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1,2-Benzothiazine 1,1-dioxide carboxylate derivatives as novel potent inhibitors of aldose reductase

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ABSTRACT

Due to the importance of aldose reductase (ALR2) as a potential drug target in the treatment of diabetic complications, there are increasing interests in design and synthesis of ALR2 inhibitors. Here, we prepared 1,2-benzothiazine 1,1-dioxide acetic acid derivatives and investigated their inhibition activity. Most of these derivatives were found to be active with IC_{50} values ranging from 0.11 μM to 10.42 μM, and compound **8d**, 2-[2-(4-bromo-2-fluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid, showed the most potent inhibition activity. Further, SAR and docking studies suggest that in comparison with the α,β-unsaturated derivatives, the saturated carboxylic acid derivatives had a greater binding affinity with the enzyme and thus an enhanced inhibition activity. Therefore, development of more powerful ARIs based on benzothiazine 1,1-dioxide by stereo-controlled synthesis could be expected.

1. Introduction

Diabetes mellitus is a chronic disease caused by deficiency in production of insulin by pancreas, and by resistance to insulin's effects. Such a deficiency results in increased concentrations of glucose in the blood, which in turn triggers the polyol pathway and then damages many of the body's systems such as eyes, kidneys, nerves, heart, and blood vessels. Increasing evidence reveals a link between glucose metabolism via the polyol pathway and long-term diabetic complications.[1–3] Aldose reductase (EC 1.1.1.21, ALR2) catalyses the NADPH-dependent reduction of glucose to sorbitol in the first step of this metabolic pathway.[4–6] Therefore, it has received great attention as a target enzyme in the search for drugs able to prevent or delay the onset and progression of diabetic complications, independently of glycaemic levels.

Animal model studies demonstrated that diabetic complications can be ameliorated by the use of aldose reductase inhibitors (ARIs).[7] In the past years, a range of structurally different compounds have been reported as ARIs (Fig. 1), such as alrestatin,[8] tolrestat,[9] epalrestat,[10] zopolrestat,[11] zenarestat,[12] ponalrestat,[13] lidorestat,[14] naptho[1,2-*d*]isothiazole derivatives,[15] sorbinil,[16] fidarestat[17] and ranirestat (AS-3201).[18] However, many of the clinically tested ARIs proved to be inadequate as drug candidates because of adverse pharmacokinetics, toxic side effects or low efficacy.[19,20] Inhibition of ALR1, an enzyme that is closely related to ALR2[21] and plays a detoxification role as it specifically metabolizes toxic aldehydes such as hydroxynonenal (HNE), 3-deoxyglucosone, and methylglyoxal,[22–24] may account for some of the undesirable side effects associated with the present ARIs. Thus, we are putting a lot of effort into the development of a larger variety of ARIs that block ALR2 strongly and specifically. In the last few years, we have designed two classes of compounds based on 1,2,4-benzothiadiazine 1,1-dioxide and pyrido[2,3-*e*]-[1,2,4]-thiadiazine 1,1-dioxide, respectively, and numerous compounds of each class have been demonstrated to be potent and selective ARIs (Fig. 1).[25,26] Both core structures have a nitrogen atom at the position-4, which is connected to the carboxylate group essential for the ARIs and therefore determines the orientation of the side chain (Fig. 1). Accordingly, it is interesting to detect effect of the substitution of a carbon atom for the N-4 of the scaffold on the aldose reductase inhibition and then our further efforts at a new design based on benzothiazine core resulted in the formation of a novel series of 1,2-benzothiazine 1,1-dioxide derivatives. Here, we report their preparation, aldose reductase inhibition activity, and docking studies.

2. Chemistry

2-[2-Benzyl-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene] acetic acid (**8a–f**) and 2-(2-benzyl-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl) acetic acid (**9a–d**) were prepared following the synthetic pathway depicted in Scheme 1 from commercially available sodium saccharin **1**. By reacting **1** with methyl

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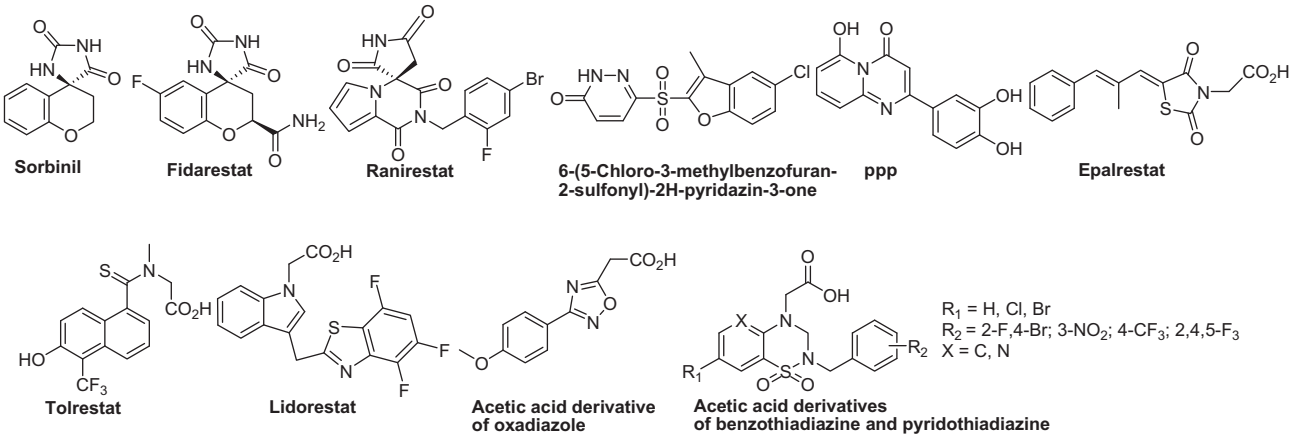
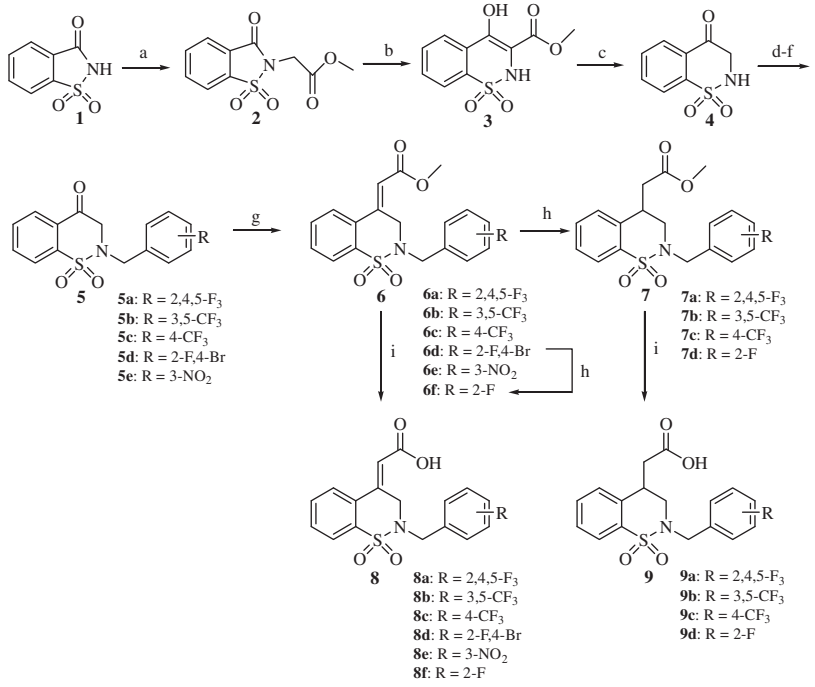


Figure 1. Chemical structures of aldose reductase inhibitors.



Scheme 1. Reagents and conditions: (a) $BrCH_2COOCH_3$, DMF, 60 °C; (b) NaOMe, MeOH, 55 °C; (c) HCl, reflux; (d) Dean and Stark apparatus, ethane-1,2-diol, *p*-TsOH, PhH, reflux; (e) substituted Bn–Br, K_2CO_3, CH_3CN, 70 °C; (f) HCl, H_2O, THF, reflux; (g) $Ph_3P{=}CHCOOCH_3$, $PhCH_3$, reflux; (h) H_2, 10% Pd/C, EtOH, AcOEt; (i) 1,4-dioxane, NaOH.

bromoacetate under mild conditions, methyl [1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3*H*)-yl]acetate **2** was obtained in excellent yield.[27] Gabriele-Colman type ring expansion of the five-membered isothiazole ring of **2** to the six-membered thiazine ring, having synchronous ring cleavage and ring closure steps, in an inert atmosphere yielded methyl 4-hydroxy-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide **3**.[28,29] Subsequently, **3** was refluxed with concentrated hydrochloric acid to get 2*H*-1,2-benzothiazin-4(3*H*)-one 1,1-dioxide (**4**). N-Substituted ketones **5a–e** were achieved by alkylation of **4** in three steps with commercially available benzylic bromides[30] and then followed by a reaction with methyl 2-(triphenylphosphoranylidene) acetate to give a series of 2-[2-benzyl-1,1-dioxido-2*H*-1,2-benzothiazine-4(3*H*)-ylidene]carboxylic acid methyl esters **6a–e**.[31] 1,4-Hydrogenation of the ester **6d** catalyzed by Pd/C resulted in a removal of bromine of the benzyl group leading to the formation of ester **6f**. The esters **6a–f** were then converted into the desired α,β-unsaturated acids **8a–f** by hydrolysis with aqueous sodium hydroxide. In order to obtain the corresponding saturated products, the α,β-double bond of esters **6a–c** and **6f** were saturated by Pd/C catalyzed 1,4-hydrogenation to form the esters **7a–c** and **7d** in good yields,[32] which were then converted to acids **9a–d** by hydrolysis. However, saturated acids corresponding to **8d–e** could be achieved neither by the 1,4-hydrogenation of precursors **6d–e** due to the presence of bromine and nitro groups which are more sensitive to the reduction reaction, nor via other synthetic procedures. All acids thus prepared were characterized as stereoisomeric mixtures.

3. Results and discussion

3.1. Aldose reductase inhibition and SAR studies

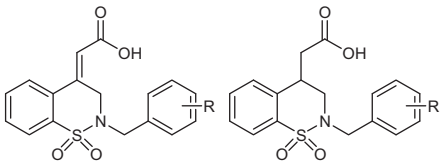
All newly synthesized acetic acid derivatives of 1,2-benzothiazine 1,1-dioxide were tested for their potential inhibitory effect on ALR2 isolated from rat lenses. In order to evaluate their selectivity for ALR2 inhibition, these compounds, which showed potent ALR2 inhibition activity, were also subjected to test for their inhibition activity against ALR1, isolated from rat kidneys. IC_{50} values

were determined by linear regression analysis of the log of the concentration–response curve. Their effectiveness was evaluated with respect to epalrestat as a potent inhibitor of ALR2. Table 1 lists the results of these biological evaluations. Full details of the biological methods employed are included in the Experimental section.

As shown in Table 1, most of 1,2-benzothiazine 1,1-dioxide acetic acid derivatives (**8a–f**, **9a–d**) showed significantly ALR2 inhibitory activity. Of these acid derivatives, 2-[2-(4-bromo-2-fluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene] carboxylic acid (**8d**) was the most active compound having an IC_{50} value of 0.11 μM. In contrast, **8b** was the lowest effective compound, producing 32% inhibition at 10 μM dose. Acids **8a**, **8e** and **9a** were shown to be potent ARIs, displaying IC_{50} values in the range between 0.14 and 0.59 μM. The remaining five compounds including **8c**, **8f**, **9b**, **9c**, and **9d** displayed varying levels of efficiency with IC_{50} values in the low micromolar ranging from 1.73 μM to 10.42 μM.

In structure–activity relationship (SAR) study at the N-2 position, our reported results from the comparison of derivatives of benzothiadiazine and pyridothiadiazine have shown that effects of substitution groups at the benzyl ring on ALR2 inhibition activity were in the rank order of 2,4,5-F_3 > 2-F,4-Br > 3-NO_2 > 4-CF_3 (Fig. 1).[25,26] Herein, it was found that the order of ALR2 inhibition activity for the benzothiazine derivatives was shown as 2-F,4-Br > 3-NO_2 > 2,4,5-F_3 > 2-F > 4-CF_3 > 3,5-$(CF_3)_2$ evidenced by comparing biological activities of compounds in series **8a–f** (**8d** > **8e** > **8a** > **8f** > **8c** > **8b**) and **9a–d** (**9a** > **9d** > **9c** > **9b**), respectively. 2-F,4-Br substitution (**8d**) on the benzyl ring structure of the benzothiazine was favored for the inhibition activity over the others and 3-NO_2 substitution (**8e**) had a close activity. Further more, both the benzothiazine derivatives **8d–e** were found to appear higher inhibition efficiency whereas 2,4,5-F_3 substituted benzothiazine derivative **8a** displayed lower efficiency when comparing them with their benzothiadiazine counterpart having the same substituent at the N-2 position (Fig. 1).[25] This indicates that at least in each case of 2-F,4-Br and 3-NO_2 substitution the α,β-unsaturated (C-4 double bond) moiety of **8d–e** may make orientation of the carboxylic acid side chain favored over the N-4 moiety of benzothiadiazine derivatives (Fig. 1)[25] for the interaction between the carboxylate and the anion site of ALR2.[33]

Table 1
Biological activity data for 1,2-benzothiazine 1,1-dioxide carboxylic acid derivatives



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9

No.	R	ALR2 IC_{50}[a] (μM)	ALR1 (% of inhibition)[b]
8a	2,4,5-F_3	0.59 (0.27–0.90)	18
8b	3,5-$(CF_3)_2$	32%[b]	c
8c	4-CF_3	6.13 (2.84–9.41)	c
8d	2-F,4-Br	0.11 (0.01–0.21)	11
8e	3-NO_2	0.14 (0.05–0.23)	26
8f	2-F	2.13 (1.20–3.06)	c
9a	2,4,5-F_3	0.34 (0.09–0.59)	28
9b	3,5-$(CF_3)_2$	10.42 (5.97–14.87)	c
9c	4-CF_3	5.30 (3.59–7.02)	c
9d	2-F	1.73 (8.70–13.87)	c
Epalrestat		0.12 (0.05–0.19)	c

[a] IC_{50} (95% CL) values represent the concentration required to produce 50% enzyme inhibition.
[b] The inhibitory effect was estimated at a concentration of 10 μM.
[c] Not determined.

In further SAR study at the C-4 position of benzothiazine derivatives, it was found that saturated carboxylic acids showed a significantly enhanced ALR2 inhibition activity over their corresponding α,β-unsaturated compounds. This was evidenced by comparing biological activities to each other for four pairs of compounds containing **8a** and **9a**, **8b** and **9b**, **8c** and **9c**, and **8f** and **9d** (Table 1) although saturated compounds corresponding to **8d–e** could not be synthetically obtained and thus used for the evidence. The compound of **9** series in each pair proved an increased activity than the other, for example, **9a** (IC_{50} = 0.34 μM) was more active than **8a** (IC_{50} = 0.59 μM). This result indicates that the acetic acid side chain linking to the saturated C-4 may take a more favorable orientation on the saturated C-4 for the interaction with the anion site of the enzyme.

As shown in Table 1, compounds **8a**, **8d**, **8e** and **9a** with significantly potent ALR2 inhibition activity (IC_{50} = 0.59, 0.11, 0.14 and 0.34 μM) were tested for their inhibition ability against ALR1. They were found to be slightly active (IC_{50} >10 μM), demonstrating their selectivity for ALR2.

3.2. Molecular modeling

To get a better understanding of the ALR2 inhibitory potency of the newly synthesized compounds at a molecular level and to propose a binding mode that explains the above-described SARs, docking experiments were performed on the human ALR2/NADP+/IDD594 complex (PDB entry code 1US0) using the automated docking program SYBYL 7.1. Optimized structures of compounds **8a–f** and **9a–d** were flexibly docked to the active site, which is comprised of three binding subsites. The first is the conserved anion-binding site located between the nicotinamide ring of the coenzyme and active site residues Tyr48, His110, and Trp111. The second is the hydrophobic pocket lined by residues Trp20, Phe122, and Trp219. The third is the specificity pocket formed by residues Trp111, Thr113, Phe122, Ala299, and Leu300.[33]

Analysis of the docking results revealed that most of the compounds were occupying the active site of ALR2, as the carboxyl head of the inhibitors, except for **8e**, formed hydrogen bonds with the anion-binding site of the enzyme. However, **8e** was bound to the anion site with its nitro group but not carboxylate displaying a different pattern from typical carboxylic inhibitors. Scores docked for **8d–e** were favorable when compared to those for the other compounds in **8** series. This is well consist with the study result from the ALR2 inhibition assay in which **8d–e** showed the most active. In addition, it was found that docking score for each of compounds **9** was more favorable than that for the counterpart in **8**, which is in agreement with the result of the enzyme inhibition experiment (Table 1), strongly suggesting that the saturated acids (**9**) are favored as inhibitors over the α,β-unsaturated acids. Analysis of docking poses for **8a** and **9a** may explain why saturated acids **9** have greater inhibitory activities. In the docking of **9a**, as shown in Figure 2c and d, the carboxyl head forms tight hydrogen bonds with the OH of Tyr48 (3.09 Å), the Nε2 atom of His110 (2.87 Å), and the Nε1 of Trp111 (2.70 Å), and a salt bridge with the positively charged nicotinamide moiety of the cofactor $NADP^+$ (N–O = 4.06 Å) within the anion-binding site. Further, the benzyl ring gets deeply trapped in the hydrophobic cage of the specificity pocket and in turn π-stacks perfectly against the indole moiety of Trp111. These interactions anchor the inhibitor tightly within the enzyme active site. Whereas in the docking of **8a**, despite a similar interaction with the anion-binding site was present, no such π-stacking interaction was found between the benzyl group and the side chain of Trp111 so that the benzyl side chain was pointed in an opposite direction but not the deep of the specificity pocket (Fig. 2a and b).

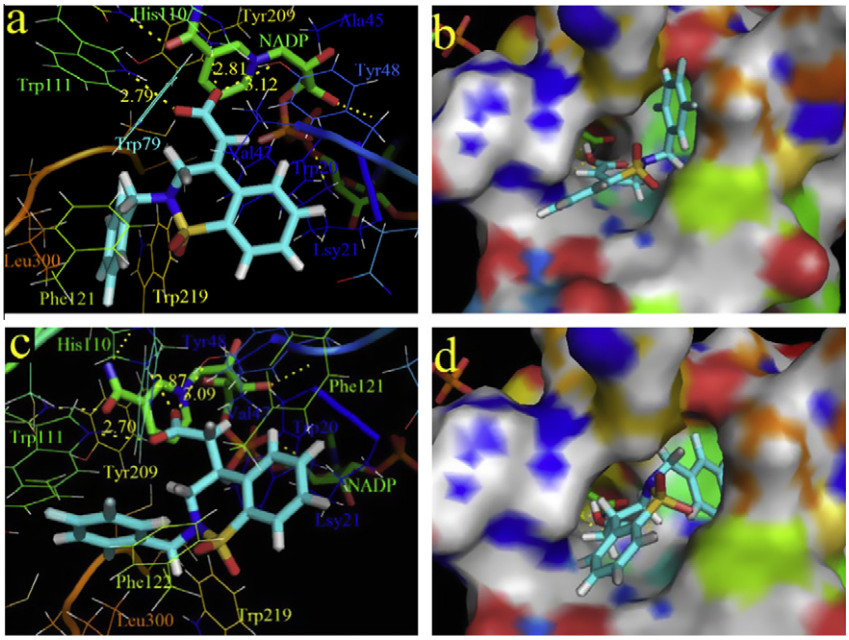

Figure 2. Docking of the inhibitors **8a** (panels a and b) and **9a** (panels c and d) into the active site of ALR2. (a and c) Protein structures are shown as cartoon diagrams with selected and labeled residues shown in line representation, and ligands and NADP are shown as stick models. Docked poses of compounds are shown in cyan (C), red (O), blue (N), yellow (S), and gray (F). (b and d) Protein residues are in surface representation.

4. Conclusions

The present effort to develop novel ARIs based on 1,2-benzothiazine 1,1-dioxide resulted in the formation of two groups of compounds containing α,β-unsaturated and saturated carboxylic acid (acetic acid) derivatives. They showed ALR2 inhibition activity with IC_{50} values in the range from 0.11 μM to10.42 μM. The most active was found to be 2-[2-(4-bromo-2-fluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene] carboxylic acid (**8d**) which is α,β-unsaturated. However, SAR combined with the docking studies demonstrated that the saturated carboxylic acid derivatives had a greater binding affinity with the enzyme and thus an enhanced inhibition activity in comparison with the α,β-unsaturated derivatives although saturated compound corresponding to α,β-unsaturated **8d** was not obtained. Consequently, we suggest that the configuration of C-4 plays an important role for the ALR2 inhibition of benzothiazine-based ARIs and the acetic acid side chain in the saturated compounds may take a favorable orientation on the C-4 position for the binding interaction or cause the benzyl side chain to point in the deep direction of specificity pocket. Given that all carboxylic acid compounds prepared in the present study were the stereoisomeric mixtures, we speculate that single stereoisomers provided in future by stereo-controlled synthetic strategy may be more powerful and selective ARIs.

5. Experimental section

5.1. Chemistry

Melting points were recorded on a X-4 microscopic melting point apparatus and were uncorrected. All reactions were routinely checked by TLC on silica gel Merck 60F254. The NMR spectra were recorded on a Bruker Advance 400 spectrometer (400 MHz, Bruker (Beijing) Tech. and Serv. Co., Ltd) or Bruker Advance 500 spectrometer (500 MHz, Beijing Normal University, China); chemical shifts are given in δ units (ppm) relative to internal standard TMS and refer to $CDCl_3$ or DMSO-d_6 solutions. MS was performed with a Varian 500-MS ion trap mass spectrometer equipped with an ESI source (Varian, USA).

The following HPLC methods were used to determine purity of acetic acid derivatives and epalrestat using Hitachi L2000 liquid chromatograph (Hitachi High-Technologies Corporation, Japan). All acetic acid derivatives and epalrestat tested in biological assays were ⩾95% pure. Method: column, C18, 5 μ, 250 mm × 4.6 mm; mobile phase: H_2O (0.1% TFA)/MeOH = 1:9, for 6 min; room temperature; flow rate: 1 mL/min; detection: 250 nm.

The sodium saccharin, methyl bromoacetate, 4-bromo-2-fluorobenzyl bromide, 3-nitrobenzyl bromide, 4-(trifluoromethyl)benzyl bromide, 2,4,5-trifluorobenzyl bromide and 3,5-bis(trifluoromethyl)benzyl bromide, used to obtain the target inhibitors, were from Alfa Aesar.

5.2. Methyl [1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3*H*)-yl] acetate (2)

A mixture of sodium saccharin (60.3 g, 250 mmol), DMF (150 ml) and methyl bromoacetate (38.3 g, 250 mmol) was taken in a round bottom flask and was heated at 60 °C for 1 h. Contents were then cooled to room temperature and poured over ice cooled water (1000 ml) resulting in the formation of a white solid, which was filtered and washed with cold water. The solid was dried and recrystallized from methyl alcohol to get the product as a white solid (40.2 g, 61%); mp 113–115 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.75 (s, 3H), 4.69 (s, 2H), 8.06 (m, 1H), 8.11 (m, 1H), 8.19 (d, J = 6.0 Hz, 1H), 8.40 (d, J = 6.0 Hz, 1H).

5.3. Methyl 4-hydroxy-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide (3)

Sodium metal (6.9 g, 300 mmol) and dry methanol (300 ml) was allowed to reflux until all the metal dissolved. To this solution, **2** (30.6 g, 120 mmol) was added in a single portion under inert conditions. Temperature of the mixture was maintained at 60 °C for 30 min till the completion of precipitation. The contents were then suddenly poured over an ice-water mixture. HCl (10%) was added

to the mixture till the pH became approximately 3. The precipitates formed were filtered and washed with cold water. The solid was dried and recrystallized from methyl alcohol to get the product as a white solid (16.9 g, 55%); mp 163–165 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.96 (s, 3H), 6.24 (s, 1H), 7.76 (m, 2H), 7.93 (m, 1H), 8.11 (d, J = 7.5 Hz, 1H), 11.44 (s, 1H).

5.4. 2*H*-1,2-Benzothiazin-4(3*H*)-one 1,1-dioxide (4)

A mixture of **3** (10.2 g, 40.0 mmol) was added to 150 mL of concentrated hydrochloric acid and refluxed for 8 h. The crude material was then poured into ice-water mixture and the precipitate was collected by suction filtration, washed with cold water and dried in vacuo to give the desired product (5.3 g, 67%); mp 160–162 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.96 (d, J = 7.5 Hz, 2H), 5.43 (d, J = 6.5 Hz, 1H), 7.76 (m, 1H), 7.82 (m, 1H), 7.91 (m, 1H), 8.11(d, J = 7.5 Hz, 1H).

5.5. General synthetic procedure for 5a–e

To a solution of **4** (43.1 g, 220 mmol), in benzene (300 mL) were added *p*-toluenesulfonic acid (PTSA) (2.6 g, 14.0 mmol) and ethylene glycol (31 mL, 550 mmol). The reaction mixture was refluxed in a Dean and Stark apparatus for 3 h. The crude material was then poured into ice-water mixture and the precipitate was collected by suction filtration, washed with cold water and dried in vacuo to leave the product, which was used without further purification. A mixture of the dioxolane (4.9 g, 20 mmol), potassium carbonate (3 g), and appropriate benzyl bromide (20 mmol) in acetonitrile (50 mL) was heated at 70 °C for 2 h. After evaporation of the solvent under reduced pressure, the resulting dioxolane was added to 40 mL tetrahydrofuran and 30 mL 10% hydrochloric acid and stirred at 80 °C for 6 h. The reaction mixture was poured into water with vigorous stirring. After 30 min, filtration afforded the desired product, which was used without further purification.

5.5.1. 2-(2,4,5-Trifluorobenzyl)-2*H*-1,2-benzothiazin-4(3*H*)-one 1,1-dioxide (5a)

Pale yellow powder (4.7 g, 70%); mp 126–128 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.48 (s, 2H), 4.54 (s, 2H), 7.50 (m, 2H), 7.84–7.96 (m, 4H).

5.5.2. 2-[3,5-Bis(trifluoromethyl)benzyl]-2*H*-1,2-benzothiazin-4(3*H*)-one 1,1-dioxide (5b)

Pale yellow powder (4.9 g, 59%); mp 106–108 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.68 (s, 2H), 4.70 (s, 2H), 7.74 (m, 2H), 7.81 (m, 2H), 7.91 (m, 2H), 7.96 (m, 1H).

5.5.3. 2-[4-(Trifluoromethyl)benzyl]-2*H*-1,2-benzothiazin-4(3*H*)-one 1,1-dioxide (5c)

Pale yellow powder (5.0 g, 71%); mp 103–105 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.59 (s, 2H), 4.61 (s, 2H), 7.46 (m, 2H), 7.66 (m, 2H), 7.89 (m, 2H), 7.99 (m, 2H).

5.5.4. 2-(4-Bromo-2-fluorobenzyl)-2*H*-1,2-benzothiazin-4(3*H*)-one 1,1-dioxide (5d)

Pale yellow powder (5.8 g, 75%); mp 102–105 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.49 (s, 2H), 4.50 (s, 2H), 7.35 (m, 2H), 7.43 (d, J = 9.5 Hz, 1H), 7.86 (m, 3H), 7.93 (m, 1H).

5.5.5. 2-(3-Nitrobenzyl)-2*H*-1,2-benzothiazin-4(3*H*)-one 1,1-dioxide (5e)

Pale yellow powder (4.4 g, 67%); mp 143–145 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.64 (s, 2H), 4.68 (s, 2H), 7.60 (m, 1H), 7.80 (m, 1H), 7.87 (m, 2H), 7.97 (m, 2H), 8.14 (m, 2H).

5.6. General synthetic procedure for 6a–e

To a stirred solution of triphenylphosphine (52.4 g, 0.2 mol) in benzene (300 mL) was added dropwise a solution of methyl bromoacetate (30.4 g, 0.2 mol) in benzene (100 mL) over 1 h. The reaction was allowed to stir for 4 h. The solid phosphonium bromide product was filtered and washed with hexane to provide a white solid. The crude solid was dissolved in water (600 mL) and sodium hydroxide (20.2 g, 0.2 mol) was added. After 1 h, the precipitate was filtered and washed with water to provide a white solid. The crude product was recrystallized from benzene/petroleum ether to provide methyl 2-(triphenylphosphoranylidene) acetate (57.4 g, 86% for two steps).

A solution containing the appropriate **5** (3 mmol) and methyl (triphenylphosphoranylidene) acetate (4.5 mmol) in toluene (30 mL) was heated at 100 °C for 2 h. After evaporation of the solvent, the residue was purified by column chromatography (ethyl acetate–hexane; 1:3) to give the product.

5.6.1. 2-[2-(2,4,5-Trifluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid methyl ester (6a)

White powder (0.65 g, 55%); mp 103–104 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.60 (s, 3H), 3.73 (s, 2H), 4.94 (s, 2H), 7.01 (s, 1H), 7.42 (m, 1H), 7.54 (m, 1H), 7.60 (m, 2H), 7.74 (m, 1H), 7.90 (d, J = 8.0 Hz, 1H).

5.6.2. 2-[2-(3,5-Bis(trifluoromethyl)benzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid methyl ester (6b)

White powder (0.70 g, 49%); mp 139–141 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.59 (s, 3H), 3.72 (s, 2H), 5.15 (s, 2H), 7.08 (s, 1H), 7.56 (m, 1H), 7.62 (m, 1H), 7.76 (m, 1H), 7.91 (m, 3H), 8.05 (s, 1H).

5.6.3. 2-[2-(4-(Trifluoromethyl)benzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid methyl ester (6c)

White powder (0.76 g, 62%); mp 158–160 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.66 (s, 3H), 4.23 (s, 2H), 4.86 (s, 2H), 6.75 (s, 1H), 7.53 (m, 3H), 7.67 (m, 3H), 7.90 (m, 1H), 8.05 (m, 1H).

5.6.4. 2-[2-(4-Bromo-2-fluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid methyl ester (6d)

White powder (0.79 g, 60%); mp 119–121 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.63 (s, 3H), 3.76 (s, 2H), 4.98 (s, 2H), 7.02 (s, 1H), 7.31 (m, 1H), 7.45 (m, 1H), 7.54 (m, 1H), 7.65 (m, 2H), 7.78 (m, 1H), 7.93 (d, J = 8.0 Hz, 1H).

5.6.5. 2-[2-(3-Nitrobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid methyl ester (6e)

White powder (0.63 g, 54%); mp 137–139 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.64 (s, 3H), 3.77 (s, 2H), 5.14 (s, 2H), 7.11 (s, 1H), 7.59 (m, 1H), 7.71 (m, 2H), 7.81 (m, 2H), 8.00 (m, 1H), 8.20 (m, 2H).

5.7. 2-[2-(2-Fluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid methyl ester (6f)

To a suspension of 10% palladium on carbon (0.27 g) in methanol (10 mL) was added a solution of **6d** (0.35 g, 0.8 mmol) in EtOAc (10 mL). The mixture was stirred at room temperature for 12 h under a hydrogen atmosphere. The catalyst was filtered off through Celite pad and the filtrate was concentrated and purified via flash chromatograph to obtain the pure product as a white powder (0.27 g, 92%); mp 70–72 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.58 (s, 3H), 3.72 (s, 2H), 4.97 (s, 2H), 6.98 (s, 1H), 7.16 (m, 1H), 7.21 (m, 1H), 7.29 (m, 1H), 7.35 (m, 1H), 7.52 (d, J = 8.0 Hz, 1H,), 7.59 (m, 1H), 7.74 (m, 1H), 7.90 (d, J = 8.0 Hz, 1H).

5.8. General synthetic procedure for 7a–c

The title compounds were obtained as described for **6f** starting from **5a–c** (1 mmol).

5.8.1. 2-[2-(2,4,5-Trifluorobenzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl]acetic acid methyl ester (7a)

White powder (0.35 g, 88%); mp 103–105 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.63 (m, 1H), 2.79 (m, 1H), 3.52 (s, 3H), 3.58 (m, 1H), 3.61–3.70 (m, 2H), 4.10 (d, J = 14.0 Hz, 1H), 4.54 (d, J = 14.0 Hz, 1H), 7.50 (m, 2H), 7.60 (m, 2H), 7.65 (m, 1H), 7.79 (d, J = 8.0 Hz, 1H).

5.8.2. 2-(2-(3,5-Bis(trifluoromethyl)benzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl) acetic acid methyl ester (7b)

White powder (0.45 g, 93%); mp 102–105 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.62 (m, 1H), 2.77 (m, 1H), 3.57 (s, 3H), 3.62 (m, 1H), 3.66 (m, 1H), 3.74 (m, 1H), 4.38 (d, J = 15.2 Hz, 1H), 4.69 (d, J = 15.2 Hz, 1H), 7.51 (m, 2H), 7.62 (m, 1H), 7.81 (d, J = 8.0 Hz, 1H), 8.05 (s, 2H), 8.09 (s, 1H).

5.8.3. 2-[2-(4-(Trifluoromethyl)benzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl]acetic acid methyl ester (7c)

White powder (0.35 g, 85%); mp 101–103 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.64 (m, 1H), 2.77 (m, 1H), 3.45 (s, 3H), 3.57 (m, 1H), 3.60–3.68 (m, 2H), 4.20 (d, J = 14.8 Hz, 1H), 4.59 (d, J = 14.8 Hz, 1H), 7.51 (d, J = 6.8 Hz, 2H), 7.60 (m, 3H), 7.75 (d, J = 7.6 Hz, 2H), 7.81 (d, J = 8.0 Hz, 1H).

5.8.4. 2-[2-(2-Fluorobenzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl]acetic acid methyl ester (7d)

The title compounds were obtained as described for **7a** starting from **6f**. White powder (0.34 g, 94%); mp 93–95 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.60 (m, 1H), 2.78 (m, 1H), 3.49 (s, 3H), 3.57 (m, 1H), 3.60–3.68 (m, 2H), 4.13 (d, J = 14.0 Hz, 1H), 4.57 (d, J = 14.0 Hz, 1H), 7.23 (m, 2H), 7.39 (m, 1H), 7.42–7.52 (m, 3H), 7.61 (t, 1H), 7.80 (d, J = 7.6 Hz, 1H).

5.9. General synthetic procedure for 8a–f

A mixture of the appropriate **6** (0.5 mmol), 1,4-dioxane (5 mL) and saturated aqueous sodium hydroxide (8 mL) was stirred at room temperature for 12 h. The alkaline suspension was adjusted to acidic with 0.1 N HCl and extracted threefold with ethyl acetate (3 × 30 mL). The combined organic layers were dried over $MgSO_4$ and filtered. The filtrate was concentrated to dryness under reduced pressure and the residue of the desired compound was recrystallised in methanol to give pure product.

5.9.1. 2-[2-(2,4,5-Trifluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid (8a)

Colorless crystal (0.13 g, 69%); purity: 98.9%; mp 174–176 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.61 (s, 2H), 4.93 (s, 2H), 6.98 (s, 1H), 7.43 (m, 1H), 7.54–7.63 (m, 3H), 7.75 (m, 1H), 7.90 (m, 1H), 12.48 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.3, 132.8, 132.5, 131.3, 130.7, 128.0, 124.6, 121.4, 118.4, 118.2, 112.6, 106.7, 106.5, 106.4, 106.2, 43.8, 35.2; MS *m/z*: negative mode 381.9 ([M−H]$^-$), positive mode 406.0 ([M+Na]$^+$).

5.9.2. 2-[2-(3,5-Bis(trifluoromethyl)benzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid (8b)

Colorless crystal (0.19 g, 82%); purity: 98.6%; mp 182–184 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.60 (s, 2H), 5.14 (s, 2H), 7.06 (s, 1H), 7.58 (m, 1H), 7.61 (m, 1H), 7.76 (m, 1H), 7.90 (m, 1H), 7.94 (s, 2H), 8.05 (s, 1H), 12.49 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.3, 140.4, 132.8, 132.6, 131.4, 130.9, 130.7, 130.3, 129.9, 128.4, 128.1, 124.6, 124.5, 121.8, 121.7, 121.4, 113.2, 49.5, 35.2; MS *m/z*: negative mode 463.9 ([M−H]$^-$), positive mode 466.0 ([M+H]$^+$), 488.1 ([M+Na]$^+$).

5.9.3. 2-[2-(4-(Trifluoromethyl)benzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid (8c)

Colorless crystal (0.15 g, 77%); purity: 97.7%; mp 170–172 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.59 (s, 2H), 5.03 (s, 2H), 7.00 (s, 1H), 7.52 (m, 3H), 7.62 (m, 1H), 7.77 (m, 3H), 7.93 (m, 1H), 12.46 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.3, 141.5, 132.9, 132.5, 131.5, 130.7, 128.6, 128.3 (2C), 127.9, 125.7, 125.6, 124.5, 121.5, 112.4, 109.5, 49.5, 35.3; MS *m/z*: negative mode 396.0 ([M−H]$^-$), positive mode 398.0 ([M+H]$^+$), 420.0 ([M+Na]$^+$).

5.9.4. 2-[2-(4-Bromo-2-fluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid (8d)

Colorless crystal (0.14 g, 77%); purity: 99.5%; mp 159–161 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.59 (s, 2H), 4.93 (s, 2H), 6.95 (s, 1H), 7.27 (m, 1H), 7.42 (m, 1H), 7.57 (m, 1H), 7.61 (m, 2H), 7.76 (m, 1H), 7.89 (m, 1H), 12.46 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.3, 132.8, 132.4, 131.8, 131.4, 130.7, 127.9, 124.5, 123.3, 123.1, 121.7, 121.4, 119.1, 118.8, 112.4, 44.0, 35.2; MS *m/z*: negative mode 423.7, 425.8 ([M−H]$^-$), positive mode 426.9, 427.9 ([M+H]$^+$), 448.9, 449.9 ([M+Na]$^+$).

5.9.5. 2-[2-(3-Nitrobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid (8e)

Colorless crystal (0.13 g, 67%); purity: 97.8%; mp 194–196 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.59 (s, 2H), 5.08 (s, 2H), 7.03 (s, 1H), 7.57 (m, 1H), 7.63 (m, 2H), 7.73 (m, 2H), 7.92 (m, 1H), 8.14 (m, 1H), 8.20 (s, 1H), 12.48 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.3, 147.8, 139.0, 134.3, 132.8, 132.5, 131.4, 130.7, 130.3, 128.0, 124.6, 122.9, 122.5, 121.5, 112.6, 49.3, 35.2; MS *m/z*: negative mode 372.9 ([M−H]$^-$), positive mode 375.0 ([M+H]$^+$), 397.0 ([M+Na]$^+$).

5.9.6. 2-[2-(2-Fluorobenzyl)-1,1-dioxido-2*H*-1,2-benzothiazin-4(3*H*)-ylidene]acetic acid (8f)

Colorless crystal (0.14 g, 83%); purity: 96.1%; mp 181–183 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.60 (s, 2H), 4.97 (s, 2H), 6.96 (s, 1H), 7.17 (m, 2H), 7.35 (m, 2H), 7.61 (m, 2H), 7.74 (m, 1H), 7.90 (d, J = 7.6 Hz, 1H), 12.47 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.3, 132.8, 132.4, 131.5, 130.7, 130.1, 127.9, 124.6, 124.5, 123.6, 123.4, 121.3, 115.6, 115.4, 112.3, 44.3, 35.2; MS *m/z*: negative mode 345.9 ([M−H]$^-$), positive mode 370.1 ([M+Na]$^+$).

5.10. General synthetic procedure for 9a–d

The title compounds were obtained as described for **8** starting from **7a–d** (0.5 mmol).

5.10.1. 2-[2-(2,4,5-Trifluorobenzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl]acetic acid (9a)

Colorless crystal (0.11 g, 58%); purity: 99.2%; mp 75–78 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.54 (m, 1H), 2.72 (m, 1H), 3.56 (m, 1H), 3.65 (m, 2H), 4.13 (d, J = 14.0 Hz, 1H), 4.52 (d, J = 14.0 Hz, 1H), 7.47 (m, 1H), 7.51 (s, 1H), 7.58 (m, 2H), 7.62 (s, 1H), 7.79 (d, J = 7.6 Hz, 1H), 12.38 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.4, 138.3, 136.0, 132.7, 128.9, 127.7, 123.9, 119.4, 119.3, 106.6, 106.4, 106.3, 106.1, 48.9, 43.5, 37.3, 30.6; MS *m/z*: negative mode 384.0 ([M−H]$^-$), positive mode 408.0 ([M+Na]$^+$).

5.10.2. 2-[2-(3,5-Bis(trifluoromethyl)benzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl]acetic acid (9b)

Colorless crystal (0.17 g, 72%); purity: 95.0%; mp 180–182 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.56 (m, 1H), 2.75 (m, 1H), 3.56

(m, 1H), 3.72 (m, 2H), 4.44 (d, J = 15.2 Hz, 1H), 4.66 (d, J = 15.2 Hz, 1H), 7.50 (m, 2H), 7.62 (m, 1H), 7.80 (d, J = 8.0 Hz, 1H), 8.05 (s, 2H), 8.06 (s, 1H) , 12.41 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.4, 140.2, 138.3, 135.9, 132.7, 130.5, 130.2, 129.9, 129.1, 128.9, 127.7, 124.6, 123.9, 121.9, 121.5, 49.8, 49.3, 37.4, 30.4; MS m/z: negative mode 466.0 ($[M-H]^-$), positive mode 468.1 ($[M+H]^+$), 490.1($[M+Na]^+$).

5.10.3. 2-[2-(4-(Trifluoromethyl)benzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl]acetic acid (9c)

Colorless crystal (0.14 g, 68%); purity: 98.6%; mp 177–178 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.58 (m, 1H), 2.75 (m, 1H), 3.59 (m, 1H), 3.67 (m, 2H), 4.28 (d, J = 15.2 Hz, 1H), 4.55 (d, J = 15.2 Hz, 1H), 7.47–7.52 (m, 2H), 7.61 (m, 3H), 7.74 (d, J = 8.0 Hz, 2H), 7.81 (d, J = 8.0 Hz, 1H), 12.41 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 172.4, 141.2, 138.4, 136.1, 132.7, 129.0, 128.8 (2C), 128.4, 127.7, 125.5, 125.4, 123.9, 109.4, 49.6, 49.3, 37.4, 30.3; MS m/z: negative mode 398.0 ($[M-H]^-$), positive mode 422.0 ($[M+Na]^+$).

5.10.4. 2-[2-(2-Fluorobenzyl)-1,1-dioxido-3,4-dihydro-2*H*-1,2-benzothiazin-yl]acetic acid (9d)

Colorless crystal (0.11 g, 63%); purity: 97.3%; mp 136–138 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.57 (m, 1H), 2.73 (m, 1H), 3.59 (m, 1H), 3.65 (m, 2H), 4.19 (d, J = 14.4 Hz, 1H), 4.52 (d, J = 14.4 Hz, 1H), 7.21 (m, 2H), 7.39 (m, 1H), 7.47–7.54 (m, 3H), 7.61 (m, 1H), 7.79 (d, J = 7.2 Hz, 1H), 12.39 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 177.4, 138.3, 136.2, 132.6, 131.4, 130.2, 128.9, 127.7, 124.6, 123.8, 122.9, 115.6, 115.4, 49.0, 44.1, 37.5, 30.3; MS m/z: negative mode 347.9 ($[M-H]^-$), positive mode 350.0 ($[M+H]^+$), 372.0 ($[M+Na]^+$).

5.11. Biology, materials, and methods

ALR2 and ALR1 were obtained from Wistar rats, 200–250 g, b.w., supplied by Vital River, Beijing, China. D,L-Glyceraldehyde, sodium D-glucuronate, and NADPH were from Sigma–Aldrich. All other chemicals were of reagent grade. Epalrestat was prepared in our laboratory according to a reported method.

ALR1 and ALR2 were prepared in accordance with the method of Kinoshita[34] and Concettina La Motta.[35] Enzyme activity was assayed spectrophotometrically on an Unico 4802S UV/VIS double beam spectrophotometer by measuring the decrease in absorption of NADPH at 340 nm, which accompanies the oxidation of NADPH catalyzed by ALR2 and ALR1.

5.12. Enzymatic assays

ALR2 activity was performed at 32 °C in a reaction mixture containing 0.25 mL of 0.10 mM NADPH, 0.25 mL of 0.1 M sodium phosphate buffer (pH 6.2), 0.1 mL of enzyme extract, 0.15 mL of deionized water, and 0.25 mL of 10 mM D,L-glyceraldehyde as substrate in a final volume of 1 mL. The reaction mixture except for D,L-glyceraldehyde was incubated at 32 °C for 10 min. The substrate was then added to start the reaction, which was monitored for 4 min.

ALR1 activity was performed at 37 °C in a reaction mixture containing 0.25 mL of 0.12 mM NADPH, 0.1 mL of enzyme extract, 0.25 mL of 0.1 M sodium phosphate buffer (pH 7.2), 0.15 mL of deionized water, and 0.25 mL of 20 mM sodium D-glucuronate as substrate in a final volume of 1 mL. The reaction mixture except for sodium D-glucuronate was incubated at 37 °C for 10 min. The substrate was then added to start the reaction, which was monitored for 4 min.

The inhibitory activity of the newly synthesized compounds against ALR2 and ALR1 was assayed adding 5 μL of the inhibitor solution to the reaction mixture described above. All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with deionized water. To correct for the nonenzymatic oxidation of NADPH, the rate of NADPH oxidation in the presence of all of the reaction mixture components except the substrate was subtracted from each experimental rate. The inhibitory effect of the synthetic compounds was routinely estimated at a concentration of 10^{-5} M (the concentration is referred to that of the compound in the reaction mixture). Those compounds found to be active were tested at additional concentrations between 10^{-5} and 10^{-7} M. Each dose-effect curve was generated using at least four concentrations of inhibitor causing an inhibition between 20% and 80% with three replicates at each concentration. The 95% confidence limits (95% CL) were calculated from t values for n −2, where n is the total number of determinations.

5.13. Docking experiments

Conformational searches and molecular docking were performed using version 7.1 of the SYBYL.

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