Full Paper

Synthesis and Anticonvulsant Activities of Some Triazolothiadiazole Derivatives

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The present study describes the synthesis and anticonvulsant activity evaluation of 6-substituted-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole derivatives (**4a**–**4x**) and their partially dehydrogenated products 5,6-dihydro-6-substituted-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole derivatives (**5a**–**5n**). The bioevaluation demonstrated that most compounds in the series of **4a**–**4x** exhibited potent anticonvulsant activity in the maximal electroshock test. Among which, 6-(4-chlorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**4h**) emerged as the most promising candidate on the basis of its favorable ED_{50} value of 23.7 mg/kg and PI value of 10.8. In addition, the potency of compound **4h** against seizures induced by pentylenetetrazole, 3-mercaptopropionic acid, and bicuculline in the chemical-induced seizure tests suggested that compound **4h** displayed broad-spectrum activity in several models, and it may exert its anticonvulsant activity through affecting the GABAergic system.

Keywords: Anticonvulsant / Maximal electroshock / Pentylenetetrazole / Synthesis / Triazolothiadiazole

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Introduction

Epilepsy, one of the most frequent neurological afflictions in men characterized by excessive temporary neuronal discharges resulting in uncontrolled convulsion, inflicts more than 2 million Americans and 60 million people worldwide [1, 2]. Although the current drugs provide adequate seizure control in many patients, it is roughly estimated that up to 28–30% of patients are poorly treated with the available antiepileptic drugs (AEDs) [3, 4]. Moreover, many AEDs have serious side effects [5–10], and lifelong medication may be required. Toxicity, intolerance, and lack of efficacy are the limitations of the current AEDs. Therefore, the continued search for safer and more effective new AEDs is necessary.

The 1,2,4-triazole nucleus has been incorporated into a wide variety of therapeutically important agents, such as antimicrobial [11–13], antiviral agents [14], anti-HIV [15, 16], anticonvulsant [15, 16], and enzyme inhibition activities [17, 18]. Moreover, the chemistry of 1,2,4-triazoles and their fused heterocyclic derivatives has received considerable attention owing to their synthetic and biological importance. In our previous study, a series of triazolothiadiazines (Fig. 1, **I**) were found to possess good anticonvulsant activity [19]. Among these compounds, 6-(4-chlorophenyl)-7*H*-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine was the most promising one with an ED_{50} value of 40.9 mg/kg and a PI value of 6.5 in the maximal electroshock test (MES).

Based on the above results and in continuation of our efforts directed toward the search for new heterocyclic compounds with anticonvulsant activities, a series of triazolothiadiazole derivatives (Fig. 1, **II**) were designed and synthesized in this study. Compounds **II**, the ring contraction analogues of compounds **I** through removal of a CH_2 in the compounds **I**, were anticipated to possess a better anticonvulsant activity. For further structure–activity relationship (SAR) establishment, another series of compounds **5a**–**5n**, the dehydrogenated products of compounds **II**, were also prepared. The structures of all the target compounds were characterized. Anticonvulsant activity and neurotoxicity were also evaluated. For explaining the possible mechanism of action, the most active compound **4h** was tested in pentyl-

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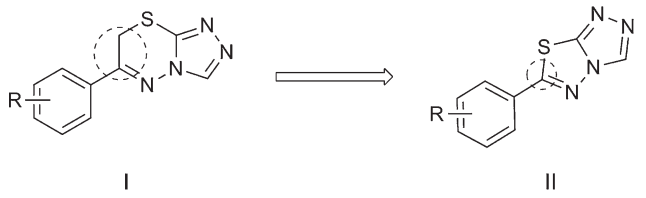


Figure 1. Structures of compounds **I** and **II**.

enetetrazole (PTZ), 3-mercaptopropionic acid (3-MP), and bicuculline (BIC) induced seizure tests.

Results and discussion

Chemistry

Target compounds were prepared according to Scheme 1. The key intermediate, 4-amino-4*H*-1,2,4-triazole-3-thiol (**3**) was prepared from thiocarbonohydrazide according to the literature [20]. The resulting **3** was further treated with fatty acids or aromatic acids in polyphosphoric acid (PPA), or aromatic aldehydes in DMF to obtain 6-substituted-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole derivatives (**4a–4x**) and 5,6-dihydro-6-substituted-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole derivatives (**5a–5n**), respectively. Structures of these compounds were confirmed by IR, MS, ^{1}H NMR, and elementary analysis.

Pharmacological evaluations

MES screening

The anticonvulsant activity and neurotoxicity of the synthesized compounds were evaluated following the standard procedures proposed by the NIH anticonvulsant drug development (ADD) program. The initial evaluation (phase I) included the maximal electroshock seizure (MES) and neurotoxicity (TOX).

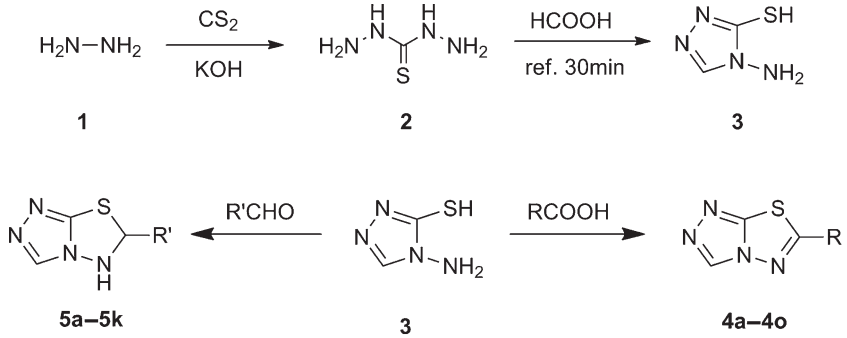
The compounds **4a–4x** and **5a–5n** were administrated intraperitoneally (i.p.) into the mice using doses of 30, 100, and 300 mg/kg and the observations were taken at the time interval of 0.5 h. Neurotoxicity was measured by the rotarod test. Their preliminary anticonvulsant activities were obtained and are listed in Tables 1 and 2.

The initial anticonvulsant evaluation of **4a–4x** indicated that all the compounds in this series were effective in i.p. MES screens except **4q** and **4r**, indicative of their ability to prevent seizure spread. At the dose of 100 mg/kg, compounds **4a–4h**, **4k**, and **4u–4x** showed complete protection, while **4j**, **4l–4m**, **4o–4p**, and **4t** showed protection in 1/3 or 2/3 of the tested mice. Compounds **4a**, **4d**, **4g**, and **4v** exhibited protection in 1/3 of the tested mice at 30 mg/kg. And compounds **4c**, **4h**, and **4w** exhibited protection in 2/3 of the tested mice at 30 mg/kg. For the series of **5a–5n**, only **5a**, **5b**, **5d**, **5f**, **5g**, and **5j** displayed poor anticonvulsant activity at the dose of 300 mg/kg.

In the neurotoxicity screen, compounds **4i**, **4l**, **4n–4s**, and **5a–5n** did not show any neurotoxicity in the maximum dose administered (300 mg/kg). Compounds **4v–4x** revealed neurotoxicity at the dose of 100 mg/kg. The rest of the compounds (majority) exhibited certain neurotoxicity at the dose of 300 mg/kg.

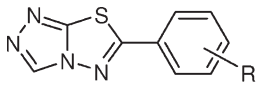
On the basis of the considerable anticonvulsant activity suggested in phase I testing, compounds **4c**, **4h**, and **4w** were subjected to phase II trials for the quantification of their anticonvulsant activity (indicated by ED_{50}) and neurotoxicity (indicated by TD_{50}) in mice. Results of the quantitative test for selected compounds, along with the data of the standard drugs carbamazepine and valproate, are shown in Table 3. The tested compounds showed a higher protective index (PI) than both standard drugs although their ED_{50} values were bigger than that of carbamazepine. Among the tested compounds, 6-(4-chlorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**4h**) gave an ED_{50} value of 23.7 mg/kg and a TD_{50} value of 254.6 mg/kg, which resulted in a higher PI value that was $TD_{50}/ED_{50} = 10.8$, when compared to carbamazepine (4.5) and valproate (1.6).

In the present study, we have synthesized a series of triazolothiadiazole derivatives **4a–4x**, and part of their dehydrogenated products **5a–5n**. The results of bioevaluation led to an understanding of the SAR of these compounds. In the first series (**4a–4x**), compounds **4a–4k**, whose phenyl ring was

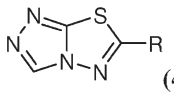


Scheme 1. The synthesis route of the target compounds.

Table 1. Phase I anticonvulsant screening of the compounds **4a–4x**



(4a–4q)



(4r–4x)

Compounds	R	MES[a] (mg/kg)			TOX (mg/kg)		
		300	100	30	300	100	30
4a	H	–	3/3[b]	1/3	1/3	0/3	–[c]
4b	2-F	–	3/3	0/3	2/3	0/3	–
4c	3-F	–	3/3	2/3	2/3	0/3	–
4d	4-F	–	3/3	1/3	1/3	0/3	–
4e	2,6-2F	–	3/3	0/3	1/3	0/3	–
4f	2-Cl	–	3/3	0/3	2/3	0/3	–
4g	3-Cl	–	3/3	1/3	1/3	0/3	–
4h	4-Cl	–	3/3	2/3	2/3	0/3	–
4i	2,4-2Cl	2/3	0/3	–	0/3	0/3	–
4j	2-Br	3/3	2/3	–	1/3	0/3	–
4k	4-Br	–	3/3	0/3	1/3	0/3	–
4l	2-NO_2	2/3	1/3	–	0/3	0/3	–
4m	3-NO_2	3/3	2/3	–	1/3	0/3	–
4n	4-NO_2	1/3	0/3	–	0/3	0/3	–
4o	2-OCH_3	2/3	1/3	–	0/3	0/3	–
4p	3-OCH_3	2/3	1/3	–	0/3	0/3	–
4q	4-OCH_3	0/3	0/3	–	0/3	0/3	–
4r	$-C_2H_5$	0/3	0/3	–	0/3	0/3	–
4s	*n*-C_3H_7	1/3	0/3	–	0/3	0/3	–
4t	*n*-C_4H_9	3/3	1/3	–	1/3	0/3	–
4u	*n*-C_5H_{11}	–	3/3	0/3	2/3	0/3	–
4v	*n*-C_6H_{13}	–	3/3	1/3	3/3	1/3	–
4w	*n*-C_7H_{15}	–	3/3	2/3	3/3	2/3	0/3
4x	*n*-C_8H_{17}	–	3/3	0/3	3/3	2/3	0/3

[a] Maximal electroshock: Doses of 30, 100, and 300 mg/kg were administrated intraperitoneally in mice; the animals were examined 0.5 h after administration.
[b] The figures n_1/n_2: the animals protected/the animals tested.
[c] The dash (–): not tested.

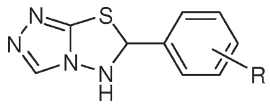
substituted with F, Cl, or Br, exhibited more potent anticonvulsant activity than those substituted with NO_2 and methoxyl (**4l–4q**). Compounds **4r–4x** were triazolothiadiazole molecules substituted by alkyl chain. The length of the alkyl chain appeared to have a direct impact on the anticonvulsant activity of these derivatives. From compounds **4r–4w**, as alkyl chain length increased, the anti-MES activity gradually increased with the compound **4w** (with the *n*-heptyl substituted group) being the most active. The trend reversed, however, when the alkyl chain had more than seven carbon atoms. Obviously, in this study the activity curve of the alkyl chain substituded derivatives is bell-shaped with a maximum activity peak. It was noteworthy that the dehydrogenation of thiadiazole in the series **4a–4x** caused a significant reduction or the complete loss of activity (e.g., compounds **5a–5n**). It seems that the conjugation and planar construction of the triazolothiadiazole play a fundamental role in anticonvulsant activity.

Chemical-induced seizure models

In this study, the majority of synthesized compounds were highly potent in the MES test, and the MES test is known to be sensitive to sodium channel inhibitors (e.g., phenytoin, carbamazepine), which suggested that the tested compounds may inhibit voltage-gated ion channels (particularly sodium channels). To further investigate the effects of the anticonvulsant activity in several different models and speculate about the possible mechanism of anticonvulsant action, compound **4h** was tested against convulsions induced by chemical substances, including PTZ, 3-MP, and BIC. Compound **4h** was administered to mice at 30 mg/kg i.p., which was higher than its ED_{50} value and far below its TD_{50} value. The reference drug carbamazepine was also administered at 30 mg/kg i.p.

In the sc-PTZ model, carbamazepine inhibited the clonic seizures, tonic seizures and death at the rates of 0%, 100%, and 100%, respectively. While compound **4h** inhibited the

Table 2. Phase I anticonvulsant screening of the compounds **5a–5n**



Compounds	R	MES[a] (mg/kg)			TOX (mg/kg)		
		300	100	30	300	100	30
5a	H	2/3	0/3[b]	–[c]	0/3	–	–
5b	2-F	1/3	0/3	–	0/3	–	–
5c	3-F	0/3	0/3	–	0/3	–	–
5d	4-F	2/3	0/3	–	0/3	–	–
5e	2-Cl	0/3	0/3	–	0/3	–	–
5f	3-Cl	1/3	0/3	–	0/3	–	–
5g	4-Cl	1/3	0/3	–	0/3	–	–
5h	2-Br	0/3	0/3	–	0/3	–	–
5i	3-Br	0/3	0/3	–	0/3	–	–
5j	4-Br	1/3	0/3	–	0/3	–	–
5k	2-OCH_3	0/3	0/3	–	0/3	–	–
5l	3-OCH_3	0/3	0/3	–	0/3	–	–
5m	4-OCH_3	0/3	0/3	–	0/3	–	–
5n	4-CH_3	0/3	0/3	–	0/3	–	–

[a] Maximal electroshock: Doses of 30, 100, and 300 mg/kg were administrated intraperitoneally in mice; the animals were examined 0.5 h after administration.
[b] The figures n_1/n_2: the animals protected/the animals tested.
[c] The dash (–): not tested.

clonic seizures, tonic seizures and lethality induced by sc-PTZ at the rates of 90%, 100%, and 100%, respectively (Table 4), which revealed that compound **4h** possessed excellent activity against sc-PTZ at the low dose of 30 mg/kg. Compound **4h** exhibited high anticonvulsant activity in the MES and sc-PTZ model which are most widely used in the search for new AEDs, which suggested that it really possesses a good anticonvulsant profile. PTZ has been reported to produce seizures by inhibiting γ-aminobutyric acid (GABA) neurotransmission [21, 22]. GABA is the main inhibitory neurotransmitter in the brain, and is widely implicated in epilepsy. Inhibition of GABAergic neurotransmission or activity has been shown to promote and facilitate seizures [23], while enhancement of GABAergic neurotransmission is known to inhibit or attenuate seizures. The findings of the present study suggest that the newly synthesized compound **4h** might inhibit or attenuate PTZ-induced seizures in mice by enhancing GABAergic neurotransmission.

Table 3. Phase II quantitative anticonvulsant data in mice

Compd.	ED_{50}[a]	TD_{50}[b]	PI (TD_{50}/ED_{50})
4c	38.0 (33.0–43.9)	264.1 (288.9–304.6)	6.9
4h	23.7 (20.5–27.3)	254.6 (218.8–296.3)	10.8
4w	24.6 (21.5–28.1)	122.2 (105.0–142.2)	5.0
Carbamazepine	9.8 (8.9–10.8)	44.0 (40.2–48.1)	4.5
Valproate	272.4 (247–338)	426.1 (369–450)	1.6

[a] The dose measured in mg/kg.
[b] Minimal neurotoxicity was determined by the rotarod test 30 min after the tested compounds were administrated.

In the 3-MP induced seizure model, both carbamazepine and **4h** inhibited the tonic seizures completely, which revealed their strong antagonistic activity against 3-MP induced seizure (Table 4). 3-MP is the competitive inhibitor of the GABA synthesis enzyme glutamate decarboxylase (GAD), and it inhibits the synthesis of GABA resulting in the decrease of GABA level in the brain [24]. Compound **4h** showed antagonism to 3-MP induced seizures, suggesting that it might activate GAD or inhibit aminotransferase (GABA-T) in the brain.

In the BIC induced seizure model, both carbamazepine and **4h** inhibited tonic seizures completely. Carbamazepine showed inhibition of clonic and tonic seizures and death at rates of 0, 100, and 80%, respectively. Compound **4h** showed inhibition of clonic and tonic seizures and death at rates of 50, 100, and 90%, respectively (Table 4). BIC is a competitive antagonist of $GABA_A$ receptor. BIC produces convulsions through its antagonism of the $GABA_A$ receptor [25]. As compound **4h** inhibited the seizures induced by BIC, it likely exerts anticonvulsant activity at least partially through $GABA_A$-mediated mechanisms.

The results of the above chemical-induced seizure models revealed that compound **4h** is a broad-spectrum anticonvulsant and that it likely exerts its anticonvulsant activity through affecting the GABAergic system.

Table 4. Effects of compound **4h** on chemically induced seizures in mice

Chemical substances	Compound	Doses (mg/kg)	Test time (h)	Clonic seizures (%)	Tonic seizures (%)	Lethality (%)
Pentylenetetrazol	DMSO	–	0.5	100	100	100
	Carbamazepine	30	0.5	100	0	0
	4h	30	0.5	10	0	0
3-Mercaptopropionic acid	DMSO	–	0.5	100	100	100
	Carbamazepine	30	0.5	100	0	0
	4h	30	0.5	100	0	0
Bicuculline	DMSO	–	0.5	100	100	100
	Carbamazepine	30	0.5	100	0	20
	4h	30	0.5	50	0	10

Conclusions

In summary, the present studies revealed that a number of triazolothiadiazole derivatives were effective in the MES screens. Especially compound **4h** showed the most activity in these compounds and it possessed higher safety than the marketed drugs carbamazepine and valproate with an ED_{50} value of 23.7 mg/kg and a PI (TD_{50}/ED_{50}) of 10.8. In addition, compound **4h** demonstrated antagonistic activity against seizures induced by PTZ, 3-MP, and BIC. These results suggested that compound **4h** is a broad-spectrum anticonvulsant and it likely exerts its anticonvulsant activity through GABA-mediated mechanisms, such as affecting GABAergic neurotransmission or the agonizing $GABA_A$ receptor in the brain.

Experimental

Chemistry

Melting points were determined in open capillary tubes and were uncorrected. IR spectra were recorded (in KBr) on IRPrestige-21. ^{1}H NMR spectra were measured on an AV-300 (Bruker, Switzerland), and all chemical shifts were given in ppm relative to tetramethylsilane. Mass spectra were measured on an AXIMA CFR Plus MALDI-TOF (Shimadzu, Japan). Elemental analyses were performed on a 204Q CHN (Perkin Elmer, USA). The chemicals were purchased from Aldrich Chemical Corporation.

*The synthesis of 4-amino-4H-1,2,4-triazole-3-thiol **3***

Thiocarbonohydrazide (**2**) 1.06 g (0.01 mol) and 20 mL formic acids were refluxed for 40 min. After removing the excess formic acid under reduced pressure, the residual mixture was purified by silica gel column chromatography (dichloromethane/methanol = 60:1) to afford compound **3**. Mp 155–157°C, yield = 37%. ^{1}H NMR ($CDCl_3$ 300 MHz) δ 5.68 (s, 2H, NH_2), 8.45 (s, 1H, CH), 13.64 (s, 1H, SH); MS *m/z* 117 (M+H).

*General procedure for preparation of compounds **4a–4x***

A mixture of aromatic acids or fatty acid (8 mmol) and PPA (10 mL) was heated to 80°C with stirring, then 4-amino-4*H*-1,2,4-triazole-3-thiol (**3**) (4 mmol) was added portionwise. The reaction mixture was heated at 100–140°C for 4–8 h with stirring, then cooled, and poured into ice-cold 10% aqueous sodium carbonate. The solid product was collected, washed with water, and dried to give compounds **4a–4x** which were purified by silica gel column chromatography.

*General procedure for preparation of compounds **5a–5n***

An equimolecular mixture of compound **3** (3 mmol) and aromatic aldehydes (3 mmol), dry DMF (5 mL) and a catalytic amount of triethyl benzyl ammonium chloride (10 mg) was taken in to a round flask. The mixture was refluxed for about 5–8 h. After cooling, the mixture was poured into ice-water with stirring. The precipitate was filtered, washed with water, dried, and recrystallized from ethanol.

*6-Phenyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **4a***

Mp 178–180°C; yield 48%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.53–7.91 (m, 5H, Ar–H), 8.98 (s, 1H, triazole-H), IR (KBr) cm^{-1}: 1610–1580 (N=C). MS *m/z* 203 (M+H). Anal. Calcd. for $C_9H_6N_4S$: C, 53.45; H, 2.99; N, 27.70. Found: C, 53.19; H, 2.76; N, 27.96.

*6-(2-Fluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **4b***

Mp 140–142°C; yield 55%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.23–7.39 (m, 2H, Ar–H), 7.35–7.64 (m, 1H, Ar–H), 8.12–8.18 (m, 1H, Ar–H), 9.01 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1612–1581 (N=C). MS *m/z* 221 (M+H). Anal. Calcd. for $C_9H_5FN_4S$: C, 49.08; H, 2.29; N, 25.44. Found: C, 49.25; H, 2.13; N, 25.67.

*6-(3-Fluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **4c***

Mp 138–140°C; yield 59%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.27–7.35 (m, 2H, Ar–H), 7.51–7.59 (m, 2H, Ar–H), 9.01 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1608–1576 (N=C). MS *m/z* 221 (M+H). Anal. Calcd. for $C_9H_5FN_4S$: C, 49.08; H, 2.29; N, 25.44. Found: C, 49.29; H, 2.08; N, 25.62.

*6-(4-Fluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole **4d***

Mp 198–200°C; yield 60%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.25 (d, 2H, J = 8.4 Hz, Ar–H), 7.90 (d, 2H, J = 8.4 Hz, Ar–H), 8.96 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1604–1577 (N=C). MS *m/z* 221

(M+H). Anal. Calcd. for $C_9H_5FN_4S$: C, 49.08; H, 2.29; N, 25.44. Found: C, 49.33; H, 2.07; N, 25.70.

6-(2,6-Difluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4e***

Mp 148–150°C; yield 60%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.14–7.19 (m, 2H, Ar–H), 7.55–7.65 (m, 1H, Ar–H), 9.05 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1622–1584 (N=C). MS *m/z* 239 (M+H). Anal. Calcd. for $C_{10}H_7FN_4S$: C, 51.27; H, 3.01; N, 23.92. Found: C, 51.45; H, 3.16; N, 23.64.

6-(2-Chlorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4f***

Mp 146–149°C; yield 49%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.27–7.61(m, 3H, Ar–H), 8.00–8.03 (m, 1H, Ar–H), 9.01 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1589–1572 (N=C). MS *m/z* 237 (M+H). Anal. Calcd. for $C_9H_5ClN_4S$: C, 45.67; H, 2.13; N, 23.67. Found: C, 45.84; H, 2.02; N, 23.88.

6-(3-Chlorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4g***

Mp 136–138°C; yield 48%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.43–7.51 (m, 2H, Ar–H), 7.76 (s, 1H, Ar–H), 7.92–8.10 (m, 1H, Ar–H), 9.04 (s, 1H, triazole-H) IR (KBr) cm^{-1}: 1571–1569 (N=C). MS *m/z* 237 (M+H). Anal. Calcd. for $C_9H_5ClN_4S$: C, 45.67; H, 2.13; N, 23.67. Found: C, 45.90; H, 2.05; N, 23.93.

6-(4-Chlorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4h***

Mp 198–201°C; yield 48%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.54 (d, 2H, J = 6.6 Hz, Ar–H), 7.83 (d, 2H, J = 6.6 Hz, Ar–H), 8.97 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1581–1574 (N=C). MS *m/z* 237 (M+H). Anal. Calcd. for $C_9H_5ClN_4S$: C, 45.67; H, 2.13; N, 23.67. Found: C, 45.95; H, 2.06; N, 23.90.

6-(2,4-Dichlorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4i***

Mp 212–214°C; yield 60%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.53–7.91 (m, 2H, Ar–H), 8.06–8.10 (m, 1H, Ar–H), 9.03 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1594–1586 (N=C). MS *m/z* 271 (M+H). Anal. Calcd. for $C_9H_4Cl_2N_4S$: C, 39.87; H, 1.49; N, 20.66. Found: C, 39.63; H, 1.35; N, 20.89.

6-(2-Bromophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4j***

Mp 158–160°C; yield 48%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.43–7.55 (m, 2H, Ar–H), 7.77–7.88 (m, 2H, Ar–H), 9.01 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1586–1570 (N=C). MS *m/z* 281 (M+H). Anal. Calcd. for $C_9H_5BrN_4S$: C, 38.45; H, 1.79; N, 19.93. Found: C, 38.74; H, 1.83; N, 19.98.

6-(4-Bromophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4k***

Mp 200–203°C; yield 43%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.70 (d, 2H, J = 6.6 Hz, Ar–H), 7.76 (d, 2H, J = 6.6 Hz, Ar–H), 8.96 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1582–1569 (N=C). MS *m/z* 281 (M+H). Anal. Calcd. for $C_9H_5BrN_4S$: C, 38.45; H, 1.79; N, 19.93. Found: C, 38.70; H, 1.91; N, 20.04.

6-(2-Nitrophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4l***

Mp 148–150°C; yield 61%. ^{1}H NMR($CDCl_3$, 300 MHz), δ 7.82–7.85 (m, 2H, Ar–H), 8.12–8.17 (m, 2H, Ar–H), 8.98 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1592–1579 (N=C). MS *m/z* 248 (M+H). Anal. Calcd. for $C_9H_5N_5O_2S$: C, 43.72; H, 2.04; N, 28.33. Found: C, 43.57; H, 2.12; N, 28.17.

6-(3-Nitrophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4m***

Mp 194–196°C; yield 62%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.78–7.84 (m, 1H, Ar–H), 8.83–8.87 (m, 1H, Ar–H), 8.46–8.49 (m, 1H, Ar–H), 8.77 (s, 1H, Ar–H), 9.03 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1581–1574 (N=C). MS *m/z* 248 (M+H). Anal. Calcd. for $C_9H_5N_5O_2S$: C, 43.72; H, 2.04; N, 28.33. Found: C, 43.50; H, 2.16; N, 28.11.

6-(4-Nitrophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4n***

Mp 188–190°C; yield 64%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 8.11 (d, 2H, J = 8.9 Hz, Ar–H), 8.44 (d, 2H, J = 8.9 Hz, Ar–H), 9.04 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1584–1569 (N=C). MS *m/z* 248 (M+H). Anal. Calcd. for $C_9H_5N_5O_2S$: C, 43.72; H, 2.04; N, 28.33. Found: C, 43.56; H, 2.08; N, 28.20.

6-(2-Methoxyphenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4o***

Mp 198–201°C; yield 45%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 4.06 (s, 3H, $-OCH_3$), 7.07–7.17 (m, 2H, Ar–H), 7.53–7.59 (m, 1H, Ar–H), 8.20–8.23 (m, 1H, Ar–H), 8.93 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1631–1575 (N=C). MS *m/z* 233 (M+H). Anal. Calcd. for $C_{10}H_8N_4OS$: C, 51.71; H, 3.47; N, 24.12. Found: C, 51.97; H, 3.30; N, 23.89.

6-(3-Methoxyphenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4p***

Mp 138–140°C; yield 56%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 3.97 (s, 3H, $-OCH_3$), 7.12–7.16 (m, 1H, Ar–H), 7.41–7.49 (m, 3H, Ar–H), 8.95 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1628–1573 (N=C). MS *m/z* 233 (M+H). Anal. Calcd. for $C_{10}H_8N_4OS$: C, 51.71; H, 3.47; N, 24.12. Found: C, 51.88; H, 3.25; N, 23.86.

6-(4-Methoxyphenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4q***

Mp 180–182°C; yield 57%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 3.91 (s, 3H, $-OCH_3$), 7.03 (d, 2H, J = 8.5 Hz, Ar–H), 7.81 (d, 2H, J = 8.5 Hz, Ar–H), 8.91 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1634–1564 (N=C). MS *m/z* 233 (M+H). Anal. Calcd. for $C_{10}H_8N_4OS$: C, 51.71; H, 3.47; N, 24.12. Found: C, 51.89; H, 3.51; N, 23.85.

6-Ethyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4r***

Mp 80–82°C; yield 75%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 1.46 (t, 3H, J = 7.5 Hz, $-CH_3$), 3.06 (q, 2H, J = 7.5 Hz, $-CH_2-$), 8.87 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1628–1526 (N=C). MS *m/z* 155 (M+H). Anal. Calcd. for $C_5H_6N_4S$: C, 38.95; H, 3.92; N, 36.34. Found: C, 39.17; H, 3.76; N, 36.59.

6-Propyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4s***

Mp 43–44°C; yield 78%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 1.08 (t, 3H, J = 7.4 Hz, $-CH_3$), 1.82–1.94 (m, 2H, $-CH_2-$), 2.98 (q, 2H, J = 7.5 Hz, $-CH_2-$), 8.86 (s, 1H, triazole-H). IR (KBr) cm^{-1}:

1624–1529 (N=C). MS *m/z* 169 (M+H). Anal. Calcd. for $C_6H_8N_4S$: C, 42.84; H, 4.79; N, 33.31. Found: C, 42.56; H, 4.87; N, 33.58.

6-Butyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4t***

Mp 78–80°C; yield 86%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.98 (t, 3H, J = 7.0 Hz, $-CH_3$), 1.44–1.53 (m, 2H, $-CH_2-$), 1.76–1.86 (m, 2H, $-CH_2-$), 2.99 (t, 2H, J = 7.6 Hz, $-CH_2-$), 8.84 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1622–1530 (N=C). MS *m/z* 183 (M+H). Anal. Calcd. for $C_7H_{10}N_4S$: C, 46.13; H, 5.53; N, 30.74. Found: C, 45.89; H, 5.41; N, 30.97.

6-Pentyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4u***

Mp 58–60°C; yield 69%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.93 (t, 3H, J = 6.7 Hz, $-CH_3$), 1.36–1.44 (m, 4H, $-CH_2-CH_2-$), 1.79–1.88 (m, 2H, $-CH_2-$), 3.00 (t, 2H, J = 7.6 Hz, $-CH_2-$), 8.86 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1621–1533 (N=C). MS *m/z* 197 (M+H). Anal. Calcd. for $C_8H_{12}N_4S$: C, 48.96; H, 6.16; N, 28.55. Found: C, 49.21; H, 6.04; N, 28.48.

6-Hexyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4v***

Mp 44–46°C; yield 66%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.88 (t, 3H, J = 6.8 Hz, $-CH_3$), 1.29–1.45 (m, 6H, $-(CH_2)_3-$), 1.79–1.86 (m, 2H, $-CH_2-$), 2.98 (t, 2H, J = 7.6 Hz, $-CH_2-$), 8.83 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1619–1535 (N=C). MS *m/z* 211 (M+H). Anal. Calcd. for $C_9H_{14}N_4S$: C, 51.40; H, 6.71; N, 26.64. Found: C, 51.27; H, 6.90; N, 26.83.

6-Heptyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4w***

Mp 50–52°C; yield 65%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.88 (t, 3H, J = 6.8 Hz, $-CH_3$), 1.26–1.45 (m, 8H, $-(CH_2)_4-$), 1.76–1.86 (m, 2H, $-CH_2-$), 2.98 (t, 2H, J = 7.6 Hz, $-CH_2-$), 8.85 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1618–1539 (N=C). MS *m/z* 225 (M+H). Anal. Calcd. for $C_{10}H_{16}N_4S$: C, 53.54; H, 7.19; N, 24.98. Found: C, 53.76; H, 7.14; N, 24.73.

6-Octyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***4x***

Mp 48–50°C; yield 70%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 0.85–0.90 (t, 3H, J = 6.9 Hz, $-CH_3$), 1.28–1.44 (m, 10H, $-(CH_2)_5-$), 1.79–1.84 (m, 2H, $-CH_2-$), 2.98 (t, 2H, J = 7.6 Hz, $-CH_2-$), 8.84 (s, 1H, triazole-H). IR (KBr) cm^{-1}: 1616–1541 (N=C). MS *m/z* 239 (M+H). Anal. Calcd. for $C_{11}H_{18}N_4S$: C, 55.43; H, 7.61; N, 23.51. Found: C, 55.69; H, 7.74; N, 23.28.

5,6-Dihydro-6-phenyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole ***5a***

Mp 156–158°C; yield 74%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.46–7.56 (m, 3H, Ar–H), 7.85–7.88 (m, 2H, Ar–H), 8.09 (s, 1H, S–CH), 10.36 (s, 1H, triazole-H), 11.52 (s, 1H, NH). IR (KBr) cm^{-1}: 1597–1553 (N=C). MS *m/z* 205 (M+H). Anal. Calcd. for $C_9H_8N_4S$: C, 52.92; H, 3.95; N, 27.43. Found: C, 53.24; H, 4.08; N, 27.21.

6-(2-Fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5b***

Mp 168–170°C; yield 79%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.20–7.29 (m, 2H, Ar–H), 7.33–7.43 (m, 1H, Ar–H), 8.07–8.10 (m, 1H, Ar–H), 8.12 (s, 1H, S–CH), 10.54 (s, 1H, triazole-H), 11.69 (s, 1H, NH). IR (KBr) cm^{-1}: 1603–1554 (N=C). MS *m/z* 223 (M+H). Anal. Calcd. for $C_9H_7FN_4S$: C, 48.64; H, 3.17; N, 25.21. Found: C, 48.88; H, 3.04; N, 25.02.

6-(3-Fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5c***

Mp 164–166°C; yield 83%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.34–7.39 (m, 1H, Ar–H), 7.67–7.75 (m, 2H, Ar–H), 8.05–8.09 (m, 1H, Ar–H), 8.06 (s, 1H, S–CH), 10.52 (s, 1H, triazole-H), 11.22 (s, 1H, NH). IR (KBr) cm^{-1}: 1600–1558 (N=C) MS *m/z* 223 (M+H). Anal. Calcd. for $C_9H_7FN_4S$: C, 48.64; H, 3.17; N, 25.21. Found: C, 48.90; H, 3.12; N, 25.07.

6-(4-Fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5d***

Mp 172–174°C; yield 81%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.18 (d, 2H, J = 8.6 Hz, Ar–H), 7.68 (d, 2H, J = 8.6 Hz, Ar–H), 8.07 (s, 1H, S–CH), 10.39 (s, 1H, triazole-H), 11.39 (s, 1H, NH). IR (KBr) cm^{-1}: 1599–1555 (N=C). MS *m/z* 223 (M+H). Anal. Calcd. for $C_9H_7FN_4S$: C, 48.64; H, 3.17; N, 25.21. Found: C, 48.81; H, 3.21; N, 25.13.

6-(2-Chlorophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5e***

Mp 190–192°C; yield 65%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.34–7.41 (m, 1H, Ar–H), 7.46–7.48 (m, 2H, Ar–H), 8.09–8.13 (m, 1H, Ar–H), 8.16 (s, 1H, S–CH), 10.82 (s, 1H, triazole-H), 10.95 (s, 1H, NH). IR (KBr) cm^{-1}: 1593–1547 (N=C). MS *m/z* 239 (M+H). Anal. Calcd. for $C_9H_7ClN_4S$: C, 45.29; H, 2.96; N, 23.47. Found: C, 45.53; H, 3.07; N, 23.32.

6-(3-Chlorophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5f***

Mp 156–158°C; yield 65%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.50–7.53 (m, 1H, Ar–H), 7.58–7.60 (m, 1H, Ar–H), 7.69–7.73 (m, 1H, Ar–H), 7.88–7.90 (m, 1H, Ar–H), 8.01 (s, 1H, S–CH), 10.52 (s, 1H, triazole-H), 11.21 (s, 1H, NH). IR (KBr) cm^{-1}: 1594–1544 (N=C). MS *m/z* 239 (M+H). Anal. Calcd. for $C_9H_7ClN_4S$: C, 45.29; H, 2.96; N, 23.47. Found: C, 45.46; H, 3.11; N, 23.25.

6-(4-Chlorophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5g***

Mp 204–206°C; yield 61%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.46 (d, 2H, J = 8.5 Hz, Ar–H), 7.81 (d, 2H, J = 8.5 Hz, Ar–H), 8.06 (s, 1H, S–CH), 10.51 (s, 1H, triazole-H), 11.61 (s, 1H, NH). IR (KBr) cm^{-1}: 1593–1545 (N=C). MS *m/z* 239 (M+H). Anal. Calcd. for $C_9H_7ClN_4S$: C, 45.29; H, 2.96; N, 23.47. Found: C, 45.60; H, 3.02; N, 23.21.

6-(2-Bromophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5h***

Mp 178–180°C; yield 66%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.32–7.44 (m, 2H, Ar–H), 7.65–7.69 (m, 1H, Ar–H), 8.10–8.14 (m, 1H, Ar–H), 8.15 (s, 1H, S–CH), 10.91 (s, 1H, triazole-H), 11.20 (s, 1H, NH). IR (KBr) cm^{-1}: 1594–1549 (N=C). MS *m/z* 283 (M+H). Anal. Calcd. for $C_9H_7BrN_4S$: C, 38.18; H, 2.49; N, 19.79. Found: C, 37.94; H, 2.55; N, 19.97.

6-(3-Bromophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5i***

Mp 170–172°C; yield 68%. ^{1}H NMR ($CDCl_3$, 300 MHz), δ 7.34–7.39 (m, 1H, Ar–H), 7.65–7.74 (m, 1H, Ar–H), 7.76–8.18 (m, 2H, Ar–H),

8.25 (s, 1H, S–CH), 10.53 (s, 1H, triazole-H), 11.04 (s, 1H, NH). IR (KBr) cm^{-1}: 1594–1548 (N=C). MS *m/z* 283 (M+H). Anal. Calcd. for $C_9H_7BrN_4S$: C, 38.18; H, 2.49; N, 19.79. Found: C, 37.99; H, 2.61; N, 18.02.

6-(4-Bromophenyl)-5,6-dihydro-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5j***

Mp 200–203°C; yield 79%. ^1H NMR ($CDCl_3$, 300 MHz), δ 7.63 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.73 (d, 2H, $J = 8.1$ Hz, Ar–H), 8.25 (s, 1H, S–CH), 10.51 (s, 1H, triazole-H), 10.63 (s, 1H, NH). IR (KBr) cm^{-1}: 1695–1544 (N=C). MS *m/z* 283 (M+H). Anal. Calcd. for $C_9H_7BrN_4S$: C, 38.18; H, 2.49; N, 19.79. Found: C, 37.87; H, 2.63; N, 18.10.

5,6-Dihydro-6-(2-methoxyphenyl)-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5k***

Mp 204–206°C; yield 72%. ^1H NMR ($CDCl_3$, 300 MHz), δ 3.93 (s, 3H, $-OCH_3$), 6.96–7.07 (m, 2H, Ar–H), 7.48–8.05 (m, 2H, Ar–H), 8.58 (s, 1H, S–CH), 10.44 (s, 1H, triazole-H), 10.51 (s, 1H, NH). IR (KBr) cm^{-1}: 1604–1551 (N=C). MS *m/z* 235 (M+H). Anal. Calcd. for $C_{10}H_{10}N_4OS$: C, 51.27; H, 4.30; N, 23.91. Found: C, 51.48; H, 4.16; N, 23.73.

5,6-Dihydro-6-(3-methoxyphenyl)-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5l***

Mp 192–194°C; yield 75%. ^1H NMR ($CDCl_3$, 300 MHz), δ 3.88 (s, 3H, $-OCH_3$), 7.09–7.11 (m, 1H, Ar–H), 7.42–7.46 (m, 3H, Ar–H), 8.08 (s, 1H, S–CH), 10.38 (s, 1H, triazole-H), 10.94 (s, 1H, NH). IR (KBr) cm^{-1}: 1603–1549 (N=C). MS *m/z* 235 (M+H). Anal. Calcd. for $C_{10}H_{10}N_4OS$: C, 51.27; H, 4.30; N, 23.91. Found: C, 51.50; H, 4.19; N, 23.64.

5,6-Dihydro-6-(4-methoxyphenyl)-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5m***

Mp 210–212°C; yield 70%. ^1H NMR ($CDCl_3$, 300 MHz), δ 3.89 (s, 3H, $-OCH_3$), 7.00 (d, 2H, $J = 8.6$ Hz, Ar–H), 7.82 (d, 2H, $J = 8.6$ Hz, Ar–H), 8.04 (s, 1H, S–CH), 9.90 (s, 1H, triazole-H), 10.18 (s, 1H, NH). IR (KBr) cm^{-1}: 1603–1549 (N=C). MS *m/z* 235 (M+H). Anal. Calcd. for $C_{10}H_{10}N_4OS$: C, 51.27; H, 4.30; N, 23.91. Found: C, 51.52; H, 4.23; N, 23.69.

5,6-Dihydro-6-(4-methylphenyl)-[1,2,4]triazolo[3,4-b]-[1,3,4]thiadiazole ***5n***

Mp 178–180°C; yield 64%. ^1H NMR ($CDCl_3$, 300 MHz), δ 2.43 (s, 3H, $-CH_3$), 7.28 (d, 2H, $J = 8.1$ Hz, Ar–H), 7.75 (d, 2H, $J = 8.1$ Hz, Ar–H), 8.02 (s, 1H, S–CH), 10.26 (s, 1H, triazole-H), 10.79 (s, 1H, NH). IR (KBr) cm^{-1}: 1601–1552 (N=C). MS *m/z* 219 (M+H). Anal. Calcd. for $C_{10}H_{10}N_4S$: C, 55.02; H, 4.62; N, 25.67. Found: C, 55.27; H, 4.48; N, 25.41.

Pharmacology

The MES test and the rotarod test were carried out by the methods described in the Antiepileptic Drug Development Program (ADD) of the National Institutes of Health (USA) [26, 27]. All compounds were tested for anticonvulsant activities with KunMing mice in the 18–22 g weight range purchased from the Laboratory of Animal Research, College of Pharmacy, Yanbian University. The test compounds were dissolved in DMSO. In phase-I screening (Tables 1 and 2), each compound was administered at the dose levels of 30, 100, and 300 mg/kg for evaluating preliminarily the anticonvulsant activity and neurotoxicity at 30 min interval after administration. In the MES test, seizures were elicited with a 60 Hz alternating current of 50 mA intensity in mice. The current was applied via corneal electrodes for 0.2 s. The protection against the spread of MES-induced seizures was defined as the abolition of the hind limb tonic extension spasm. Anticonvulsant drug-induced neurologic deficit was detected in mice by using the rotarod test.

For determination of the median effective dose (ED_{50}) and the median toxic dose (TD_{50}), the phase-II screening was prepared (see Table 3). Groups of 10 mice were given a range of intraperitoneal doses of the tested compound until at least three points were established in the range of 10–90% seizure protection or minimal observed neurotoxicity. From these data, the respective ED_{50}, TD_{50} values, and 95% confidence intervals were calculated by probit analysis.

In chemically induced seizures (see Table 4), mice were given doses of convulsant drugs that could induce seizures at least 97% of animals. The doses used were: PTZ, 85 mg/kg; 3-mercaptopropionic acid, 40 mg/kg; and BIC, 5.4 mg/kg. The test compounds and standard AEDs were administered i.p. in a volume of 30 mg/kg to groups of 10 mice 30 min before injection of PTZ (s.c.), 3-mercaptopropionic acid (i.p.) and BIC (s.c.). The mice were placed in individual cages and observed for 30 min, the number of clonic seizures, tonic seizures, and the lethality were recorded [28, 29].

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The authors have declared no conflict of interest.

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