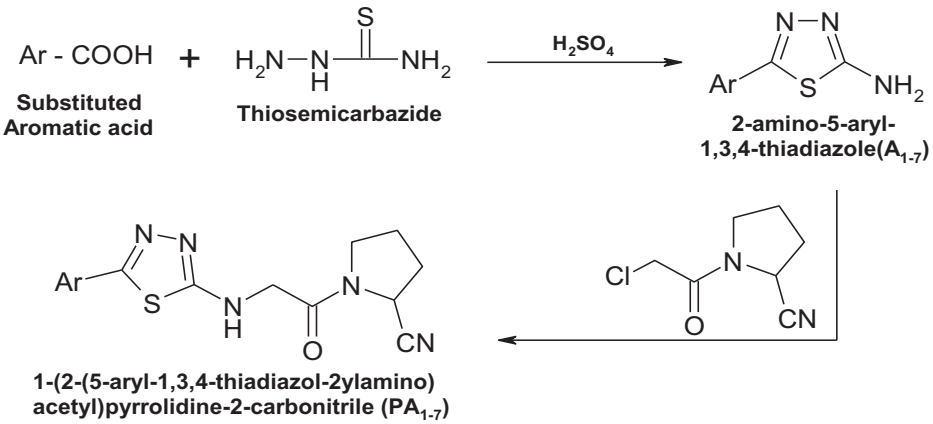



**Figure 3:** Synthesis of 1-(2-(5-aryl-1,3,4-thiadiazol-2-ylamino)acetyl)pyrrolidine-2-carbonitrile derivatives ($PA_{1-7}$)

room temperature for 2 hr. An ammonium bicarbonate (12.4 g, 0.1573 mol) added portion wise (over 5 min) to this mixture and the temperature of the mixture was maintained at 5–10°C. The resultant mixture was stirred at room temperature for 45 min and then concentrated under vacuum at 40°C. The residue was stirred in toluene (60 mL) at room temperature for 1 h. After filtration, the filtrate was concentrated under vacuum at 40°C to afford an oily mass which was stirred in hexane (20 ml) at room temperature for 30 min. The mixture was cooled to 0-5°C and allowed to stand at the same temperature for 30 min. The resulting crystalline solid was filtered and washed with cold hexane to give the target compound. (Yield: 82 %). M.P. 53°C. $R_f$ : 0.48, IR (KBr, $cm^{-1}$): 2925, 2812 (>$CH_2$ of $CH_2Cl$), 2870 (>CH-), 2229(CN), 1687(C=O of $COCH_2Cl$), 1412(C-N), 1492 (>$CH_2$ of Pyrrolidine), 1302, 754 (C-Cl). $^1$H NMR (400 MHz, DMSO) δ: 2.1 (2H, $C_4$ of Pyrrolidine), 2.25-2.26 (2H, $C_3$ of Pyrrolidine), 3.5–3.36 (2H, $C_5$ of Pyrrolidine), 4.76 (2H, $CH_2Cl$), 4.05-4.06 (1H, C*H*CN) m/z = 173 $[M+H]^+$.

**General Procedure for Synthesis of 2-amino-5-aryl-1, 3, 4-thiadiazole ($A_{1-7}$) [8]**

In round bottomed flask aromatic carboxylic acid (0.05 M) and thiosemicarbazide (0.05 M) were added and dissolved in ethanol (25 ml) by shaking. Concentrated sulphuric acid (1 ml) was added to this flask with shaking and the reaction mixture was heated under reflux for 2 hrs on a boiling water bath. Ethanol was removed after completion of the reaction (monitored by TLC) to a possible extent by distillation and the residue was cooled and triturated with crushed ice. The solid crude product was filtered, washed with small portion of cold water and dried. It was purified by recrystallization from hot alcohol.

**Representative Procedure for Preparation of 1-(2-(5-phenyl-1,3,4-thiadiazol-2-ylamino) acetyl)pyrrolidine-2-carbonitrile ($PA_1$)**

A solution of (S)-1-(2-chloroacetyl) pyrrolidine-2-carbonitrile (2.70 mmol) in THF (5.0 ml) was added drop-by-drop to an ice-cooled stirred suspension of $A_1$ (2.50 mmol) and $K_2CO_3$ (2.70 mmol) in acetone (20 ml). The reaction mixture was stirred at room temperature overnight. The resulting mixture was filtered to remove insoluble materials and concentrated under reduced pressure. To this ice cooled solution 1, 4 - dioxane (5.0 ml) was added. The reaction mixture was stirred at 0°C for 1 h and then evaporated to yield the title compound.

**DPP 4 Inhibition Assay**

DPP-4 inhibitor screening assay employs fluorogenic substrate, Gly-Pro-Aminomethylcoumarin (AMC), to measure DPP 4 activity. Cleavage of the peptide bond by DPP releases the free AMC group, resulting in fluorescence that can be analyzed using an excitation wavelength of 350-360 nm and emission wavelength of 450-465 nm.

Assay was carried out using DPP-4 inhibitor screening assay kit (Cayman's DPP 4 Inhibitor Screening Assay Kit) as per the manufacturer's instructions. Briefly the experiment was performed in a white half volume 96 well plate. 30 µl of assay buffer, 10 µl of DPP-4 enzyme, 10 µl of dimethoxy sulphoxide (DMSO) and 50 µl of DPP substrate were added to 100% initial activity wells. 40 µl of assay buffer, 10 µl of DMSO and 50 µl of DPP substrate were added to background wells. 30 µl of assay buffer, 10 µl of DPP-4 enzyme, 10 µl of extract (sample) and 50 µl of DPP substrate were added to inhibitor wells. The plate was incubated for 30 min at 37°C and the fluorescence was recorded using multimode micro plate reader at excitation and emission wavelengths of 355 and 455 nm respectively.

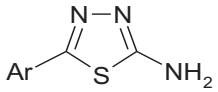
**RESULTS AND DISCUSSION**

The desired compound was synthesized as per the scheme, where the 1-(2-chloroacetyl)pyrrolidine-2-carbonitrile(1) in THF react with suspension of 2-amino-5-aryl-1, 3, 4-thiadiazole to give 1-(2-(5-aryl-1,3,4-thiadiazol-2-ylamino) acetyl) pyrrolidine-2-carbonitrile derivatives. The structures of the synthesized compounds were confirmed by IR, NMR, Mass and CHN analysis.

**Spectral Characterization of 1-(2- chloroacetyl) pyrrolidine-2-carbonitrile (1)**

(Yield: 82 %). M.P. 53°C. $R_f$ : 0.48, IR (KBr, $cm^{-1}$): 2925, 2812 (>$CH_2$ of $CH_2Cl$), 2870 (>CH-), 2229(CN), 1687(C=O of $COCH_2Cl$), 1412(C-N), 1492 (>$CH_2$ of Pyrrolidine), 1302, 754 (C-Cl). $^1$H NMR (400 MHz, DMSO) δ: 2.1 (2H, $C_4$ of Pyrrolidine), 2.25-2.26 (2H, $C_3$ of Pyrrolidine), 3.5–3.36 (2H, $C_5$ of Pyrrolidine), 4.76 (2H, C*H*$_2$Cl), 4.05-4.06 (1H, C*H*CN) m/z = 173 $[M+H]^+$.

**Table 1: Physical Data of 2-amino-5-ary-1, 3, 4-thiadiazole derivatives ($A_{1-7}$)**

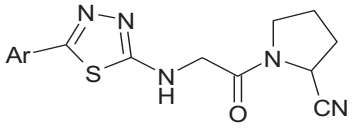


2-amino-5-aryl-1,3,4-thiadiazole

| Compound | Ar | Molecular Formula | Molecular Weight | Rf* | Melting Point (°C) |
|---|---|---|---|---|---|
| A1 | $C_6H_5$- | $C_8H_7N_3S$ | 177 | 0.34 | 223-225 |
| A2 | 4-Cl-$C_6H_5$- | $C_8H_6N_3SCl$ | 211 | 0.56 | 230-232 |
| $A_3$ | 4-$CH_3$-$C_6H_5$- | $C_9H_9N_3S$ | 191 | 0.39 | 215-217 |
| $A_4$ | 4-$OCH_3$-$C_6H_5$- | $C_9H_9N_3SO$ | 207 | 0.53 | 186-188 |
| $A_5$ | 4-F-$C_6H_5$- | $C_8H_6N_3SF$ | 195 | 0.47 | 236-238 |
| $A_6$ | 4-OH-$C_6H_5$- | $C_8H_7N_3SO$ | 193 | 0.68 | 139-141 |
| $A_7$ | 4-Br-$C_6H_5$- | $C_8H_6N_3SBr$ | 256 | 0.26 | 226-228 |

* Mobile Phase: Toluene: Ethyl Acetate: Formic Acid (5:3:1)

**Table 2: Physical Data of 1-(2-(5-aryl-1,3,4-thiadiazol-2-ylamino) acetyl) pyrrolidine-2-carbonitrile ($PA_{1-7}$)**



1-(2-(5-aryl-1,3,4-thiadiazol-2-ylamino)acetyl) pyrrolidine-2-carbonitrile ($PA_{1-7}$)

| Compound | Ar | Molecular Formula | Molecular Weight | Rf* | Melting Point (°C) | % Yield* |
|---|---|---|---|---|---|---|
| $PA_1$ | $C_6H_5$- | $C_{15}H_{15}N_5OS$ | 313 | 0.64 | 264-266 | 58 |
| $PA_2$ | 4-Cl-$C_6H_5$- | $C_{15}H_{14}N_5OSCl$ | 347 | 0.53 | 258-260 | 60 |
| $PA_3$ | 4-$CH_3$-$C_6H_5$- | $C_{16}H_{17}N_5OS$ | 327 | 0.57 | 271-273 | 63 |
| $PA_4$ | 4-$OCH_3$-$C_6H_5$- | $C_{16}H_{17}N_5O_2S$ | 343 | 0.37 | 227-229 | 78 |
| $PA_5$ | 4-F-$C_6H_5$- | $C_{15}H_{14}N_5OSF$ | 331 | 0.58 | 282-284 | 53 |
| $PA_6$ | 4-OH-$C_6H_5$- | $C_{15}H_{15}N_5O_2S$ | 329 | 0.41 | 190-192 | 58 |
| $PA_7$ | 4-Br-$C_6H_5$- | $C_{15}H_{14}N_5OSBr$ | 392 | 0.49 | 258-260 | 53 |

* Mobile Phase: Toluene: Ethyl Acetate: Formic Acid (5:3:1)

**Table 3: *In-Vitro* Screening of Molecules for DPP-4 Inhibition**

| S. No. | Compound | R | % DPP-4 Inhibition | |
|---|---|---|---|---|
| | | | 1 µM | 10 µM |
| 1 | $PA_1$ | $C_6H_5$- | 5.4 | 11.6 |
| 2 | $PA_2$ | 4-Cl-$C_6H_5$- | 7.4 | 13.9 |
| 3 | $PA_3$ | 4-$CH_3$-$C_6H_5$- | 5.9 | 12.1 |
| 4 | $PA_4$ | 4-$OCH_3$-$C_6H_5$- | 4.4 | 8.3 |
| 5 | $PA_5$ | 4-F-$C_6H_5$- | 8.7 | 21.5 |
| 6 | $PA_6$ | 4-OH-$C_6H_5$- | 4.9 | 11.7 |
| 7 | $PA_7$ | 4-Br-$C_6H_5$- | 7.7 | 14.6 |
| 8 | Standard | vidagliptin | 12.8 | 68.1 |

**Spectral Characterization of 1-(2-(5-phenyl-1,3,4-thiadiazol-2-ylamino) acetyl)pyrrolidine-2-carbonitrile ($PA_1$)**

(Yield: 79 %), M.P. 189-190°C, FT-IR (KBr, $cm^{-1}$): 3339 (-NH-), 3060 (Aromatic CH), 2937 (>$CH_2$ of -$COCH_2$-), 2887 (>CH-), 2236 (CN), 1689(C=O of -$COCH_2$-), 1613 (C=N), 1590(Aromatic C=C), 1452 (>$CH_2$ of Pyrrolidine), 676 (C-S) $^1$H NMR (400 MHz, DMSO) δ: 1.48 (2H, $C_4$ of Pyrrolidine), 2.1 (2H, $C_5$ of Pyrrolidine), 2.24-2.29 (2H, $C_3$ of Pyrrolidine), 3.8 (1H, C*H*CN), 3.45 (2H, $CH_2CO$), 8.05-8.39 (5H, Aro. CH), 8.7 (1H, NH) m/z =313 $[M + H]^+$.

The percentage of residual activity of DPP 4 was determined for 1-(2-(4-Oxo-3,4-Dihydroquinazolin-2-ylamino)Acetyl) Pyrrolidine-2-Carbonitrile by comparing the activity of DPP 4 in the presence and absence of the tested compound. DPP 4 activity was not affected at the used DMSO concentration. Negative controls lacking DPP 4 were used as background. A standard DPP 4 inhibitor, vildagliptin, was used as a positive control. All measurements were conducted in duplicates. The percentage DPP-4 inhibition was calculated at 1 µM and 10 µM as given in Table 3.

The results shows *in-vitro* screening of molecules for DPP-4 inhibition and the values indicated that the synthesized molecule have weak to moderate inhibitory action against DPP 4 enzyme compared to vildagliptin. Compound $PA_2$, $PA_4$ and $PA_7$ showed comparatively good activity and produce more inhibition of enzyme.

## CONCLUSION

In this research work, seven novel DPP-4 inhibitors were synthesized. All the synthesized compounds were assayed by *in-vitro* biological screening and were found to give less than 25 % inhibition of DPP 4 during *in-vitro* enzyme inhibition assay up to 10 µM concentration except compound $PA_5$. This compound gave 8.7% and 21.5% DPP 4 inhibition at 1 µM and 10 µM concentrations respectively. It was also reveal that increasing electron withdrawing efficacy at -4 the position of aromatic ring increases the activity.

Further, 1-(2-(5-phenyl-1,3,4-thiadiazol-2-ylamino) acetyl)pyrrolidine-2-carbonitrile derivative could be served as useful clues for developing next generation of antidiabetes medicines via inhibiting DPP-4 activity. The information provides new scaffold which may also lead to the discovery of other possible DPP-4 inhibitors, which could improve treatment of diabetes.

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