Synthesis and Antimicrobial and Pharmacological Properties of New Thiosemicarbazide and 1,2,4-Triazole Derivatives

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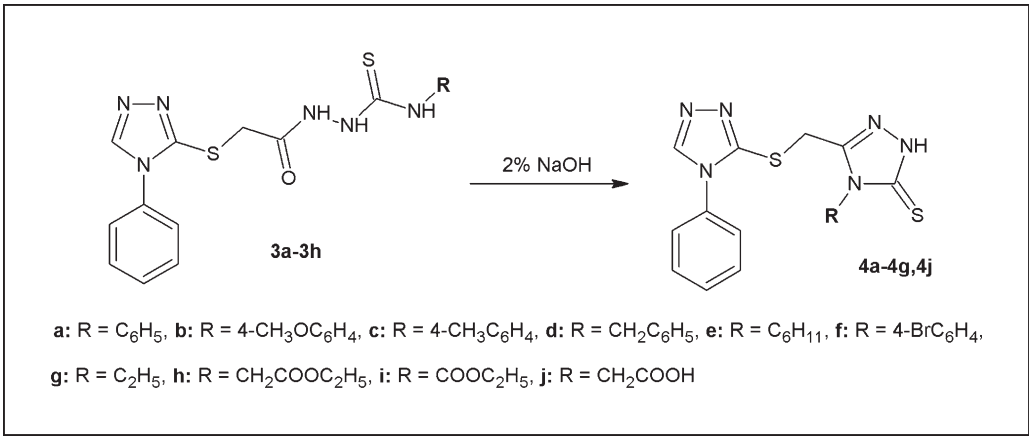

Reaction of 4-phenyl-4*H*-1,2,4-triazole-3-thione with ethyl bromoacetate has led to the formation of ethyl [(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetate **1**, the structure of which was confirmed by X-ray analysis. In the next reaction with 80% hydrazide hydrate, appropriate hydrazide **2** was obtained, which in reaction with isothiocyanates was converted to new acyl derivatives of thiosemicarbazides **3a–3h**. The cyclization of these compounds in alkaline media has led to formation of new derivatives of 5-{[(4-phenyl-4*H*-1,2,4-triazole-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazole-3(2*H*)-thiones **4a–4g**, **4j**. The structure of the compounds was confirmed by elementary analysis and IR, ^{1}H-NMR, ^{13}C-NMR, and MS spectra. Compounds **3a–3h** and **4a–4g** were screened for their antimicrobial activities, and the influence of the compounds **4a, 4b**, and **4e–4g** on the central nervous system of mice in behavioral tests was examined.

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INTRODUCTION

Cyclization of thiosemicarbazide derivatives leads to 1,2,4-triazole derivatives in alkaline media [1,2], where as in acidic media [3,4] to 1,3,4-thiadiazole derivatives.

In previous papers [2–5], it was stated that the reaction of cyclization was affected not only by pH of the medium but also by the nature of substituents in thiosemicarbazide derivatives. The course of cyclization in alkaline and acidic media for thiosemicarbazide derivatives of formic, benzoic, and nicotinic acids was investigated [5].

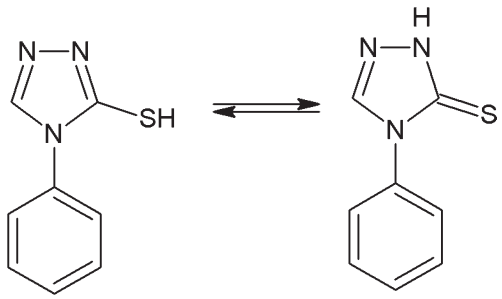
Cyclization of thiosemicarbazide derivatives of formic and nicotinic acids in alkaline and in acidic media leads to 1,2,4-triazole derivatives. In the cyclization reaction of benzoic acid thiosemicarbazide derivatives in the presence of alkaline media 1,2,4-triazole system was obtained, whereas in acidic media 1,3,4-thiadiazole derivatives were obtained [5].

4-Phenyl-4*H*-1,2,4-triazole-3-thione, which can exist in two tautomeric forms (Scheme 1), was a starting material for synthesis.

In various reactions, depending on the conditions used, this compound can lead either to the S- or N-derivatives. In this article, the reaction of 4-phenyl-4*H*-1,2,4-triazole-3-thione with ethyl bromoacetate was investigated. Based on the results of the previous papers [5,6], the elemental and spectral analysis as well as the X-ray crystallography, it was revealed and confirmed that reaction leads to the formation of S-derivative **1** (Fig. 1).

This compound was converted to hydrazide **2**, then in reaction with isothiocyanates appropriate

Scheme 1. Thiol/thione tautomerism forms of 4-phenyl-4*H*-1,2,4-triazole-3-thiol.



thiosemicarbazide derivatives were obtained **3a–3h**. Cyclization of these compounds in alkaline media leads to new derivatives of 1,2,4-triazole **4a–4g**.

The reactions were performed according to Scheme 2, and the substituents are presented in Table 1.

Depending on the nature of substituents, the 1,2,4-triazole derivatives show various pharmacological activities. Some of them, obtained in previous papers and investigated on experimental animals, had potential action on central nervous system (CNS) [7]. Others

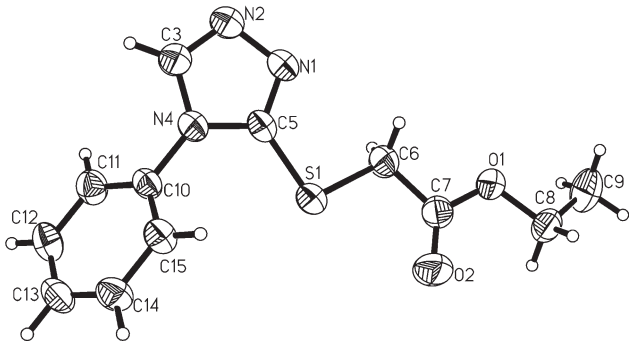


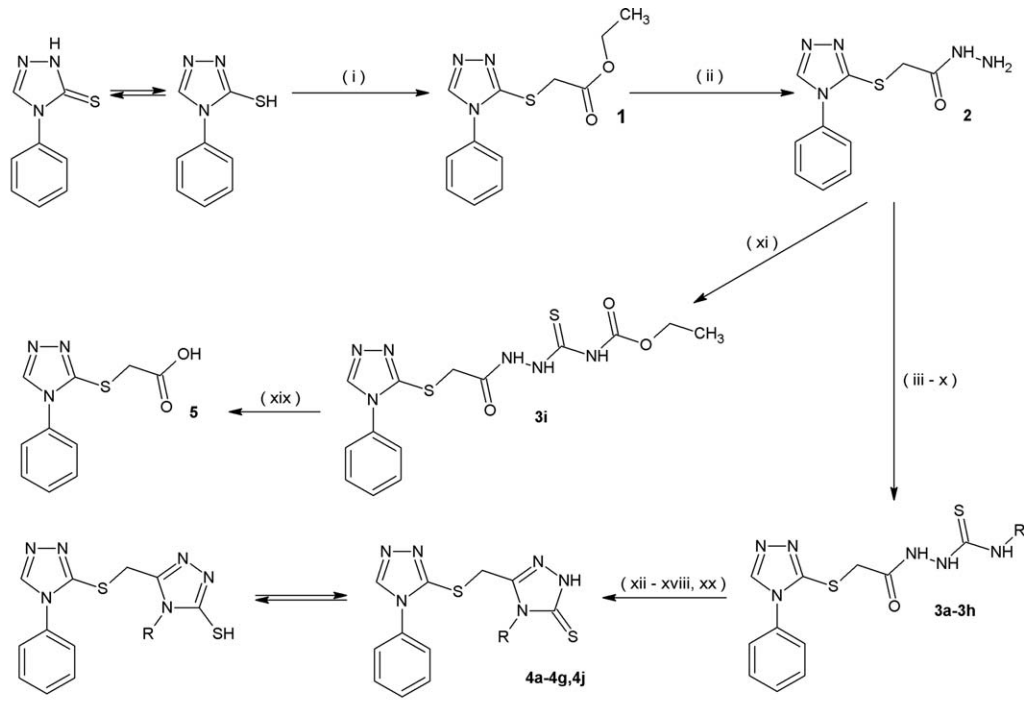
Figure 1. Molecular structure with atom numbering scheme for compound **1**. Displacement ellipsoids are drawn at the 50% probability level.

show analgesic [8], antifungal [9], antibacterial [10–12], antiphlogistic [13], and antituberculous [14,15] action.

Pharmacological experiments (on CNS on mice) were carried out for compounds **4a**, **4b**, and **4e–4g**.

All tested compounds were screened for *in vitro* antibacterial activity by the agar well diffusion method, and for agents **3d**, **3f**, **3g**, and **3h** showing potential inhibitory effect on the growth of bacteria minimal inhibitory

Scheme 2. Synthesis of new thiosemicarbazide and 1,2,4-triazole derivatives.

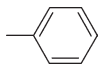
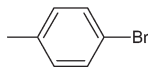
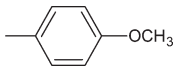
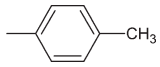
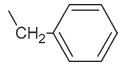
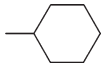


a: R = C_6H_5, **b:** R = 4-$CH_3OC_6H_4$, **c:** R = 4-$CH_3C_6H_4$, **d:** R = $CH_2C_6H_5$, **e:** R = C_6H_{11}, **f:** R = 4-BrC_6H_4, **g:** R = C_2H_5, **h:** R = $CH_2COOC_2H_5$
i: R = $COOC_2H_5$, **j:** R = CH_2COOH

Reagents and conditions: (i) NaOEt then $BrCH_2COOC_2H_5$, mix at 25°C for 4h, left at 25°C for 12 h and heat at boiling point for 2 h (65.9%);
(ii) EtOH then 80% $NH_2NH_2 \cdot H_2O$, left at 25°C for 24 h (78%); (iii) C_6H_5NCS, 50°C, 12 h (91.5%);
(iv) 4-$CH_3OC_6H_4NCS$, 50°C, 12 h (88.9%); (v) 4-$CH_3C_6H_4NCS$, 80°C, 12 h (88.5%); (vi) $C_6H_5CH_2NCS$, 40°C, 12 h (93.9%);
(vii) $C_6H_{11}NCS$, 70°C, 12 h, (84,5%); (viii) 4-BrC_6H_4NCS, 95°C, 12 h (79.2%); (ix) C_2H_5NCS, 50°C, 12 h (76.3%);
(x) $C_2H_5OOCCH_2NCS$, 40°C, 12 h (65.2%); (xi) $C_2H_5OOCNCS$, 45°C, 12 h (75,1%); (xii-xx) 2% NaOH, heat at boiling point for 2 h (77.8-95.1%).

Table 1

Substituents of compounds **3a–4j**.

Compounds	R	Compounds	R
3a, 4a		**3f, 4f**	
3b, 4b		**3g, 4g**	C_2H_5
3c, 4c		**3h**	$CH_2COOC_2H_5$
3d, 4d		**3i**	$COOC_2H_5$
3e, 4e		**4j**	CH_2COOH

concentration (MIC) values were estimated by microdilution technique [16,17].

RESULTS AND DISCUSSION

4-Phenyl-4*H*-1,2,4-triazole-3-thione was the starting material for synthesis of new derivatives, which consist of two 1,2,4-triazole systems connected with *S*-methylene group.

This compound was obtained (using the method described earlier) by cyclization of 1-formyl-4-phenyl thiosemicarbazide in alkaline media. Reaction with ethyl bromoacetate in the presence of sodium ethanolate gave ethyl [(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl] acetate **1** (Fig. 1), which was converted to hydrazide **2** on reaction with 80% hydrazine hydrate.

Reactions of hydrazide **2** with aliphatic, aromatic, ethoxycarbonyl, and ethoxycarbonylmethyl isothiocyanates were carried out by heating substrates in an oil bath; temperatures were selected experimentally ($t = 40–95°C$).

New thiosemicarbazide derivatives **3a–3g** in cyclization reaction with 2% aqueous solution of sodium hydroxide leads to a new group of 5-{[4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazol-3(2*H*)-thione **4a–4g** derivatives.

The cyclization of thiosemicarbazide in alkaline media, which contain ethoxycarbonylmethyl group **3h**, was accompained by hydrolysis of ester group and led to formation of 4-carboxymethyl-5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione **4j**.

The hydrolysis was also observed in the case of cyclization of 4-ethoxycarbonyl-1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}thiosemicarbazide **3i** in alkaline media, which led to formation of [(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl] acetic acid **5**. This compound was described earlier [18,19], but it was obtained in a different way.

The test of cyclization of 1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}-1-phenyl thiosemicarbazide **3a** in acidic media (acetic acid) gave the derivative with 1,2,4-triazole system **4a**.

The structure of the obtained compounds was confirmed by elementary analysis, IR, and ^{1}H-NMR spectra. Some of them were also submitted to ^{13}C-NMR and MS spectral analysis.

In the IR spectra of 1,2,4-triazole system **4a–4g, 4j**, the following characteristic absorption bands were observed: 1513–1530 cm^{-1} corresponding to C—N group and in the range of 1608–1624 cm^{-1} corresponding to C=N group.

^{1}H-NMR spectra of the thiosemicarbazide derivatives **3a–3i** show three proton signals typical for the NH group in the δ 8.30–10.45 ppm range. Whereas for the new compounds of 1,2,4-triazole system **4a–4g**, **4j** one proton signal of the NH group was observed in the δ 13.63–13.99 ppm range.

Table 2

Antinociceptive activity compounds **4a**, **4b**, **4e** and **4f** in the writhing syndrome test in mice.

Compound	Part of LD_{50}	Mean writhing number	Inhibition (%)
Control **4a**	–	47.9 ± 4.7	–
	0.025	41.4 ± 6.2	14
	0.05	15.5 ± 4.5**	68**
	0.1	12.0 ± 5.0**	75**
Control **4b**	–	57.2 ± 5.1	–
	0.00625	58.2 ± 6.2	–
	0.0125	26.4 ± 8.4**	54**
	0.025	28.1 ± 5.6**	51**
	0.05	10.0 ± 4.4**	82.5**
	0.1	10.9 ± 4.3**	81**
Control **4e**	–	64.8 ± 5.9	–
	0.0125	62.0 ± 6.5	4
	0.025	47.8 ± 8.8*	26*
	0.05	29.1 ± 5.5**	55**
	0.1	5.6 ± 1.8**	91**
Control **4f**	–	46.8 ± 2.6	–
	0.0125	40.7 ± 2.3	13**
	0.025	35.5 ± 4.3*	24*
	0.05	34.3 ± 5.9*	27*
	0.1	25.9 ± 3.2**	44**

Compounds were given 30 min before the test.
% of inhibition obtained by comparison with control group.
* $P < 0.05$ *vs*. the control group.
** $P < 0.001$.

Table 3

The influence of assayed **3d, 3f, 3g, 3h** compounds on the growth of gram-positive bacteria recorded as the average diameter of the growth inhibition zone (including 8 mm of the well) in the agar well diffusion technique and on the basis of MIC values in the broth microdilution method (OD_{600}).

	3d		3f		3g		3h	
Species	Zone of growth inhibition (mm)	MIC (mg/L)	Zone of growth inhibition (mm)	MIC (mg/L)	Zone of growth inhibition (mm)	MIC (mg/L)	Zone of growth inhibition (mm)	MIC (mg/L)
Staphylococcus aureus ATCC 25923	8	>1000	17	500	17	500	16	1000
Staphylococcus aureus ATCC 6538	8	>1000	15	125	8	>1000	8	>1000
Staphylococcus epidermidis ATCC 12228	15	>1000	17	250	17	500	16	1000
Bacillus subtilis ATCC 6633	25	1000	8	500	54	500	32	1000
Bacillus cereus ATCC 10876	17	>1000	16	250	11	>1000	9	>1000
Micrococcus luteus ATCC 10240	22	1000	30	125	50	250	50	250

^{13}C-NMR spectra were performed for compounds **3a**, **3f**, **3g**, **4a**, **4f**, and **4g**, whereas MS spectra for **3f**, **3g**, **4a**, **4f**, and **4g** compounds.

The behavioral study showed that the compounds **4a**, **4b**, **4e**, and **4f** weakly affected the CNS of mice. Compound **4g** was without effect. The compounds **4a**, **4b**, **4e**, and **4f** showed antinociceptive properties (Table 2). The most active compounds were **4b** in doses of 0.0125–0.1 LD_{50} and **4e** in doses of 0.025–0.1 lethal dose $(LD)_{50}$. These doses induced a decrease (by 54–81% and 26–91%, respectively) in a number of mice exhibiting pain reactivity in the ''writhing syndrome'' test. In the remaining tests, none of the compounds produced a statistically significant effect. In our research, we have shown that the antinociceptive activity of **4b** (4-(4-methoxyphenyl)-5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione) and **4e** (5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]-methyl}-4-cyclohexyl-4*H*-1,2,4-triazole-3(2*H*)-thione) is interesting and should be examined in more detail.

Among Gram-positive species of bacteria, the most sensitive to all of the assayed compounds was *Micrococcus luteus* American Type Culture Collection (ATCC) 10240 (growth inhibition zone from 22 to 50 mm, MIC = 125–1000 mg/L). The growth of *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 6538, and *Staphylococcus epidermidis* ATCC 12220 were totally or partially inhibited by the following compounds: **3f** (inhibitory zone from 15 to 17 mm, MIC 125–500 mg/L), **3g** (inhibitory zone from 8 to 17 mm, MIC 500–1000 mg/L), and **3h** (inhibitory zone from 8 to 16 mm, MIC = 1000 or >1000 mg/L), similarly growth of *Bacillus subtilis* ATCC 6633 and *B. cereus* ATCC 10876 by compounds **3d** (inhibitory zone from 17 to 25 mm, MIC = 1000 or >1000 mg/L), **3g** (inhibitory zone from 11 to 54 mm, MIC = 500 or >1000 mg/L), and **3h** (inhibitory zone from 9 to 32 mm, MIC = 1000 or >1000 mg/L). For comparison, MIC values for reference strains of tested bacteria were 0.015–0.49 mg/L for gentamicin and 0.49–1.95 mg/L for cefuroxime.

It was found that the most effective compound against Gram-positive microorganisms was **3f** with diameter of the growth inhibition zone from 8 to 30 mm at 5000 mg/L concentration and MIC values from 125 to 500 mg/L (Table 3). No activity of the tested compounds against Gram-negative bacteria or fungi was found.

Our results should be of value to further detailed studies on the biological activity of this group of compounds. Especially **3f** agent described in this article could be regarded as leading structure at seeking of the compounds with increased antibacterial activity against potentially pathogenic or opportunistic Gram-positive bacteria, including Staphylococci (coagulase-positive *S. aureus* and coagulase-negative *S. epidermidis*).

EXPERIMENTAL

Chemistry. Melting points were determined in Fisher-Johns (Pittsburgh, PA) blocks and presented without any corrections. The IR spectra (ν, cm^{-1}) were recorded in KBr tablets using a Specord IR-75 spectrophotometer (Germany). The ^{1}H-NMR spectra were recorded on a Bruker Avance 300 apparatus (Bruker BioSpin GmbH, Rheinstetten/Karlsruhe, Germany) in dimethyl sulfoxide (DMSO)-d_6 with tetramethylsilane (TMS) as internal standard. The ^{13}C-NMR spectra were recorded on a Bruker Avance 300. Chemical shifts are given in ppm (δ scale). The MS spectra were recorded on a ThermoFinnigan Trace TSQGC MS apparatus (Waltham, MA). Chemicals were purchased from Merck Co. (Whitehouse Station, NJ) or Lancaster (Windham, NH) and used without further purification.

The purity of obtained compounds was checked by thin layer chromatography (TLC) on aluminum oxide 60 F_{254} plates (Merck Co.), in a $CHCl_3/C_2H_5OH$ (10:1, v/v) solvent system with UV visualization ($\lambda = 254$ nm).

Ethyl [(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl] acetate (1). Sodium [0.23 g (0.01 mol)] was added to 5 mL of anhydrous ethanol, placed in a three-necked flask equipped with reflux condenser closed with a tube of $CaCl_2$ and mercury stirrer. The content was mixed till sodium dissolved completely and then 1.77 g (0.01 mol) of 4-phenyl-4*H*-1,2,4-triazole-3-thione was added. Then 1.22 mL ethyl bromoacetate was added drop by drop. The content of the flask was mixed for 4 h and left at room temperature for 12 h. Then 10 mL anhydrous ethanol was added and heated for 1 h. The mixture was filtered off inorganic compounds. After cooling, the precipitate was filtered off and crystallized from ethanol.

Yield: 1.73 g, (65.9%). m.p.: 66–68°C. For $C_{12}H_{13}N_3O_2S$ (263.31) calculated: C: 54.76%, H: 4.97%, N: 15.96%; found: C: 54.73%, H: 4.95%, N: 15.95%. IR (KBr): 3098 (CH_{ar}), 2913, 1417 (CH_{al}), 1735 (C=O), 1556 (C=N). ^{1}H-NMR (DMSO-d_6): 1.17 (t, 3H, CH_3, $J = 7.2$ Hz); 4.09 (s, 2H, CH_2); 4.06–4.13 (q, 2H, CH_2, $J = 7.2$ Hz); 7.51–7.65 (m, 5H, $5CH_{ar}$); 8.87 (s, 1H, CH).

[(4-Phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl] acetohydrazide (2). Hydrazine hydrate (0.63 mL of 80%) was added to 2.63 g (0.01 mol) of compound **1** in 5 mL of anhydrous ethanol. The mixture was left at room temperature for 24 h. The precipitate of hydrazide **2** was filtered off, dried, and crystallized form ethanol.

Yield: 1.94 g, (78%). m.p 132–134°C. For $C_{10}H_{11}N_5OS$ (249.29) calculated: C: 48.14%, H: 4.44%, N: 28.08%; found: C: 48.16%, H: 4.41%, N: 28.05%. ^{1}H-NMR (DMSO-d_6): 3.89 (s, 2H, CH_2); 4.29 (s, 2H, NH_2); 7.51–7.61 (m, 5H, $5CH_{ar}$); 8.86 (s, 1H, CH); 9.33 (s, 1H, NH).

Derivatives of 1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl] acetyl}thiosemicarbazide (3a–3i)

General procedure. A mixture of hydrazide (**2**) 2.49 g (0.01 mol) and 0.01 mol appropriate isothiocyanate was heated in an oil bath at 40–95°C for 12 h. The product was washed with diethyl ether to remove unreacted isothiocyanate and with water to remove unreacted hydrazide (**2**). Then it was filtered off, dried, and crystallized from ethanol.

1-{[(4-Phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}-4-phenyl thiosemicarbazide (3a). Yield: 3.51 g, (91.5%). Temperature of reaction: 50°C for 12 h. m.p.: 168–170°C. For $C_{17}H_{16}N_6OS_2$ (384.48) calculated: C: 53.06%, H: 4.19%, N: 21.85%; found: C: 53.09%, H: 4.20%, N: 21.87%. ^{1}H-NMR (DMSO-d_6): 3.99 (s, 2H, CH_2); 7.13–7.65 (m, 10H, $10CH_{ar}$); 8.90 (s, 1H, CH); 9.74, 9.76, 10.43 (3s, 3H, 3NH). ^{13}C-NMR: 34.8 (CH_2); 125.2, 125.4, 128.0, 129.6, 129.9 ($6\times CH_{ar}$); 133.2, 139.0 ($2\times C_{ar}$); 145.4 ($CH_{triazole}$); 149.2 (N=C—S—); 166.9 (C=O); 180.8 (C=S).

1-{[(4-Phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}-4-(4-methoxyphenyl)thiosemicarbazide (3b). Yield: 3.68 g, (88.9%). Temperature of reaction: 50°C for 12 h. m.p.: 174–176°C. For $C_{18}H_{18}N_6O_2S_2$ (414.50) calculated: C: 52.11%, H: 4.37%, N: 20.27%; found: C: 52.10%, H: 4.39%, N: 20.28%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.74 (s, 3H, CH_3); 3.98 (s, 2H, CH_2); 6.90–7.35 (dd, 4H, 4-$CH_3OC_6H_4$, $J = 6$ Hz); 7.52–7.61 (m, 5H, $5CH_{ar}$); 8.89 (s, 1H, CH); 9.63, 9.65, 10.38 (3s, 3H, 3NH).

1-{[(4-Phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}-4-(*p*-tolyl)thiosemicarbazide (3c). Yield: 3.52 g, (88.5%). Temperature of reaction: 80°C for 12 h. m.p.: 188–190°C. For $C_{18}H_{18}N_6OS_2$ (398.50) calculated: C: 54.2%, H: 4.55%, N: 21.1%; found: C: 54.18%, H: 4.56%, N: 21.11%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.28 (s, 3H, CH_3); 3.98 (s, 2H, CH_2); 7.11–7.38 (dd, 4H, 4-$CH_3C_6H_4$, $J = 9$ Hz); 7.52–7.62 (m, 5H, $5CH_{ar}$); 8.89 (s, 1H, CH); 9.67, 9.70, 10.41 (3s, 3H, 3NH).

4-Benzyl-1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}thiosemicarbazide (3d). Yield: 3.73 g (93.9%). Temperature of reaction: 40°C for 12 h. m.p.: 164–166°C. For $C_{18}H_{18}N_6OS_2$ (398.50) calculated: C: 54.2%, H: 4.55%, N: 21.1%; found: C: 54.18%, H: 4.53%, N: 21.09%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.96 (s, 2H, CH_2); 4.79 (s, 2H, CH_2); 7.18–7.64 (m, 10H, $10CH_{ar}$); 8.86 (s, 1H, CH); 8.81, 9.51, 10.37 (3s, 3H, 3NH).

4-Cyclohexyl-1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}thiosemicarbazide (3e). Yield: 3.29 g, (84.5%). Temperature of reaction: 70°C for 12 h. m.p.: 166–168°C. For $C_{17}H_{22}N_6OS_2$ (390.53) calculated: C: 52.24%, H: 5.67%, N: 21.51%; found: C: 54.26%, H: 5.65%, N: 21.50%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.07–1.76 (m, 10H, $5CH_2$); 3.86 (s, 2H, CH_2); 4.08 (s, 1H, CH); 7.52–7.69 (m, 5H, $5CH_{ar}$); 8.94 (s, 1H, CH); 9.26, 9.52, 10.16 (3s, 3H, 3NH).

4-(4-Bromophenyl)-1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}thiosemicarbazide (3f). Yield: 3.67 g, (79.2%). Temperature of reaction: 95°C for 12 h. m.p.: 178–180°C. For $C_{17}H_{15}N_6BrOS_2$ (463.37) calculated: C: 44.02%, H: 3.25%, N: 18.13%; found: C: 44.0%, H: 3.26%, N: 18.15%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.97 (s, 2H, CH_2); 7.51–7.62 (m, 9H, $9CH_{ar}$); 8.90 (s, 1H, CH); 9.81, 9.88, 10.45 (3s, 3H, 3NH). ^{13}C-NMR: 34.7 (CH_2); 125.2, 125.4, 129.6, 129.9, 130.8 ($5\times CH_{ar}$); 117.5, 133.1, 138.4 ($3\times C_{ar}$); 145.4 ($CH_{triazole}$); 149.2 (—N=C—S—); 166.9 (C=O); 180.8 (C=S). MS *m/e* (%): 463 (M^{+}, 0.1); 287 (0.7); 249 (3.1); 213 (80); 190 (7); 176 (100); 157 (16); 134 (20); 104 (12); 91 (27); 77 (35).

4-Ethyl-1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}thiosemicarbazide (3g). Yield: 2.56 g, (76.3%). Temperature of reaction: 50°C for 12 h. m.p.: 171–173°C. For $C_{13}H_{16}N_6OS_2$ (336.43) calculated: C: 46.37%, H: 4.79%, N: 24.97%; found: C: 46.38%, H: 4.80%, N: 24.98%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.11 (t, 3H, CH_3, $J = 6$ Hz); 3.50–3.59 (q, 2H, CH_2, $J = 6.6$ Hz); 3.90 (s, 2H, CH_2); 7.53–7.65 (m, 5H, $5CH_{ar}$); 8.94 (s, 1H, CH); 8.30, 9.32, 10.25 (3s, 3H, 3NH). ^{13}C-NMR: 14.6 (CH_3); 34.1 (—S—CH_2—); 38.6 (—CH_2—CH_3); 125.3, 129.7, 129.9 ($3\times CH_{ar}$); 133.1 (C_{ar}); 145.5 ($CH_{triazole}$); 149.5 (—N=C—S—); 166.7 (C=O); 181.2 (C=S). MS *m/e* (%): 336 (M^{+}, 1); 321 (0.5); 257 (3); 249 (2); 218 (20); 204 (7); 190 (6); 176 (100); 149 (7); 135 (5); 104 (10); 91 (18); 87 (24); 77 (30).

4-Ethoxycarbonylmethyl-1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl} thiosemicarbazide (3h). Yield: 2.57 g (65.2%). Temperature of reaction: 40°C for 12 h. m.p.: 109–112°C. For $C_{15}H_{18}N_6O_3S_2$ (394.47) calculated: C: 45.67%, H: 4.60%, N: 21.30%; found: C: 45.65%, H: 4.61%, N: 21.28%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.19 (t, 3H, CH_3, $J = 7.2$ Hz); 3.97 (s, 2H, CH_2); 4.07–4.14 (q, 2H, CH_2); 4.24 (d, 2H, CH_2); 7.46–7.65 (m, 5H, $5CH_{ar}$); 8.91 (s, 1H, CH); 8.86, 9.68, 10.46 (3s, 3H, 3NH).

4-Ethoxycarbonyl-1-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetyl}thiosemicarbazide (3i). Yield: 2.85 g, (75.1%). Temperature of reaction: 45°C for 12 h. m.p.: 172–174°C. For $C_{14}H_{16}N_6O_3S_2$ (380.44) calculated: C: 44.16%, H: 4.23%, N:

22.08%; found: C: 44.15%, H: 4.21%, N: 22.07%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.23 (t, 3H, CH_3, J = 6.9 Hz); 4.09 (s, 2H, CH_2); 4.14–4.21 (q, 2H, CH_2, J = 6.9 Hz); 7.51–7.64 (m, 5H, $5CH_{ar}$); 8.89 (s, 1H, CH); 9.96, 11.06, 11.37 (3s, 3H, 3NH).

Derivatives of 5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]-methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione (4a–4g, 4j)

General procedure. A mixture of thiosemicarbazide (**3a–3h**; 0.01 mol) and 20 mL of 2% aqueous solution of sodium hydroxide was refluxed for 2 h. Then, the solution was neutralized with diluted hydrochloric acid, and the formed precipitate was filtered off and crystallized from ethanol.

5-{[(4-Phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4-phenyl-4*H*-1,2,4-triazole-3(2*H*)-thione (4a). Yield: 3.12 g, (85.3%). m.p.: 160–162°C. For $C_{17}H_{14}N_6S_2$ (366.46) calculated: C: 55.68%, H: 3.85%, N: 22.93%; found: C: 55.69%, H: 3.85%, N: 22.91%. IR (KBr, cm^{-1}): 3110 (CH_{ar}); 2918, 1458 (CH_{al}); 1608 (C=N); 1513 (C—N). ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.12 (s, 2H, CH_2); 7.25–7.58 (m, 10H, $10CH_{ar}$); 8.89 (s, 1H, CH); 13.83 (s, 1H, NH). ^{13}C-NMR: 27.8 (CH_2); 128.2, 129.1, 129.3, 129.5, 129.6, 129.7 ($6\times CH_{ar}$); 133.1 ($2\times C_{ar}$); 145.8 ($CH_{triazole}$); 147.1 (CH_2—C); 148.0 (C—S); 168.2 (C=S). MS *m/e* (%): 366 (M^+, 0.2); 266 (0.15); 222 (0.04); 190 (0.54); 176 (75); 149 (14); 135 (8); 104 (9); 91 (28); 77 (100).

5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4-(4-methoxyphenyl)-4*H*-1,2,4-triazole-3(2*H*)-thione (4b). Yield: 3.58 g, (90.1%). m.p.: 168–170°C. For $C_{18}H_{16}N_6OS_2$ (396.49) calculated: C: 54.48%, H: 4.06%, N: 21.19%; found: C: 54.48%, H: 4.08%, N: 21.17%. IR (KBr, cm^{-1}): 3095 (CH_{ar}); 2922, 1461 (CH_{al}); 1618 (C=N); 1515 (C—N). ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.81 (s, 3H, CH_3); 4.10 (s, 2H, CH_2); 7.03 (d, 2H, $2CH_{ar}$, 4-$CH_3OC_6H_4$, J = 9 Hz); 7.16 (d, 2H, $2CH_{ar}$, 4-$CH_3OC_6H_4$, J = 9 Hz); 7.35–7.59 (m, 5H, $5CH_{ar}$); 8.89 (s, 1H, CH); 13.77 (s, 1H, NH).

5-{[(4-Phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4-(4-tolyl)-4*H*-1,2,4-triazole-3(2*H*)-thione (4c). Yield: 3.52 g, (92.7%). m.p.: 166–168°C. For $C_{18}H_{18}N_6S_2$ (380.49) calculated: C: 56.77%, H: 4.23%, N: 22.08%; found: C: 56.78%, H: 4.22%, N: 22.08%. IR (KBr, cm^{-1}): 3095 (CH_{ar}); 2922, 1459 (CH_{al}); 1610 (C=N); 1526 (C—N). ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.37 (s, 3H, CH_3); 4.10 (s, 2H, CH_2); 7.10–7.32 (dd, 4H, $4CH_{ar}$, 4-$CH_3C_6H_4$, J = 8.1 Hz); 7.34–7.58 (m, 5H, $5CH_{ar}$); 8.89 (s, 1H, CH); 13.80 (s, 1H, NH).

4-Benzyl-5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione (4d). Yield: 3.61 g, (95.1%). m.p.: 130–132°C. For $C_{18}H_{16}N_6S_2$ (380.49) calculated: C: 56.77%, H: 4.23%, N: 22.08%; found: C: 56.77%, H: 4.22%, N: 22.07%. IR (KBr, cm^{-1}): 3088 (CH_{ar}); 2926, 1459 (CH_{al}); 1615 (C=N); 1529 (C—N). ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.31 (s, 2H, CH_2); 5.26 (s, 2H, CH_2); 7.19–7.56 (m, 10H, $10CH_{ar}$); 8.90 (s, 1H, CH); 13.83 (s, 1H, NH).

4-Cyclohexyl-5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]-methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione (4e). Yield: 3.32 g, (89.4%). m.p.: 102–104°C. For $C_{17}H_{20}N_6S_2$ (372.51) calculated: C: 54.76%, H: 5.38%, N: 22.55%; found: C: 54.75%, H: 5.37%, N: 22.55%. IR (KBr, cm^{-1}): 3100 (CH_{ar}); 2918, 1455 (CH_{al}); 1610 (C=N); 1527 (C—N). ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.06–1.75 (m, 10H, $5CH_2$); 3.58 (s, 2H, CH_2); 4.44 (s, 1H, CH); 7.37–7.64 (m, 5H, $5CH_{ar}$); 8.95 (s, 1H, CH); 13.95 (s, 1H, NH).

4-(4-Bromophenyl)-5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione (4f). Yield: 3.72 g, (83.6%). m.p.: 147–149°C. For $C_{17}H_{13}BrN_6S_2$ (445.46) calculated: C: 45.8%, H: 2.93%, N: 18.86%; found: C: 45.8%, H: 2.92%, N: 18.88%. IR (KBr, cm^{-1}): 3088 (CH_{ar}); 2924, 1470 (CH_{al}); 1624 (C=N); 1527 (C—N). ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.14 (s, 2H, CH_2); 7.23 (d, 2H, $2CH_{ar}$, 4-BrC_6H_4, J = 8.7 Hz); 7.35–7.59 (m, 5H, $5CH_{ar}$); 7.72 (d, 2H, $2CH_{ar}$, 4-BrC_6H_4, J = 8.7 Hz); 8.89 (s, 1H, CH); 13.87 (s, 1H, NH). ^{13}C-NMR: 27.8 (CH_2); 125.4, 129.5, 129.7, 130.4, 132.3 ($5\times CH_{ar}$); 122.9, 132.4, 133.0 ($3\times C_{ar}$); 145.8 ($CH_{triazole}$); 147.0 (CH_2—C); 147.9 (C—S); 168.1 (C=S). MS *m/e* (%): 445 (M^+, 0.03); 366 (0.01); 287 (0.01); 268 (0.2); 257 (0.03); 213 (0.07); 190 (0.17); 176 (75); 149 (15); 135 (9); 104 (10); 91 (28); 77 (100).

4-Ethyl-5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione (4g). Yield: 2.93 g, (94.5%). m.p.: 184–186°C. For $C_{13}H_{14}N_6S_2$ (318.42) calculated: C: 48.98%, H: 4.43%, N: 26.38%; found: C: 48.99%, H: 4.44%, N: 26.39%. IR (KBr, cm^{-1}): 3101 (CH_{ar}); 2922, 1463 (CH_{al}); 1612 (C=N); 1530 (C—N). ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.36 (t, 3H, CH_3, J = 9 Hz); 4.15–4.22 (q, 2H, CH_2, J = 7.2 Hz); 4.53 (s, 2H, CH_2); 7.37–7.61 (m, 5H, $5CH_{ar}$); 9.00 (s, 1H, CH); 13.63 (s, 1H, NH). ^{13}C-NMR: 13.1 (CH_3); 27.4 (—S—CH_2—); 38.5 (—CH_2—CH_3); 125.8, 129.6, 129.7 ($3\times CH_{ar}$); 133.1 (C_{ar}); 145.9 ($CH_{triazole}$); 147.3 (CH_2—C); 148.1 (C—S); 166.7 (C=S). MS *m/e* (%): 318 (M^+, 25); 302 (1); 290 (2); 258 (2.5); 218 (3); 204 (12); 190 (3); 176 (100); 149 (9); 135 (6); 104 (8); 91 (11); 77 (25).

4-Carboxymethyl-5-{[(4-phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]methyl}-4*H*-1,2,4-triazole-3(2*H*)-thione (4j). Yield: 2.71 g, (77.8%). m.p.: 123–125°C. For $C_{13}H_{12}N_6O_2S_2$ (348.40) calculated: C: 44.81%, H: 3.47%, N: 24.12%; found: C: 44.82%, H: 3.47%, N: 24.11%. ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.26 (s, 2H, CH_2); 4.64 (s, 2H, CH_2); 7.35–7.62 (m, 5H, $5CH_{ar}$); 8.84 (s, 1H, CH); 10.52 (s, 1H, OH); 13.83 (s, 1H, NH).

[(4-Phenyl-4*H*-1,2,4-triazol-3-yl)sulfanyl] acetic acid (5). Compound **5** was obtained using the same method described earlier for derivatives **4a–4g**, **4j**. That is, a mixture of thiosemicarbazide (**3i**; 0.01 mol) and 20 mL of 2% aqueous solution of sodium hydroxide was refluxed for 2 h. Then, the solution was neutralized with diluted hydrochloric acid and the formed precipitate was filtered off and crystallized from ethanol.

Yield: 1.88 g, (80.2%). m.p.: 184–186°C. For $C_{10}H_9N_3O_2S$ (235.26) calculated: C: 51.0%, H: 3.58%, N: 17.87%; found: C: 51.0%, H: 3.6%, N: 17.9%. ^{1}H-NMR (DMSO-d_6): 4.04 (s, 2H, CH_2); 7.49–7.64 (m, 5H, $5CH_{ar}$); 8.86 (s, 1H, CH); 12.91 (s, 1H, OH).

X-ray crystallography. Crystal data for compound **1**: space group, $P2_1/n$, a = 7.726(1) Å, b = 19.313(3) Å, c = 9.222(2) Å, β = 113.92(2)°, V = 1257.9(4) Å^3, Z = 4, d_{calc} = 1.390 g cm^{-3}, μ = 0.255 mm^{-1}.

A crystal with approximate dimensions of 0.41 × 27 × 0.14 mm^3 was mounted on a glass fiber in a random orientation. Single-crystal diffraction data were measured at room temperature in the ω/2θ mode on the Oxford Diffraction Xcalibur diffractometer using the graphite-monochromated MoK_α radiation. The stability of intensities was monitored by measurement of three standards for every 100 reflections. Crystal structure was solved by direct methods using SHELXS97 [20] and refined by the full-matrix least-squares on F^2 using the SHELXL97 [21]. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were positioned geometrically and allowed to ride on their parent

atoms, with $U_{iso}(H) = 1.2\ U_{eq}(C)$. Final discrepancy factors are $R_1 = 0.0438$, $wR_2 = 0.0954$ for $I > 2\sigma(I)$, GOOF = 1.019, $\Delta\rho_{min,\ max} = -0.22/0.21$ e Å^{-3}.

Pharmacology. The experiments were carried out on male Albino-Swiss mice weighing 20–24 g. The animals were housed in colony cages with free access to food (standard laboratory pellets, Bacutil Motycz, Poland) and tap water and maintained in the natural light–dark cycle. The tested groups, consisting of eight animals, were randomly assigned. The experiments were performed between 8:00 a.m. and 2:00 p.m. The investigated compounds (**4a**, **4b**, **4e**, **4f**, and **4g**) were administered intraperitoneally (i.p.) in doses equivalent to 0.00625, 0.0125, 0.025, 0.05, and 0.1 of their LD_{50} as suspensions in 1% Tween 80 in a volume 10 cm^3/kg at 30 min before tests. Control mice received the equivalent volume of solvent. The Bioethical Committee of Lublin Medical University approved all experimental procedures applied in this study. The following behavioral tests were performed.

Chimney test. The effect of above investigated compounds in a dose of 0.1 of their LD_{50} on motoring impairment was quantified with the chimney test [22]. All animals were pertained 24 h before the test. The mice were inserted into a plastic tube (25 cm long, 3 cm in inner diameter). When the mouse reached the end, the tube was positioned upright, and motor impairment was indicated when the animal was unable to climb backward up the tube within 60 s. It was quantified as the percentage of animals that failed to complete the test.

Body temperature. The rectal body temperature in mice (Ellab thermometer) was recorded 15, 30, 45, 60, 90, and 120 min after the administration of the investigated compounds in a dose of 0.1 of their LD_{50}.

Hole board test. To determine the effects of the compounds on the explorative activity, the hole board test of Boissier *et al*. [23] was taken. The mice were placed on the board 30 min after administration of compounds in a dose of 0.1 of their LD_{50}. The number of holes explored was counted for 5 min.

Four plate test. Anxiolytic activity was measured by the ''four plate'' test in mice according to Aron *et al*. [24]. Thirty minutes after the injection of compounds in a dose of 0.1 of their LD_{50}, the number of punished crossings was counted for 1 min.

Passive avoidance task. The passive avoidance task is considered to be a measure of long-term memory in rodents [25]. On the first day, 30 min after injection of compounds in a dose of 0.1 of their LD_{50}, the animals were placed in an illuminated box (10 × 13 × 15 cm^3) connected to a dark box (25 × 20 × 15 cm^3), equipped with an electric grid floor. Having entered the dark box, the mice were punished by an electric foot shock (0.6 mA, 2 s). Animals not entering the dark box within 60 s were excluded from the subsequent experiment. The next day (24 h later), animals were again placed individually in the illuminated box and observed up to 3 min. The time from placement in the illuminated box to entry into the dark box was taken as an indication of the degree of long-term memory impairment. In the control group, the animals did not enter the dark compartment within 3 min.

Forced swimming test. Thirty minutes after the injection of compounds in a dose of their LD_{50}, the mice were individually placed and forced to swim in a glass cylinder (27 × 16 cm^2) containing 15 cm of water (25°C). A mouse was considered immobile when it floated in the water in an upright position and made only small movements to keep its head above water. The total immobility time of mice was measured during the last 4 min of the 6-min test [26].

Thiopental sleeping time. Thiopental (75 mg/kg i.p.) was given 30 min after the injection of the all compounds in a dose of 0.1 of their LD_{50}. The sleeping time of mice (from disappearance to return of the righting reflex) was measured.

Antinociceptive activity. Pain reactivity was measured by the writhing syndrome test in mice according to Witkin *et al*. [27]. Thirty minutes after the injection of compounds, **4a** (0.025–0.1 LD_{50}), **4b** (0.00625–0.1 LD_{50}), **4e** and **4f** (0.0125–0.1 their LD_{50}), **4g** (0.1 LD_{50}), the animals were injected with 0.6% acetic acid. The number of writhing episodes was counted for 30 min.

Pentetrazole-induced seizures. Thirty minutes after administration of compounds in a dose of 0.1 their LD_{50}, the animals were injected subcutaneously (sc) with pentetrazole (110 mg/kg) and were observed during 30 min. The number of mice developing clonic and tonic seizures as well as mortality was recorded in that period.

Head twitches. Head-twitch responses induced by L-5-hydroxytryptophan (L-5-HTP) were measured in mice according to Corne *et al*. [28]. The compounds in a dose of 0.1 of their LD_{50} were injected 30 min before L-5-HTP (165 mg/kg). The number of head-twitch episodes of mice was counted during 60 min after the injection of L-5-HTP.

Microbiology. The reference strains of ATCC, including six Gram-positive bacteria (*S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12220, *B. subtilis* ATCC 6633, *B. cereus* ATCC 10876, *M. luteus* ATCC 10240), four Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, and *Pseudomonas aeruginosa* ATCC 9027), and three strains of yeasts belonging to *Candida* spp. (*C. albicans* ATCC 10231, *C. albicans* ATCC 2091, *C. parapsilosis* ATCC 22019) were used.

Microbial suspensions were prepared in sterile saline (0.85% NaCl) with an optical density of McFarland standard 0.5 (150 × 10^6 colony forming units per mL). All stock solutions of the tested compounds were dissolved in DMSO. The medium containing DMSO at the final concentrations and without any of tested compounds served as control, and no microbial growth inhibition was observed. Gentamicin and cefuroxime were used as control antimicrobial agents. Mueller-Hinton agar or Mueller-Hinton agar supplemented with 2% glucose was used for examination of antibacterial or antifungal activity, respectively.

In the first step, all tested compounds were screened for *in vitro* antimicrobial activity by the agar well diffusion method based on the appearance of the growth inhibition zone surrounding the well ($d = 8$ mm) containing the tested chemicals at 5000 mg/L concentration (80 μL/well separately). The sterile swabs were used to spread the microbial suspensions onto the medium surface. The plates were preincubated at room temperature for 1.5 h to allow the diffusion of solution into the medium and then were incubated at 37°C for 18 h (for bacteria) or at 30°C for 24–48 h (for fungi).

Subsequently, MIC of the compounds **3d**, **3f**, **3g**, and **3h**, showing some antibacterial activity using the agar well diffusion method, was estimated by microdilution technique [optical density $(OD)_{600}$] and Mueller-Hinton broth containing from 1.95 to 1000 mg/L of the tested agents. MIC is usually defined as the lowest concentration of compound at which there was no visible growth of tested microorganisms. Microdilution

broth technique was developed using 96-well microplates, which were inoculated with a 1:10 diluted microbial suspension of optical density of 0.5 McFarland standard. About 20 μL of bacterial suspension was put into 180 μL of medium containing twofold dilution of the tested compounds. After incubation (at 37°C for 18 h), the optical density (OD_{600}) measurements were determined for bacterial culture in broth medium, and the MIC values were determined by comparison with the bacterial growth in control (compound free) medium.

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