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Synthesis of 2,6-dicarbethoxy-3,5-diaryltetrahydro-1,4-thiazine-1,1-dioxidesenhances glucose uptake activity by *in vitro* method

S. Anand[1]*, N.Edayadulla[2], P.Prabu[2]

[1]Department of Chemistry, Saveetha Engineering College, Thandalam, Chennai-602105, Tamil Nadu, India
[2]Department of Chemistry, VELTECH, Avadi, Chennai-600062, Tamil Nadu, India

Abstract: Diabetes is a chronic metabolic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post-prandial blood sugar levels. Increasing epidemic of type 2 diabetes is anticipated to rise to two-fold from the current estimate of 150 million by 2025. Heterocyclic compounds containing nitrogen and sulphur have potential pharmacological properties. Herein we have reported the synthesis and anti-diabetic activity 2,6-dicarbethoxy-3,5-diaryltetrahydro-1,4-thiazine-1,1-dioxides. All synthesized compounds were evaluated for their anti-diabetic activity. Among all the compounds the 2,6-dicarbethoxy-3,5-diaryltetrahydro-1,4-thiazine-1,1-dioxides significantly enhanced glucose uptake activity when compared to the standard anti-diabetic agent and also found to be minimal side effect by *in vitro* assay.
Keywords : Glucose uptake activity, Cytotoxicty, Thiazine, heterocyclic compounds.

Introduction

Diabetes mellitus, the most common multi-factorial disorder in India, currently affects more than 150 million people worldwide and is predicted to affect more than 354 million by the year 2025.Insulin dependent Diabetes Mellitus is primarily due to an autoimmune mediated destruction of pancreatic islet β cells, resulting in a deficiency of insulin in circulation [1]. The peptide hormone insulin lowers blood glucose levels by facilitating glucose uptake, mainly into skeletal muscle and fat tissue, and by inhibiting the endogenous glucose production in the liver. NIDDM, which more than 90% of diabetes patients suffer from, is characterized by the resistance of target tissues to insulin stimulation. Insulin resistance occurs when a normal dose of the hormone insulin is incapable of eliciting metabolic responses. Development of insulin resistance is a multistep process having strong genetic and environmental influences although the exact sequence remains unknown.

Heterocyclic compounds are the mainstay of anti-diabetic therapy for many years. Several drugs such as sulphonylureas and a few biaguanides are valuable in the treatment for hyperglycemia in NIDDM, but they are unable to lower glucose concentration to within normal range and reinstate a normal pattern of glucose

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homeostasis permanently. These therapies are restricted by their pharmacokinetic properties, secondary failure rates and other side effects. These drugs have side effects and thus searching for a new class of compounds is crucial to overcome these problems. Nevertheless, there is continuous search for alternative drugs; management of diabetes without any side effects is still a challenge to the medicinal chemist. Heterocyclic compounds represent one of the most active classes of compounds possessing a wide spectrum of biological activities, including antibacterial, antifungal, antidiabetic and other biological activities. Compounds like 1, 3, 4-thiadiazole, benzthiazole, 3-1Benzthiazolyl1-1-3-thiazolidine-4-ones, pyrazole derivative, Indole derivatives, pyrimidine derivative [2]. Heterocyclic ring like pyridine ring also plays important role in antidiabetic activity of some drugs such as rosiglitazone and pioglitazone. In general, pyridine ring and substituted thiazolidinediones are essential for antidiabetic activity [3]. Heterocyclic compounds are very widely distributed in nature and are essential to life in various ways. Most of the sugars and their derivatives, including vitamin C exist in the form of five-membered (furan) or six-membered (pyran) rings containing one oxygen atom [4]. The higher polarity and water solubility of the heterocyclic substances is based on the substitution of one carbon atom by nitrogen, sulfur or oxygen [5]. These chemical properties lead to increased bioavailability and mobility as compared to the homologous polycyclic aromatic hydrocarbons [6]. The present work illustrates about the various categories of heterocyclic compounds and their structural modifications with structural activity relationships that are vital for the regulation of diabetes. The article shows that wide varieties of heterocyclic compounds have been synthesized and have shown antidiabetic potentials. In continuation of our drug discovery research on heterocyclic compounds, herein we have synthesized our earlier reported compounds and evaluated them for their *in-vitro* anti-diabetic activity using *in vitro* based approach [7, 8, 9].

Experiments

Biological activity

L6 skeletal muscle cell culture

L6 skeletal muscle cell culture (obtained from ATCC-CRL-1458) was maintained in DMEM with 10% FCS and supplemented with penicillin (120 units/ml), streptomycin (75 μg/ml), gentamycin (160μg/ml) and amphotericin B (3 μg/ml) in 5% CO2 environment. For differentiation of L6 cells they were transferred to DMEM with 2% FCS for 4 days post-confluence. The extent of differentiation was established by observing multinucleate of cells. In the present experiment, 90% of the myoblasts were fused into myotubes. L6 muscle cell line is a well established in vitro model to study the glucose transport and metabolism and hence used for this study.

Measurement of 2-deoxy-D-[1-3H] glucose

L6 myoblast cells grown in 24-well plate (BD Falcon) were subjected to glucose uptake as reported [10] In brief; differentiated myotubes were serum starved for 5 h and were incubated with the compounds for 24 hours and stimulated with Insulin (100nM) for 20 min. After experimental incubation, cells were rinsed once with HEPES-buffered Krebs Ringer phosphate solution (118mM NaCl, 5mM KCl, 1.3mM CaCl2, 1.2mM MgSO4, 1.2mM KH2PO4 and 30mM HEPES-pH 7.4) and were subsequently incubated for 15 min in HEPES-buffered solution containing 0.5μCi/ml 2-deoxy-D-[1-3H] glucose. The uptake was terminated by aspiration of media. Cells were washed thrice with ice cold HEPES buffer solution and lysed in 0.1% SDS. The lysates were transferred to 96 well plates (Packard) with glass fiber paper and air dried overnight. This plate was used to measure the cell-associated radioactivity by liquid scintillation counting. All the assays were performed in duplicates and repeated thrice for concordance. Results were expressed as % Glucose uptake with respect to solvent control (control was more or less similar to solvent control). Rosiglitazone (50 μM) was used as the positive control.

Assessment of cytotoxicity by lactate dehydrogenase (LDH) release assay

Lactate dehydrogenase (LDH) release assay was performed [11] using a cytotox 96 assay kit (Promega). This assay quantitatively measures the LDH, a stable cytosolic enzyme that is released upon cell lysis. The assay was done with 0.2 $\times 10^6$ cells/0.2ml/well, seeded in 96 well cell culture plates. Cells on treatment with different concentrations (from 1 ngto 10 μg/ml) of the compounds in L6 myoblast, was

measured at a time point of 24 hours. Triton X-100 was used to induce maximal lysis. The plate was read at 492 nm in a scanning multiwell spectrophotometer.

Chemistry

General Procedures

All the chemicals and solvent were purchased from commercial sources. Solvents and reagents were dried and purified according to the literature methods. Melting points were determined in open capillary tubes and are uncorrected. The structures of synthesized compounds were assigned based on their analytical and spectroscopic properties. IR spectra were recorded on a Schimadzu FT-IR spectrophotometer using KBr pellets. ^{1}H- NMR spectra were recorded on a BrukerAvance 300 MHz instrument, using $CDCl_3$/DMSO-d6 as solvent and TMS as internal standard; the chemical shifts (δ) are reported in ppm and coupling constants (*J*) are given in Hz. Signal multiplicities are represented by s (singlet), d (doublet), t (triplet) and m (multiplet). Elemental analyses were performed on a Perkin-Elmer 2400 Series-II Analyzer. Analytical figures were within ± 0.4% of the theoretical values. Thin layer chromatography (TLC) was performed to monitor the progress of the reaction and purity of the compounds, spot being located under iodine vapour.

Synthesis of diethyl 2,2'-thiodiacetate (2)

To a refluxing alcoholic solution of ethyl chloroacetate,1(2.0 mol), solution of sodium sulfide (1.0 mol) in water was added and the mixture was refluxed for an hour. The course of the reaction was monitored by TLC. After completion of the reaction, the solvent was removed in vacuo. Then the residue was treated with cold water and neutralized with dil. HCl. The separated oily product was extracted with dichloromethane (3 x 25 mL) and on evaporation of the solvent afforded diethyl 2,2'- thiodiacetate(2)as a thick oil in 70 % yield; it answered positive test for sulfur.

Synthesis of diethyl 2,2'-sulfonyldiacetate, 3

To a stirred solution of diethyl 2,2'-thiodiacetate, **2** (1.0 mol) in methanol (20 ml), a pinch of selenium dioxide, 30 % H_2O_2 (10 ml) was added dropwise and stirring continued while maintaining the temperature of the reaction mixture at 20^{o}C for about 2 h. The contents were extracted with dichloromethane (3x 25 ml) and removal of the solvent afforded diethyl 2,2'-sulfonyldiacetate **3** as a thick oil. Yield: 88 %; ^{1}H NMR (300 MH_Z, $CDCl_3$):δ 1.36 (t, 6H, -OCH_2CH_3), 4.32 (q, 4H, -OCH_2CH_3), 4.39 (s, 4H, CH_2).

General procedure for the synthesis of 2,6-dicarbethoxy-3,5-diaryltetrahydro-1,4-thiazine-1,1-dioxides (4a-e)

A mixture of diethyl 2,2'-sulfonyldiacetate,**3** (0.01 mol), aromatic aldehydes (0.02 mol), ammonium acetate (0.02 mol) and a few drops of alcohol were made as a homogeneous paste. Then it was stirred with 10 ml of water at room temperature for about 3 hour and kept as such overnight. Then the reaction mixture was diluted with excess of water, the solid separated which was filtered, washed with water and dried; crystallization by aqueous alcohol afforded pure 2,6-dicarbethoxy-3,5-diaryltetrahydro-1,4-thiazine-1,1-dioxides, (4a-e) in good yield.

2,6-Dicarbethoxy-3,5-diphenyltetrahydro-1,4-thiazine-1,1-dioxide (4a)

White solid; m.p. 184 ^{o}C (lit.184 ^{o}C);IR (KBr)cm^{-1}: 3328 (NH str.), 1730 (ester C=O), 1323 (S=O unsym. str.), 1129 (S=O sym. str.). 1H NMR (300 MHZ, CDCl3): δ 1.02 (t, 6H, -OCH2*CH3*), 2.10 (s, 1H, NH) 4.08 (q, 4H, -O*CH2*CH3), 4.22 (d, 2H, *J*=10.5 Hz, H-2/H-6), 4.72 (d, 2H, *J*=10.5 Hz, H-3/H- 5), 7.24-7.44 (m, 10H, Ar-H). 13C NMR (75 MHz): δ 13.72 (OCH2*CH3)*, 61.94(C-3,C-5), 62.49(O*CH2*CH3), 72.69 (C-2, C-6), 127.87, 128.78, 129.12, 130.33, 137.70 (*ipso* carbon), 161.09 (C=O).

2,6-Dicarbethoxy-3,5-bis(4-nitrophenyl)tetrahydro-1,4-thiazine-1,1-dioxide (4b):

White solid; m.p. 164^o C;IR (KBr) cm^{-1} : 3328 (NH str.), 1732 (ester C=O), 1321 (S=O unsym. str.), 1142 (S=O sym. str.). 1H NMR (300 MHZ, CDCl3): δ 1.03 (t, 6H, -OCH2*CH3*), 2.0 (broad s, 1H, NH) 4.09 (q, 4H, -O*CH2*CH3), 4.27 (d, 2H, *J*=10.2 Hz, H-2/H-6), 4.73 (d, 2H, *J*=10.5 Hz, H-3/H-5), 7.27-7.46 (m, 8H, Ar- H).

13C NMR (75 MHz): δ 13.68 (OCH2*CH3)*, 61.94 (C-3,C-5), 62.48 (O*CH2*CH3), 72.49 (C-2,C-6), 127.90, 128.75, 129.12, 137.51 (*ipso* carbon), 161.27 (C=O).

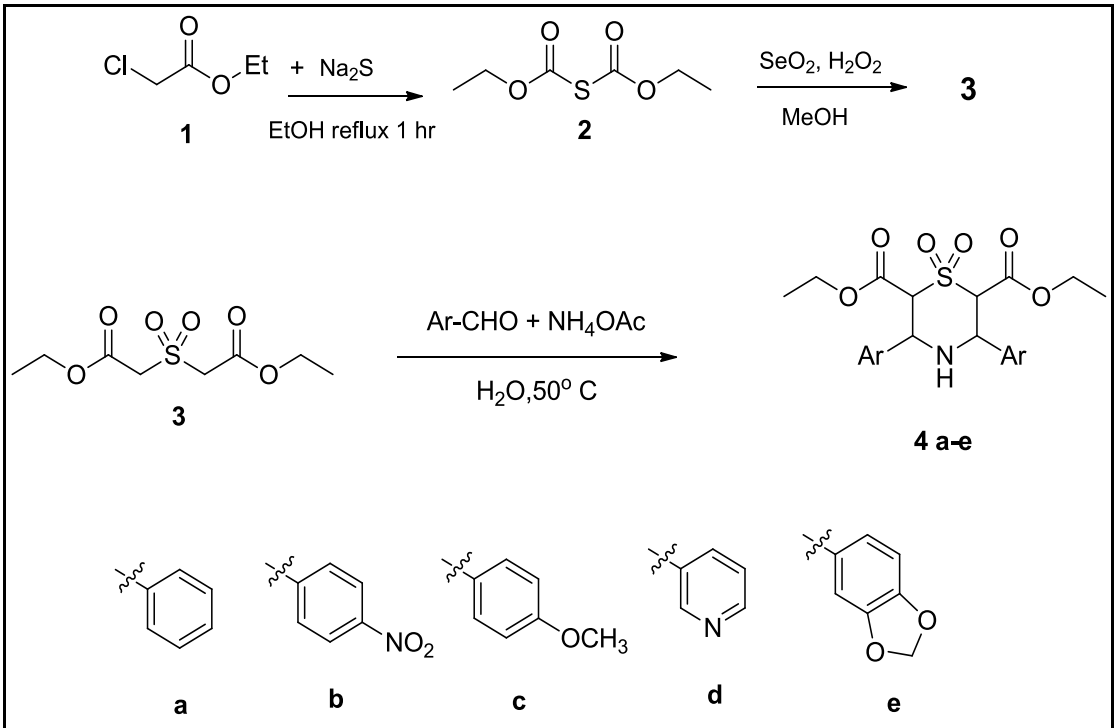
2,6-Dicarbethoxy-3,5-bis(4-methoxyphenyl)tetrahydro-1,4-thiazine-1,1-dioxide (4c):

White solid; m.p. 171 ^{o}C (lit.171-173 ^{o}C); IR (KBr)cm^{-1} : 3326 (NH str.), 1734 (ester C=O), 1316 (S=O unsym. str.), 1154 (S=O sym. str.). 1H NMR (300 MHZ, CDCl3): δ 1.06 (t, 6H, -OCH2*CH3*), 2.1 (broad s, 1H, NH), 3.82 (s, 6H, -O*C*H3), 4.08 (q, 4H, -O*CH2*CH3), 4.21 (d, 2H, *J*=10.2 Hz, H-2/H-6), 4.74 (d, 2H, *J*=10.5 Hz, H-3/H-5), 7.29-7.51 (m, 8H, Ar- H). 13C NMR (75 MHz): δ 14.18 (OCH2*CH3)*, 56.1(O*CH3*), 61.78 (C-3,C-5), 62.34 (O*CH2*CH3), 72.51 (C-2,C-6), 127.90, 128.75, 129.12, 138.14 (*ipso*), 161.56 (C=O).

2,6-Dicarbethoxy-3,5-bis(pyridin-3-yl)tetrahydro-1,4-thiazine-1,1-dioxide (4d):

White solid; m.p. 178 ^{o}C;IR (KBr)cm^{-1} : 3327 (NH str.), 1738 (ester C=O), 1356 (S=O unsym. str.), 1136 (S=O sym. str.) 1H NMR (300 MHZ, CDCl3): δ 1.04 (t, 3H, - OCH2*CH3*), 2.07 (broad s, 1H, NH) 4.09 (q, 2H, -O*CH2*CH3), 4.27 (d, 2H, *J*=10.5 Hz, H-2/H-6), 4.69 (d, 2H, *J*=10.5 Hz, H-3/H-5), 7.46-7.27 (m, 8H, Ar-H). 13C NMR (75 MHz): δ 13.61 (OCH2*CH3)*, 61.45 (C-3,C-5), 62.57 (O*CH2*CH3), 72.46 (C-2,C-6), 127.90, 133.75, 140.51 (*ipso* carbon), 144.78, 161.59 (C=O).

2,6-Dicarbethoxy-3,5-bis(1,3-benzodioxol-6-yl)tetrahydro-1,4-thiazine-1,1- dioxide (4e):Colourless solid; m.p. 189 ^{o}C;IR (KBr) cm^{-1}: 3321 (NH str.), 1734 (ester C=O), 1350 (S=O unsym. str.), 1135 (S=O sym. str.). 1H NMR (300 MHZ, CDCl3): δ 1.03 (t, 6H, -OCH2*CH3*), 2.0 (broad s, 1H, NH) 4.09 (q, 4H, -O*CH2*CH3), 4.27 (d, 2H, *J*=10.2 Hz, H-2/H-6), 4.73 (d, 2H, *J*=10.5 Hz, H-3/H-5), 6.09 (s, 4H, -OCH2O-), 6.97-7.72 (m, 6H, Ar-H). 13C NMR (75 MHz): δ 13.89 (OCH2*CH3)*, 61.94 (C-3,C-5), 62.48 (O*CH2*CH3), 72.49 (C-2,C-6), 101.7 (-O-CH2-O-),123.90, 128.75, 147.9 (*ipso* carbon), 161.06 (C=O).



Scheme1: Protocol for the synthesis of the title compounds (**4a-e**)

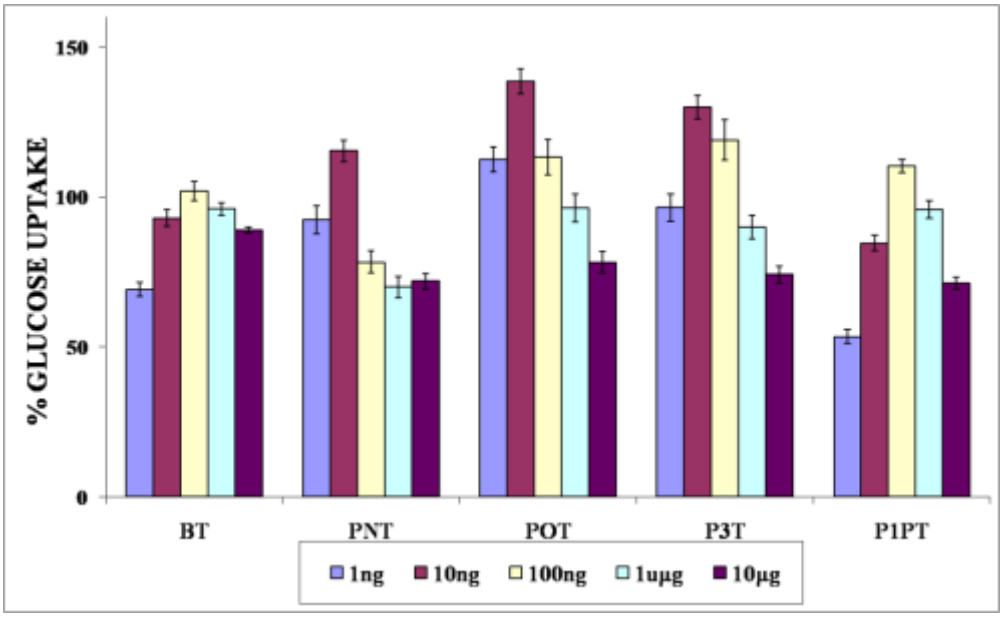
Statistical analysis

The data were expressed as mean ± SEM. The statistical significance was evaluated using one way ANOVA and Tukey's multiple comparison tests. These were applied using SPSS version 11.0 (SPSS, Cary, NC, USA) and P< 0.05 was considered to be statistically significant.

Results and discussion

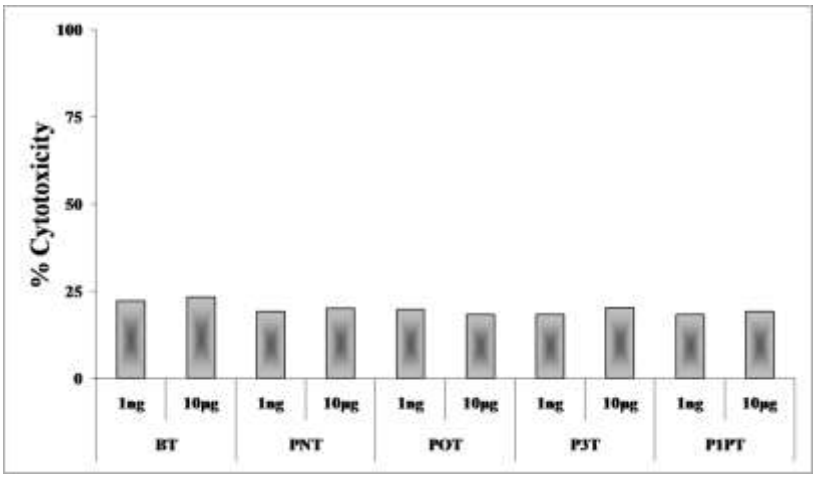
Despite considerable progress in the field of drug discovery, there is a global increase in the diabetic population. Although there are a variety of anti-hyperglycemic agents in market, their utility has become limited due to a few shortcomings like single targeted mode of action, unwanted off target side effects etc. This approaches work in a dynamic fashion with a diverse array of functions including synergistic, agonistic and antagonistic potential with maximum therapeutic efficacy and minimal side effects.The introduction of electron donating group para- methoxy 4c into the phenyl ring results in an increase in inhibitory activity. Introduction of the electron-withdrawing group nitro 4b into the phenyl ring results in a significant decreased in inhibitory activity. It is interesting to point out that instead of phenyl ring heterocyclic ring 4d exhibited potent inhibitory activity. In particular, compound 4c with a p-methoxy group at the para-position of the phenyl ring and a heteroatom 4d was found to be the most active compound in this series.

Hence, based on these considerations 2,6-dicarbethoxy-3,5-diaryltetrahydro-1,4-thiazine-1,1-dioxides derivatives 4a–4e have been synthesized and evaluated for their glucose uptake activity. Hence, the compounds was examined the effect anti-diabetic activity of 4a, 4b, 4c, 4d and 4e on glucose transport, L6 myotubes were treated with varying concentrations of the compounds at 24h.



Glucose uptake analysis of BT, PNT, POT, P3T and P1PT using 2-deoxy-d-1-[3H] glucose. L6 myotubes were treated with different concentrations of compounds for 24 h. The results were expressed as % glucose uptake with respect to the solvent control (DMSO). All the data were expressed as mean ± S.E.M. of triplicates of two independent experiments.*$P < 0.05$.

Adose dependent increase in glucose uptake activity was observed at 24h. The compounds 4a, 4b, 4c and 4d significantly increased glucose uptake activity when compared to Rosiglitazone as positive control. Further, to determine the cell cytotoxicity, LDH release was measured at 24 hours. The results showed that the compound 4a is slightly increased in toxic at high concentration. But rest of the other compounds is nontoxic even at higher doses.



Effect of Cytotoxicity of compounds measured on L6 myotubes as indicated doses at 24 h. Cytotoxicity was expressed as % LDH release. Data represent the means ± S.E.M. of triplicates of three independent experiments.*$P < 0.05$ as compared with untreated control group.

Conclusions

In summary, 2,6-dicarbethoxy-3,5-diaryltetrahydro-1,4-thiazine-1,1-dioxidesderivatives 4a-4ehave been synthesized and evaluated for their glucose uptake activity. The compounds significantly enhance glucose uptake activity was found to be 4b (10ng), 4c(10ng), 4d (10ng) and 4e(100ng) and these concentrations were used for further studies.

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