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Synthesis, in vitro and in silico studies of a PPARγ and GLUT-4 modulator with hypoglycemic effect

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ABSTRACT

Compound {4-[({4-[(*Z*)-(2,4-dioxo-1,3-thiazolidin-5-ylidene)methyl]phenoxy}acetyl)amino]phenoxy}acetic acid (**1**) was prepared and the in vitro relative expression of PPARγ, GLUT-4 and PPARα, was estimated. Compound **1** showed an increase of 2-fold in the mRNA expression of PPARγ isoform, as well as the GLUT-4 levels. The antidiabetic activity of compound **1** was determined at 50 mg/Kg single dose using a non insulin dependent diabetes mellitus (NIDDM) rat model. The in vivo results indicated a significant decrease of plasma glucose levels, during the 7 h post-administration. Also, we performed a molecular docking of compound **1** into the ligand binding pocket of PPARγ, showing important short contacts with residues Ser289, His323 and His449 in the active site.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily, belonging to the ligand activated transcription factors that exist in three isoforms: PPARα, PPARγ, and PPARβ/δ.[1]

Each isoform regulates tissue-specific target genes acting as lipid sensors and regulators of glucose homeostasis, such as the fatty acid transport protein 1 (FATP1) and the solute carrier family 2 (facilitated glucose transporter), member 4 (GLUT-4).[2] The fibrate drugs (e.g., clofibrate and fenofibrate) which are hypolipidemic agents, exert their effect by agonist action on PPARα. On the other hand, thiazolidine-2,4-diones (rosiglitazone and pioglitazone) function as insulin-sensitizing drugs, through the activation of PPARγ.[3,4]

Metabolic syndrome, an unwanted group of disorders that includes obesity, hyperglycemia, dyslipidemia and hypertension, contributes to the etiology of insulin resistance and diabetes.[2] Therefore, the development of new compounds which interact with PPARs constitutes an important strategy for the treatment of diabetes and metabolic syndrome working in an effective, specific and efficient manner.

Multitarget therapies, which at the same time control hyperglycemia and inhibit progression of cardiovascular complications, may be attractive options for the therapeutic treatment of diabetes. PPARα/γ dual agonists are new class of drugs which have been developed to target both PPARs in order to produce antidiabetic and hypolipidemic effects.[5] This type of compounds are examples of the novel multitarget paradigm of drug discovery.[6] The PPARα/γ dual agonist tesaglitazar (Fig. 1) is well-known to reduce triglycerides, to elevate cardioprotective HDL levels and consequently improve insulin sensitivity.[7–9]

In our ongoing research on PPARα/γ dual agonists derivatives with cardioprotective and antidiabetic activities,[9] we report in this Letter the preparation of {4-[({4-[(*Z*)-(2,4-dioxo-1,3-thiazolidin-5-ylidene)methyl]phenoxy}acetyl)amino]phenoxy}acetic acid (**1**) and its potential ethyl ester prodrug (**2**), as well as the in vitro relative expression of PPARα, PPARγ and GLUT-4. We also describe the molecular docking of **1** in the active site of PPARγ, and its in vivo hypoglycemic effect using a streptozotocin–nicotinamide rat model of non-insulin dependent diabetes mellitus (NIDDM).

The design of compound **1** was based on the pharmacophore of PPAR dual agonist:[9](a) An acidic head group, such as thiazolidine-2,4-dione, carboxylic acid, or related bioisosteres; (b) a central aromatic backbone; (c) an extra-lipophilic aromatic region; (d) a

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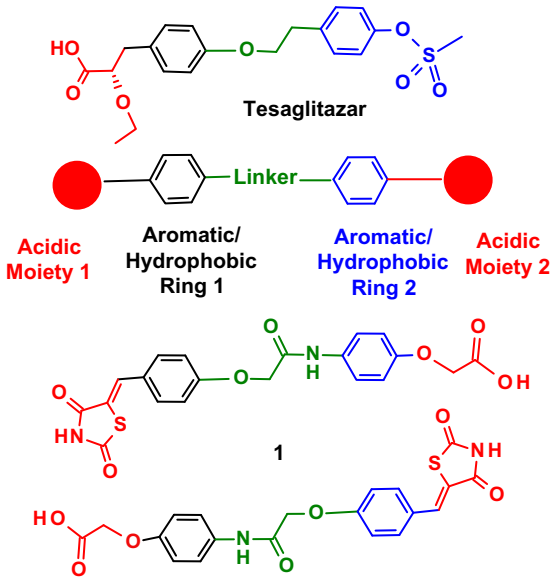


Figure 1. Pharmacophore in PPARα/γ dual agonist tesaglitazar and the requirements fulfilled by compound **1** in two manners.

flexible spacer that connects regions (b) and (c), and allow the structure to adopt a specific conformation (Fig. 1).[9]

The herein designed compound **1** matches the pharmacophoric pattern for PPARα/γ dual agonist in two manners: **1** has two acid heads, two aromatic/hydrophobic rings and a flexible linker (Fig. 1).

Compound **1** was prepared starting from 4-nitrophenol (**3**) and ethyl bromoacetate (**4**) via Williamson synthesis to give nitroether **5**. This compound was reduced under catalytic hydrogenation with 5% Pd/C affording aniline **6**, which was immediately reacted with α-chloroacetyl chloride (**7**), in presence of triethylamine and dichloromethane as solvent, to give α-chloroacetamide **8**. The later compound treated with 4-hydroxybenzaldehyde (**9**) under SN^2 conditions led to the ether–aldehyde **10**, which was condensed with thiazolidine-2,4-dione, following Knoevenagel reaction conditions to afford compound **2** in good yields. The ethyl ester hydrolysis of **2** with 3.5 equiv of LiOH gave desired quantitative phenoxyacetic acid **1** (Scheme 1). All compounds were purified by recrystallization. The chemical structures of the synthesized compounds were confirmed on the basis of their spectroscopic and spectrometric data (NMR and mass spectra), and their purity ascertained by microanalysis.[10,11]

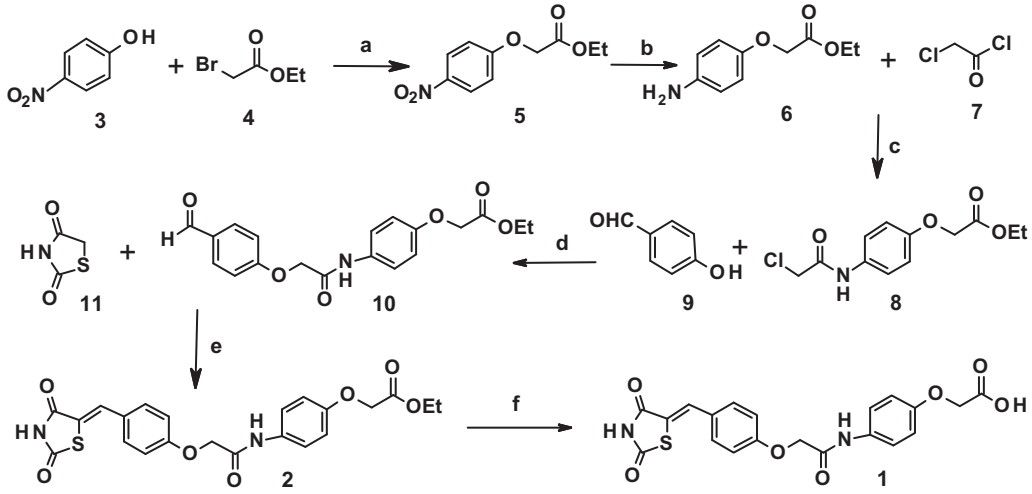
To determine the effect of compounds on PPARs and GLUT-4 expression, 3T3-L1 fibroblasts were cultured in 6-well plates. After 2 days of confluence the cells were differentiated to the adipocyte phenotype. After that, cells were treated during 24 h with 10 μM of compounds **1** and **2**, pioglitazone (10 μM) and fenofibrate (100 μM). The expression analysis was performed using SYBR Green. Relative changes in the expression level of one specific gene ($\Delta\Delta C_t$) were calculated as ΔC_t of the test group minus ΔC_t of the control group and then presented as $2^{-\Delta\Delta C_t}$.

Results summarized in Figures 2 and 3 show that compounds **1** and **2** increases significantly (about 2-fold) the levels of relative expression for PPARγ and GLUT-4 mRNA's. These findings are relevant, since the activation of PPARγ could decrease the glucose serum levels in diabetic patients as a result of a reduction of insulin resistance. In contrast, no change in the relative expression for PPARα was observed with treatment of compounds **1** or **2** (Fig. 4).

Skeletal muscle glucose uptake is the rate-limiting step of glucose utilization, and insulin-dependent and insulin-independent signaling pathways physiologically regulate it, both leading to the translocation of GLUT-4 glucose transporter to the plasma membrane.[9,12] Our data suggest that compounds **1** and **2**, could induce GLUT-4 expression.

The antidiabetic activity in vivo was measured using a streptozotocin–nicotinamide NIDDM rat model.[9,13–15] For this analysis, we employed compound **2** which is the potential prodrug (ethyl ester) form of **1**. Compound **2** was employed to increase the bioavailability of **1** according to the following rationale: Most of the fibrates are prodrugs that, after extensive metabolism by hydrolysis, lead to the free carboxylic acid, which is the bioactive form.

Glibenclamide was used as hypoglycemic control (5 mg/Kg). The antidiabetic activity of **2** was determined using a 50 mg/Kg intragastric single dose. Compound **2** demonstrated significant hypoglycemic activity ($p < 0.05$) compared to control, by lowering glycemia ranging from 20% to 36% (Table 1). The effect was sustained during the 7 h of experiment and it was comparable with the hypoglycemic action showed by glibenclamide (a secretagogue). It is important to mention that pioglitazone (an insulin sensitizer) did not show any hypoglycemic effect under these conditions of the experiment, in accordance with the research



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) K_2CO_3, acetone, reflux; (b) H_2, Pd/C 5%, ethanol; (c) Et_3N, CH_2Cl_2; (d) K_2CO_3, CH_3CN, reflux; (e) piperidine (0.3 equiv), benzoic acid (0.3 equiv), toluene, Dean–Stark apparatus; (f) LiOH (3.5 equiv), THF–H_2O.

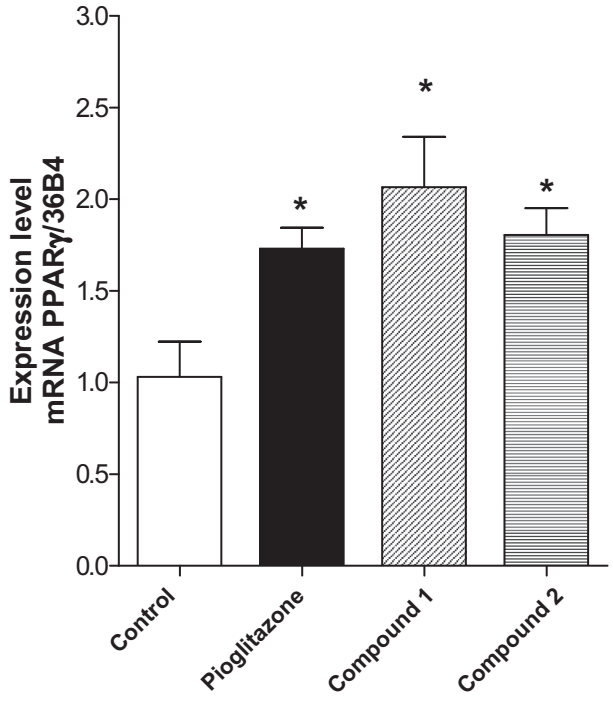


Figure 2. Effect of compounds on expression of mRNA PPARγ. Results are mean ± SEM ($n = 6$) $^{*}p < 0.01$ compared with control group.

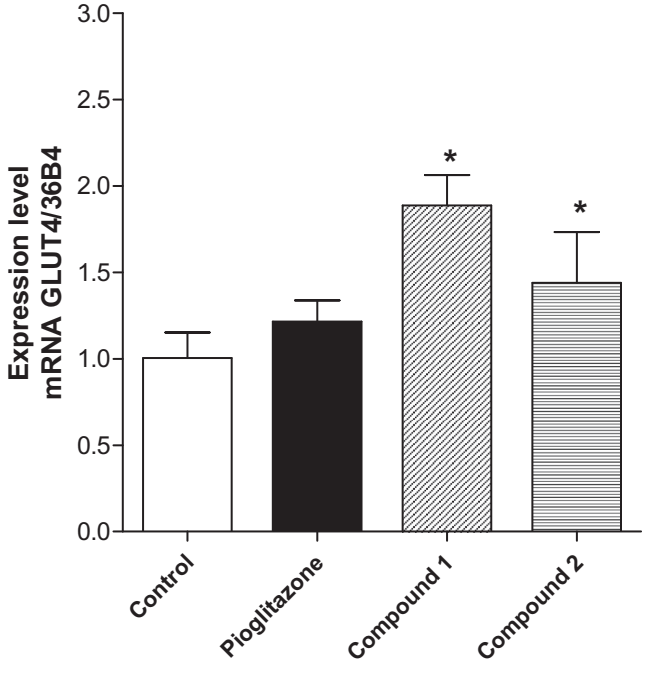


Figure 3. Effect of compounds on expression of mRNA GLUT-4. Results are mean ± SEM ($n = 6$) $^{*}p < 0.01$ compared with control group.

reported by Majithiya and co-workers,[16,17] due to streptozotocin–nicotinamide rat model is partially insulin-deficient and not insulin resistant bioassay.

As might be expected, compound **2** did not show a significant decrease of glycemia in the first hour post administration, due to the ester prodrug must be activated by hydrolysis to led compound **1**, which showed the best in vitro PPARγ and GLUT-4 effects. In the in vivo experiments, the increase of GLUT-4

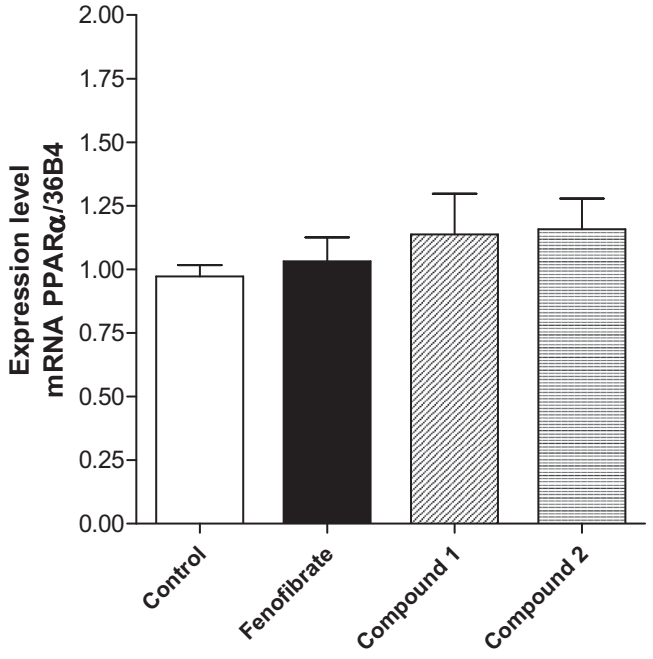


Figure 4. Effect of compounds on expression of mRNA PPARα. Results are mean ± SEM ($n = 6$).

expression in rat could be associated with a marked decrease of glucose concentrations, confirming that the level of expression of this transporter in skeletal muscle may be crucial for the regulation of total body glucose homeostasis and insulin action.

In order to gain an insight into the binding mode of **1**, it was docked into the catalytic site of the human PPARγ (PDB ID: 1I7I) using the program AutoDock 4.0. The docking protocol (see Supplementary data) was validated by docking the co-crystal ligand tesaglitazar. The root-mean square deviation (RMSD) obtained for tesaglitazar was 1.19 Å. This result showed the ability of the docking protocol to reproduce the binding mode of the co-crystal ligand.

Figures 5 and 6 show the two top ranked binding modes of **1** found by AutoDock with docking energies of −10.13 and −9.47 kcal/mol, respectively. According to the docking results, the first binding mode is characterized by internalization of carboxylic acid into the ligand binding site, making three hydrogen interactions with the catalytic residues Ser289, His323 and His449. Other polar contacts are made between the thiazolidine-2,4-dione and Arg280 and Glu259 (Fig. 5).

The second binding mode is differentiated from the first one in that carboxylic acid of **1** is located at the opposite side of the binding cavity, and the main contacts are given by the interactions between thiazolidine-2,4-dione and the catalytic residues Ser289, His323 and Tyr473 (Fig. 6).

Figure 7 depicts the 3D overlay of tesaglitazar (co-crystal ligand) and compound **1**. It is remarkable that both compounds share the same binding pattern and pharmacophoric interactions with PPARγ.

These results contribute to explain at the molecular level, the relevant activity of molecules in the in vitro and the in vivo tests. As commented above, compound **1** matches the pharmacophoric pattern of PPAR ligand in two ways: it contains two acid heads (carboxylic acid and thiazolidine-2,4-dione), two aromatic/hydrophobic rings and a flexible spacer connecting them.

With the aim of anticipating potential toxicity issues of the newly compounds herein developed, a computational prediction

Table 1
Percentage of variation of blood glucose concentration on streptozotocin–nicotinamide induced diabetic rats treated with a single dose of compound **2** (potential prodrug form of **1**, 50 mg/Kg; intragastric administration)

Compd	Dose (mg/Kg)	Percentage of variation of glycemia ±SEM (mg/dL)				
		Zero hour	First hour	Third hour	Fifth hour	Seventh hour
2	50	0 ± 0.0	−4.4 ± 3.1	−20.5 ± 6.0*	−36.7 ± 8.0*	−31.0 ± 6.0*
Gli	5	0 ± 0.0	−20.5 ± 8.0*	−36.3 ± 8.0*	−37.5 ± 7.1*	−43.6 ± 9.9*

* Values represent the mean ± SEM ($n = 6$). $p < 0.05$ compared with control group. The negative value indicates decrease in glycemia. Gli, glibenclamide.

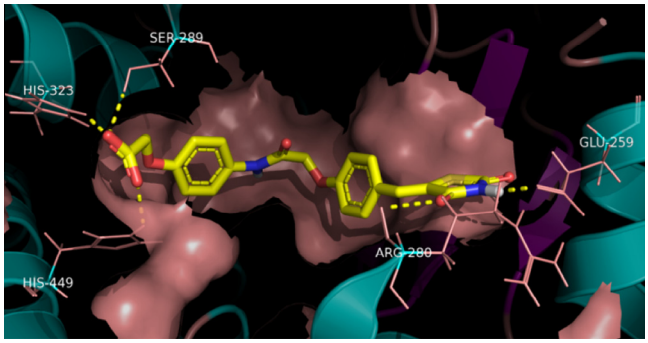


Figure 5. Binding model of **1** into the active site of PPARγ, representing the lowest binding energy (−10.13 kcal/mol). Stick representation of side chains (in pink), shows the residues that participate in the hydrogen bond network.

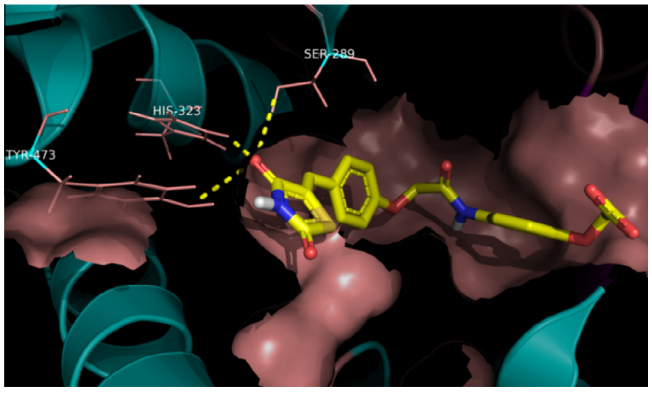


Figure 6. Binding mode of compound **1** with second lowest binding energy (−9.47 kcal/mol) into the active site of PPARγ.

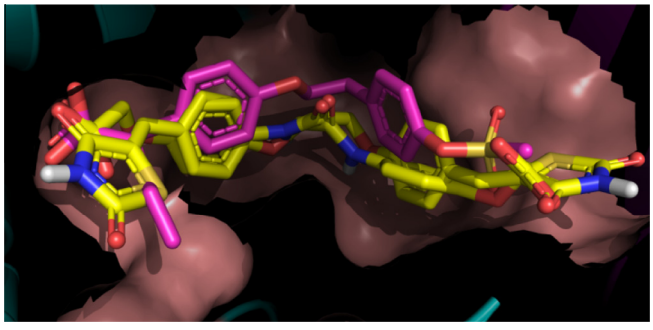


Figure 7. Overlay of bioactive conformations of tesaglitazar (pink) and compound **1** (yellow) represented in the two top ranked binding modes.

of toxicity was performed. The toxicity parameters of compound **1**, potential prodrug **2**, tesaglitazar and pioglitazone were calculated through the ACD/ToxSuite software, v. 2.95 (Table 2).

Table 2
Toxicity profiles predicted for compounds **1**, **2**, tesaglitazar and pioglitazone

Compd	LD_{50} (mg/Kg)				Probability of inhibition (IC_{50} or Ki < 10 μM) CYP450 isoform			
	Mouse		Rat					
	ip	po	ip	po	3A4	2D6	1A2	hERG
1	730	3100	850	1600	0.04	0.00	0.00	0.01
2	850	1900	760	1200	0.42	0.03	0.05	0.40
Tesaglitazar	440	930	230	3600	0.22	0.05	0.02	0.01
Pioglitazone	440	1900	400	1100	0.22	0.03	0.08	0.10

Inhibition of CYP3A4 (the major enzyme responsible for drug metabolism in human organism), can lead to drug-drug interactions and undesirable adverse effects.[9,15,18] The predictions of inhibition for the three main isoforms of CYP450 for compounds **1** and **2** were comparable to the reference antidiabetic drugs at 10 μM, showing satisfactory toxicity profiles. Cardiotoxicity of drug-like compounds associated with human ether-a-go-go related gene (hERG) channel inhibition is one of the main causes of bioactive compounds attrition.[19,20] Some thiazolidine-2,4-dione are associated with cardiovascular risks and are the leading cause for the withdrawal of these drugs from the market.[20] Compound **1** showed very low prediction of hERG channel blockage at clinically relevant concentrations (K_i < 10 μM), being considered as non-cardiotoxic. In the prediction of acute toxicity, compounds **1** and **2** demonstrated similar calculated LD_{50} than tesaglitazar and pioglitazone by different administration routes, showing very low toxicity profiles ranging from 730 to 3100 mg/Kg.

The scaffolds of multitarget compounds **1** and **2** are partially coincident with the structure of aldose reductase inhibitors reported by Maccari and co-workers.[21–23] This enzyme (an aldo-keto reductase) plays a critical role in the development of chronic diabetic problems, such as neuro-, nephro-, and retino-pathies, as well as an increased cardiovascular risk.[23] Compounds **1** and **2** were tested in silico using PASS software (Prediction of Activity Spectra for Substances)[24] showing a *Pa* (probability 'to be active') of 0.094 and 0.064 as aldose reductase inhibitors, respectively. *Pa* estimates the chance that the studied compounds are belonging to the sub-class of active compounds (resembles the structures of molecules, which are the most typical in a sub-set of 'actives' in PASS training set. When $Pa > 0.7$, the chances of finding experimental activity are rather high but the compounds found may be close structural analogs of known drugs. If we select in the range $0.5 < Pa < 0.7$, the chances for detecting experimental activity will be lower but the compounds will be less similar to known pharmaceutical agents. For $Pa < 0.5$, the chances of detecting experimental activity will be even lower.[25]

In summary, a new hypoglycemic entity has been developed as a promising lead compound for the treatment of diabetes. The synthetic strategy is highly efficient and gave high overall yield. Compound **1** is a specific PPARγ ligand as it exhibited a marked induction of the target gene GLUT-4, with predicted low toxicity profile, multitarget effect and in vivo efficacy.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.07.068. These data include MOL files and InChiKeys of the most important compounds described in this article.

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10. {4-[({4-[(*Z*)-(2,4-Dioxo-1,3-thiazolidin-5-ylidene)methyl]phenoxy}acetyl)amino]phenoxy}acetic acid (**1**). Recrystallized from ethanol. Yield: 92%, mp: >300 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (400 MHz, DMSO-d_6) δ: 4.85 (s, 2H, H-2), 4.77 (s, 2H, H-8′), 6.95 (d, 2H, H-2′, H-6′, J = 8.8 Hz), 7.17 (d, 2H, H-2″, H-6″, J = 8.8 Hz), 7.51 (d, 2H, H-3′, H-5′, J = 8.8 Hz), 7.56 (d, 2H, H-3″, H-5″, J = 8.4 Hz), 7.71 (s, 1H, H-7″), 8.51 (s, broad, 1H, COOH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ: 63.7 (C-2), 67.3 (C-8′), 113.7 (C-2′, C-6′), 115.8 (C-4″), 121.0 (C-3′, C-5′), 121.2 (C-2″, C-6″), 121.2 (C-3″, C-5″), 126.1 (C-8″), 131.7 (C-7″), 152.5 (C-4′), 160.1 (C-1′), 165.5 (C-1″), 167.5 (C-12″), 168.0 (C-10″), 172.8 (C-1, COOH) ppm. MS/FAB$^+$: m/z 429 (M^+). Anal. Calcd for $C_{20}H_{16}N_2O_7S$: C, 56.07; H, 3.76; N, 6.54. Found: C, 56.01; H, 3.70; N, 6.61. Ethyl {4-[({4-[(*Z*)-(2,4-dioxo-1,3-thiazolidin-5-ylidene)methyl]phenoxy}-acetyl)amino]phenoxy}acetate (**2**). Recrystallized from toluene. Yield: 78, mp: 250 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 1.20 (t, 3H, OCH_2CH_3), 4.16 (c, 2H, OCH_2CH_3), 4.73 (s, 2H, H-2), 4.77 (s, 2H, H-8′), 6.98 (d, 2H, H-2′, H-6′, J = 8.8 Hz), 7.15 (d, 2H, H-2″, H-6″, J = 8.8 Hz), 7.53 (d, 2H, H-3′, H-5′, J = 8.8 Hz), 7.58 (d, 2H, H-3″, H-5″, J = 8.4 Hz), 7.74 (s, 1H, H-7″), 10.05 (s, broad, 1H, CONH) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.1 (OCH_2CH_3), 60.6 (OCH_2CH_3), 64.8 (C-2), 67.0 (C-8′), 114.5 (C-2′, C-6′), 115.5 (C-4″), 120.8 (C-3′, C-5′), 121.2 (C-2″, C-6″), 121.2 (C-3″, C-5″), 126.1 (C-8″), 131.5 (C-7″), 153.7 (C-4′), 159.3 (C-1′), 165.5 (C-1″), 167.6 (C-12″), 167.9 (C-10″), 168.5 (C-1) ppm. MS/FAB$^+$: m/z 456 (M^+). Anal. Calcd for $C_{22}H_{20}N_2O_7S$: C, 57.89; H, 4.42; N, 6.14. Found: C, 57.82; H, 4.55; N, 6.32.
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