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Il Farmaco 56 (2001) 905–918

IL FARMACO

Synthesis and in vivo pharmacology of new derivatives of isothiazolo[5,4-*b*]pyridine of Mannich base type

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Received 3 March 2000; accepted 15 March 2001

Abstract

Recently we reported on 2*H*-4,6-dimethyl-2-[(4-phenylpiperazin-1-yl)methyl]-3-oxo-2,3-dihydroisothiazolo[5,4-*b*]pyridine (**V**), which exhibited high anorectic action in animal models as a result of stimulation of serotoninergic system. This paper describes the synthesis of the series **3**–**5** of analogues of **V** prepared from 2-hydroxymethyl-4,6-dimethylisothiazolopyridine (**2**) and corresponding 4-substituted-piperazines(piperidines) or tetrahydroisoquinoline. The 12 compounds obtained were screened in standard CNS tests in in vivo (mice and rats). In contrast to **V**, none of its analogues showed serotoninergic activity, whereas several of these compounds were found to be active as weak to moderate analgesic agents. According to X-ray and molecular modeling studies the different pharmacological profile of **V** and its *o*-OCH_3 analog **3a**, taken as an example, should be referred back to the conformational restriction incorporated by the *o*-substitution rather than effects of different lipophlicity or basicity of these compounds.

Keywords: 3-Oxoisothiazolo[5,4-*b*]pyridines; Mannich bases; Analgesic activity

1. Introduction

N- or *C*-[(4-phenylpiperazin-1-yl)methyl] Mannich bases of various heterocyclic ring systems produce different CNS effects, which have been associated with stimulation of various types of receptors and behavioral responses. Among these compounds, derivatives of 5-arylidenepyridazinone (**I**) [1], and 2-oxazolopyridine (**II**) [2] are potent analgesic agents. Vaugien et al. [3] described a derivative of 4,5-dihydro-2-oxazolamine (COR 3224) (**III**) which is under clinical trials as a potential antidepressant drug. Other authors reported [4] that 3-[(4-phenylpiperazin-1-yl)methyl]indole (**IV**) and related compounds with modified 4-phenylpiperazinyl residue are ligands of dopamine receptors (D_2, D_3, D_4).

Recently we prepared and tested in vivo in animal models 2*H*-4,6-dimethyl-2-[(4-phenylpiperazin-1-yl)-methyl]-3-oxo-2,3-dihydroisothiazolo[5,4-*b*]pyridine (**V**) (Fig. 1) which showed a profile of action characteristic to substances stimulating the central serotoninergic system (5-HT) [5]. Compound **V** considerably potentiated the number of the head twitch episodes in mice, and at doses of 7.7–61.6 mg/kg (ip) caused the 5-HT behavioral syndrome in rats which included: flat body posture, reciprocal forepaw treading, hindlimb-abduction and Straub's tail. Probably the serotoninergic mechanism underlies the anorectic (2 mg/kg ip, 8.5 mg/kg po) and potential antidepressive activity of **V**. However, radioligand binding experiments involving 5-HT_{1A} and 5-HT_{2A} receptors showed that **V** has no receptor affinity and suggest an indirect mechanism of serotoninergic action of this compound. It should be mentioned that *m*-chlorophenyl- and (2-pyrimidinyl) piperazinyl analogues of **V**, which have been synthesized and tested recently [6], showed no biological activity.

In the present paper, as an extension of our earlier work, we describe several new analogues of our lead

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0014-827X/01/$ - see front matter
PII: S0014-827X(01)01157-0

compound **V** modified at the phenylpiperazinyl unit and their evaluation in a series of commonly used CNS tests for pharmacological and structural comparison.

The new isothiazolopyridines (Table 1) were characterized by the presence of a 4-arylpiperazinyl residue, which possessed a substituent in the aromatic ring (**3c**–**f**). Additionally, the benzyl analogue **3g** of **V** and more complex (4-chlorobenzhydryl, piperonyl) derivatives of this analogue (**3h**,**i**) were obtained. We also prepared the structurally related to **V** derivatives with the piperazine ring replaced by piperidine (**4**) and an analogue of **V** with 1,2,3,4-tetrahydroisoquinoline (**5**), of which the *N*-atom may mimic the basic nitrogen of 4-phenylpiperazine [7]. Finally, for comparative purposes we investigated the biological properties of the isothiazolopyridines **3a**,**b** and a homologue of **V** with central ethylene chain (**3j**). These compounds have been synthesized recently and were described in our previous papers [6,8,9].

2. Chemistry

The synthesized target isothazolopyridines **3**–**5** with the exception of **3j** [9], were all prepared in one-step reaction from corresponding commercially available 4-substituted-piperazines(piperidines) or 1,2,3,4-tetrahydroisoquinoline and 2-hydroxymethyl-isothiazolopyridine (**2**) (Scheme 1). The crude products were purified by crystallization or column chromatography.

The identity of new compounds **3**–**5** (Table 1) was established by their IR, ^{1}H NMR and elemental analyses. The spectral data within the series of piperazine and piperidine derivatives, respectively **3** and **4**, did not show remarkable differences and are presented for selected compounds in Section 4. However, it should be mentioned that in ^{1}H NMR spectrum of **V** [5] and its analogues **3**, with the exception of *o*-methyl derivative **3c**, protons of the piperazine rings appeared as two multiplets with centers in ranges of 2.8–2.9 ppm (4H) and 3.05–3.3 ppm (4H). In spectrum of the *o*-methyl analogue **3c** signal of these protons appeared as a sharp singlet (8H) at $\delta = 2.9$ ppm. Similar ^{1}H NMR data of the piperazine ring protons occurs also in the *o*,*m*-dimethyl analogue **3e** prepared for comparison ($\delta = 2.9$, s, 8H). Table 1 summarizes the physical data associated with the new compounds.

The profile of biological activity of the piperazine-(**3**), piperidine-(**4**) and tetrahydroisoquinoline-(**5**) analogs of **V** was determined in commonly used behavioral CNS tests in animal models (Section 3). The results clearly indicated that all of the studied compounds (**3**–**5**), in contrast to the parent compound **V**, were devoid of serotoninergic action; they did not potentiate the head twitch response induced by 5-HTP. Instead, all derivatives in the series of piperidine (**4**) and most of those in the series of piperazine (**3**) derivatives were found to exhibit a predominant profile of analgesic activity evidenced in writhing and/or hot plate tests (Tables 7 and 8). To explain such a discrepancy observed between the biological effects of **V** and its simple analogues **3**, some physical properties [lipophilicity (Table 1), pK_a] were determined for comparison.

As seen in Table 1, the lipophilicities of isothiazolopyridines **V** and **3**, expressed as log P_{calc} (the calculated octanol–water partition coefficient) do not differ considerably. For example, the log P_{calc} values of the compounds most interesting in writhing (**3b**) or hot plate (**3a**) test were 4.95, and 4.18, respectively, whereas 4.43 for **V**.

The typical pK_a (ionization constant) values of simple 1-arylpiperazines are within the range of 7.94–9.14 [10]. These data suggest that under physiological conditions (pH 7.4) different fractions of compounds **3** and **V** may be protonated. Therefore, the concentration of the N1 (piperazine ring) protonated species of **3a**, taken as an example, and **V** at pH 7.4 was compared. Due to the poor water solubility and low stability in acidic medium of our isothiazolopyridines of Mannich base type [5], the ionization constant of **3a** (p$K_a = 7.15$), similarly as

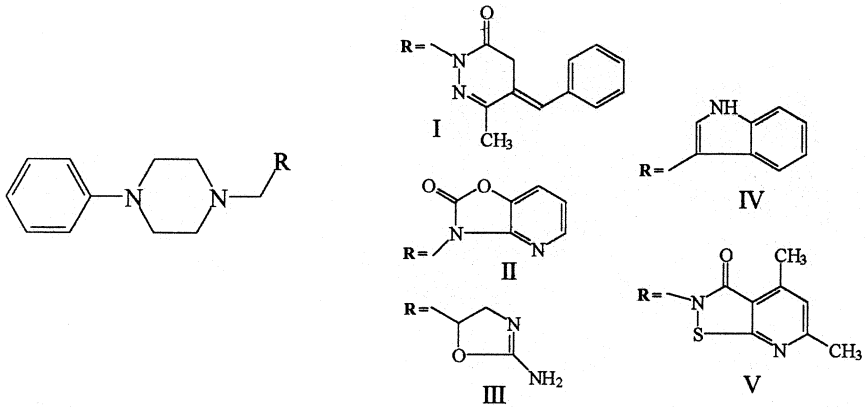
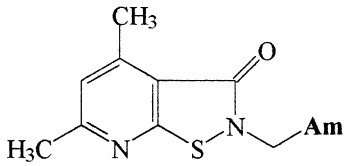


Fig. 1.

for **V** determined previously ($pK_a = 7.55$) [5], was measured in 33% alcohol–water solution (37 °C). We believe that the aqueous pK_a of both compounds are higher by about 0.3–0.35 units because it was found that alcohol weakens the basicity of aliphatic and aromatic amines (e.g. 50% alcohol leads to an average

Table 1
Physical data of isothiazolo[5,4-*b*]pyridine derivatives **3–5**



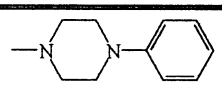
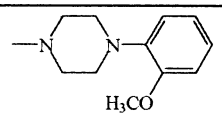
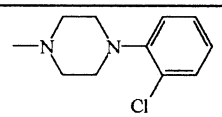
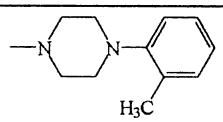
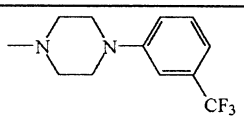
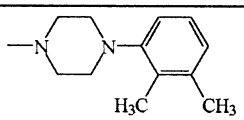
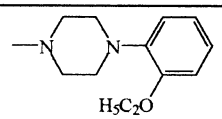
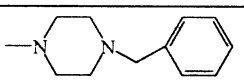
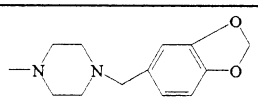
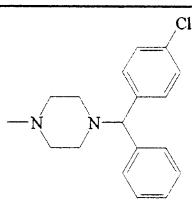
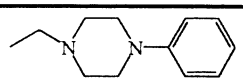
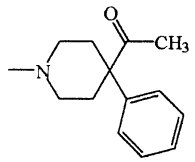
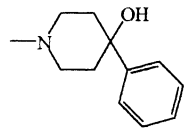
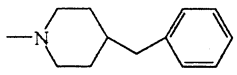
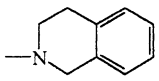
No	Am	Molecular formula; m.w.	Mp °C	$LogP_{calc.}$	Lit.
V				4.43	[5]
3a				4.18	[8]
3b				4.95	[6]
3c		$C_{20}H_{24}N_4OS$ 368.49	108-110	4.90	
3d		$C_{20}H_{21}F_3N_4OS$ 422.46	145-148	5.31	
3e		$C_{21}H_{26}N_4OS$ 382.52	158-160		
3f		$C_{21}H_{26}N_4O_2S$ 398.52	131-133		
3g		$C_{20}H_{24}N_4OS$ 368.49	105-107	4.05	
3h		$C_{21}H_{26}N_4O_3S$ 312.50	153-156	3.73	
3i		$C_{26}H_{27}ClN_4OS$ 479.03	86-88	6.42	

Table 1 (*Continued*)

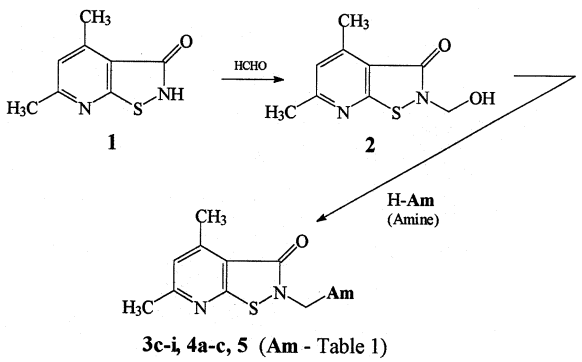
3j				4.06	[9]
4a		$C_{22}H_{25}N_3O_2S$ 395.51	126-128	4.95	
4b		$C_{20}H_{23}N_3O_2S$ 369.48	178-181	3.54	
4c		$C_{21}H_{25}N_3OS$ 367.50	106-108	5.14	
5		$C_{18}H_{19}N_3OS$ 325.42	78-80	4.23	

depression of the pK_a value ~ 0.54) [11]. Hence it is possible that in in vivo about 50–60% of **3a** may be represented by positively charged species and it is similar to the ionization percentage of **V** (about 70%) [5]. The above data displayed that the discrepancy observed in the profile of the pharmacological action of **V** and **3a** are not effects of significant differences in lipophilicity or basicity of these compounds, but it should be steric in nature, particularly within the side chain.

In order to confirm our assumption, a molecular modeling study was undertaken. Basic conformational informations were obtained from the X-ray crystal structures of **V** [12] and **3a**.

*2.1. Crystal structure of **3a** and molecular modeling study*

A perspective drawing with atomic labelling of the molecular structure of isothiazolopyridine **3a** as determined by X-ray analysis is shown in Fig. 2. The final atomic coordinates and equivalent isotropic thermal parameters for this compound are given in Table 2, whereas selected bond distances and valence angles in Table 3. The molecular packing of **3a** in the crystal is influenced by the presence of weak C–H···X hydrogen bonds: C12–H121···O3^{i} [C12···O3 = 3.479(4), H121···O3 = 2.59(3) Å and C12–H121···O3 = 154(2)°] and C5–H51···O3^{ii} [C5···O3 = 3.334(4), H51···O3 = 2.47(3) Å and C5–H51···O3 = 159(3)°], where (i) x, $-y+1/2, z+1/2$; (ii) $-x, y-1/2, -z-1/2$.



Scheme 1.

The bond lengths, angles and planarity of the isothiazolopyridine fragment in **V** and **3a** are very similar. In the molecule **3a**, the piperazine ring adopts a chair conformation with puckering parameters of $Q = 0.585(3)$ Å and $\theta = 177.4(3)°$ [13], but the deformation of this ring and partial conjugation of the lone pair of electrons at N24 with the π system of the phenyl ring, reported for **V** [12], is not observed. The *o*-methoxy substituent lies almost in the plane of the phenyl ring (O37 and C38 atoms are displaced from the best phenyl plane by $-0.012(2)$ and $-0.026(7)$ Å, respectively) and changes the position of the aromatic ring with respect to the piperazine ring, described by the torsion angle C23–N24–C31–C32 (Table 4), from partially coplanar in **V** [15.3°] to nearly perpendicular in **3a** [71.5°].

To further investigate the conformational preferences of **V** and **3a**, molecular modeling studies were undertaken. The initial geometries of the compounds were built from crystallographic data of **3a** and **V**. The torsion angles for **V** were calculated for the inverted structure $(-x, -y, -z)$ in relation to that cited in [12] and they were compared to the respective torsion angles in **3a**. The starting structures were energetically minimized and geometrically optimized using AM1 method and the parameters for a water environment (the structures were placed in the center of a box

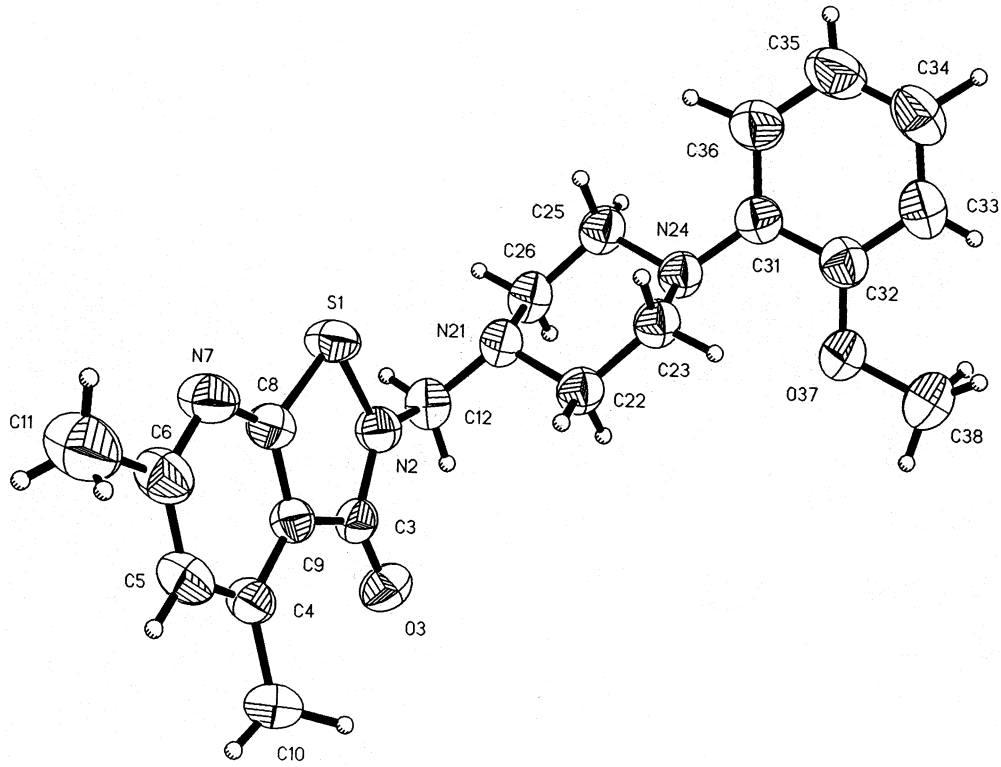


Fig. 2. A view of the molecule **3a** with the atomic labelling. Non-hydrogen atoms are represented by displacement ellipsoids of 50% probability.

Table 2
Fractional atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($Å^2 \times 10^3$) for non-H atoms (**3a**)

Atom	*x*	*y*	*z*	U_{eq} [a]
S(1)	2082(1)	644(1)	1736(1)	51(1)
O(3)	371(1)	1879(2)	−1005(2)	57(1)
O(37)	3627(1)	6188(2)	−417(2)	70(1)
N(2)	1426(1)	1616(2)	875(2)	44(1)
N(7)	1847(2)	−1415(2)	697(3)	55(1)
N(21)	2244(1)	3180(2)	1697(2)	42(1)
N(24)	3398(1)	4883(2)	1659(2)	44(1)
C(3)	863(2)	1261(2)	−286(3)	41(1)
C(4)	593(2)	−647(3)	−1529(3)	45(1)
C(5)	844(2)	−1739(3)	−1410(3)	55(1)
C(6)	1453(2)	−2102(3)	−298(4)	57(1)
C(8)	1601(2)	−367(2)	565(3)	42(1)
C(9)	987(2)	72(2)	−467(3)	39(1)
C(10)	−55(2)	−261(4)	−2736(4)	62(1)
C(11)	1688(4)	−3306(4)	−138(8)	91(2)
C(12)	1450(2)	2722(3)	1479(3)	48(1)
C(22)	2416(2)	3556(3)	392(3)	47(1)
C(23)	3260(2)	3981(3)	635(3)	45(1)
C(25)	3219(2)	4510(3)	2958(3)	49(1)
C(26)	2367(2)	4111(2)	2683(2)	47(1)
C(31)	4149(2)	5419(2)	1795(3)	47(1)
C(32)	4261(2)	6105(3)	704(3)	53(1)
C(33)	4987(2)	6642(3)	831(4)	64(1)
C(34)	5596(2)	6521(3)	2000(4)	70(1)
C(35)	5495(2)	5867(4)	3065(4)	77(1)
C(36)	4775(2)	5318(3)	2962(4)	65(1)
C(38)	3712(4)	6883(6)	−1549(7)	110(2)

[a] U_{eq} is defined as one-third of the trace of the orthogonalized U_{ij} tensor.

surrounded by 18 water molecules). Table 4 summarizes the results of these calculations, whereas Fig. 3 shows the result of the molecular superimposition of **V** and **3a**, where the isothiazolopyridine ring systems were superimposed.

The overlay clearly indicates that the conformations of both compounds do not differ considerably. A com-

Table 3
Selected bond distances (Å) and angles (°) for **3a**

Bond distances			
S(1)–N(2)	1.706(2)	C(3)–C(9)	1.466(3)
S(1)–C(8)	1.746(3)	C(4)–C(5)	1.380(4)
O(3)–C(3)	1.221(3)	C(4)–C(9)	1.404(4)
C(4)–C(10)	1.500(4)	C(5)–C(6)	1.396(4)
N(2)–C(3)	1.380(3)	C(6)–C(11)	1.504(5)
N(7)–C(8)	1.328(4)	C(8)–C(9)	1.386(4)
N(7)–C(6)	1.339(4)	C(22)–C(23)	1.505(4)
C(25)–C(26)	1.506(4)		
Bond angles			
N(2)–S(1)–C(8)	89.7(4)	N(7)–C(8)–C(9)	127.2(3)
C(32)–O(37)–C(38)	117.8(3)	N(7)–C(8)–S(1)	120.5(2)
C(3)–N(2)–C(12)	124.7(2)	C(9)–C(8)–S(1)	112.3(2)
C(3)–N(2)–S(1)	116.8(2)	C(8)–C(9)–C(4)	117.5(3)
C(8)–N(7)–C(6)	114.9(3)	C(5)–C(4)–C(9)	116.1(3)
C(8)–C(9)–C(3)	113.0(2)	O(3)–C(3)–N(2)	123.1(3)
C(36)–C(31)–N(24)	123.2(3)	O(3)–C(3)–C(9)	128.8(3)
C(32)–C(31)–N(24)	118.9(3)	C(5)–C(4)–C(10)	121.5(3)
C(9)–C(4)–C(10)	122.5(3)	C(4)–C(5)–C(6)	121.7(3)
N(7)–C(6)–C(5)	122.5(3)	N(7)–C(6)–C(11)	116.3(3)
C(5)–C(6)–C(11)	121.2(3)	O(37)–C(32)–C(33)	124.7(3)

Table 4
Comparison of the significant torsion angles for solid state **V** [a] and **3a** and AM1 energy minimized (in water environment) conformers of **V** and **3a** and their N21 protonated species ($\mathbf{VH}^+$, $\mathbf{3aH}^+$)

Torsion angle (°)	**3a** X-ray	**3a** (H_2O)	$\mathbf{3aH}^+$ (H_2O)	**V** X-ray	**V** (H_2O)	$\mathbf{VH}^+$ (H_2O)
S1–N2–C12–N21	55.8	80.5	102.4	24.5	76.8	83.8
N2–C12–N21–C22	75.6	94.4	94.1	77.9	94.6	51.7
C23–N24–C31–C32	71.5	65.8	59.9	15.3	0.0	65.3

[a] The torsion angles are calculated for inverted structure at $-x, -y, -z$ in relation to that in Ref. [12].

parison of the location of the side chain in **3a** and **V** in relation to the bicyclic system of isothiazolopyridine, described by the torsion angle S1–N2–C12–N21 [80.5 and 76.8° in water solution (Table 4), respectively], showed that the arylpiperazinyl group in both compounds extends in the same direction. As expected, the overall spatial difference of the side chains in compounds **3a** and **V** could be observed between the rotameric position of the aromatic ring in respect to the piperazine ring, described by the torsion angle C23–N24–C31–C32. In **Va**(H_2O) the orientations of the piperazine and aromatic rings are practically co-planar [0.0° (Table 4)] whereas for **3a**(H_2O) the both rings are distinctly twisted [65.8° (Table 4)].

The determined pK_a values of **V** and **3a** (7.55 [12] and 7.15 in 33% ethanol solution, respectively) suggest that bases of both compounds are easily protonated under physiological condition and $\mathbf{VH}^+$ or $\mathbf{3aH}^+$ species may also be bioactive forms. Therefore, we have built the theoretical models of the preferred N21 protonated conformations of $\mathbf{VH}^+$ and $\mathbf{3aH}^+$ using the AM1 method in a water environment. The data predicted by the AM1 method (Table 4) demonstrated that protonation of **V** and **3a** in general did not possess a drastic influence on the molecular geometry as compared with the free bases. However, it is worth noting that the spatial geometry of the arylpiperazinyl group of $\mathbf{VH}^+$, described by the value of 65.3° of the torsion angle C23–N24–C31–C32, conforms with the conformation of this fragment found in **3a** and $\mathbf{3aH}^+$ (65.8 and 59.9°, respectively). Therefore, in the last step of the conformational study we determined the energy effect of the free-rotation between the aromatic and piperazine rings, taking into account the one degree of freedom described by the torsion angle φ = C23–N24–C31–C32 (Fig. 4) using the molecular mechanics method. The energies of the conformations were minimized and all geometrical parameters optimized for each rotation with a 15° increment from -180 to 180° of φ (Fig. 4).

As expected, the calculation showed that the energy differences between rotamers of the 1-phenylpiperazinyl fragment in **V** and $\mathbf{VH}^+$ are relatively low ($\Delta E \sim 2.9$ and ~ 3.3 kcal/mol, respectively; ΔE is calculated with respect to the lowest energy conformation) and suggests, that this compound may not be pharmacologically active exactly in its minimum energy conformation. In contrast, the presence in **3a** and $\mathbf{3aH}^+$ on the phenyl ring of *o*-OCH_3 substituent induced a free rotation hindrance and the ΔE value rose to ~ 6.1 and ~ 7.4 kcal/mol for $\varphi = 180°$ and ~ 6.0 and ~ 4.6 kcal/mol for $\varphi = 0°$, respectively. This prevents the arylpiperazinyl grouping of **3a** and $\mathbf{3aH}^+$ from adopting the conformation of the co-planar type.

These fragmental data support our assumption that the observed differences in the profile of the biological action between the isothiazolopyridine **V** and its *o*-OCH_3 analog **3a**, taken as an example, are rather effect of conformational restriction incorporated by the *o*-substitution than lipophilicity or basicity of these compounds. On the other hand, the different electronic natures of the *o*-substituents [electron donating groups (CH_3, OCH_3), electron atractor (Cl)] on aromatic ring of the side chains of isothiazolopyridines **3a**–**c** may influence the observed differences in the strength of the analgesic action of these compounds, observed in writhing and/or hot plate tests (Tables 7 and 8).

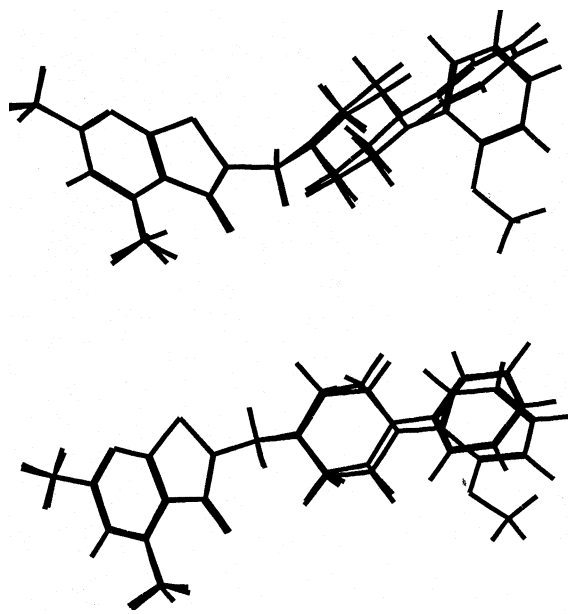
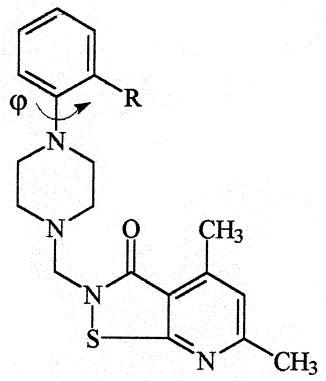


Fig. 3. Overlay low-energy conformations of compounds **V** and **3a** arranged for maximum fit at isothiazolopyridine ring system [average deviation of atoms 0.007(8) Å] obtained from AM1 computation in a water environment.



V(VH+) R = H
3a(3aH+) R = OCH_3
(N21 protonated species = **VH+**. **3aH+**)

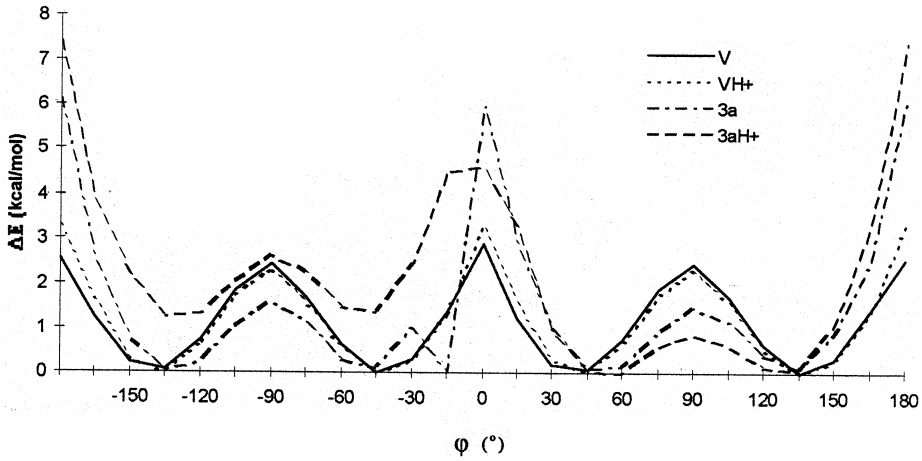


Fig. 4. The energy effect upon φ (C23–H24–C31–C32) rotation, calculated by the molecular mechanics method.

3. Pharmacological results and discussion

Twelve derivatives of isothiazolopyridine (compounds **3a**–**d**,**g**–**j**, **4a**–**c**, **5**) were subjected to the preliminary pharmacological analysis. The following pharmacological tests were performed: acute toxicity in mice, motor coordination in the rota-rod test in mice, spontaneous locomotor activity in mice, amphetamine-induced locomotor hyperactivity in mice, pain reactivity in the writhing syndrome test in mice, pain reactivity in the hot-plate test in mice, anxiolytic properties in four plates test in mice, pentetrazol-induced seizures in mice, maximal electric shock (MES) in mice, head twitches induced by 5-hydroxytryptophane (5-HTP) in mice and arterial blood pressure in rats.

3.1. Acute toxicity

The LD_{50} value of the investigated compounds after their ip administration to mice are presented in Table 5. All investigated compounds were quite toxic, with LD_{50} between 83 and 750 mg/kg.

Table 5
Acute toxicity of investigated compounds ($n = 10$)

Compound	LD_{50} (mg/kg ip)	Confidence limit
3a	154.2	[130.6–182.1]
3b	83.0	[72.1–95.5]
3c	270.3	[223.3–327.3]
3d	573.0	[342.3–952.2]
3g	107.7	[72.9–159.3]
3h	171.4	[102.7–286.1]
3I	359.0	[303.3–424.8]
3j	749.8	[578.7–971.4]
4a	219.0	[136.0–352.3]
4b	316.6	[218.8–458.1]
4c	342.4	[257.6–455.1]
5	308.1	[245.8–386.1]

Litchfield and Wilcoxon test.

Table 6
The influence of investigated compounds on the spontaneous locomotor activity in mice ($n = 10$)

Compound	Dose (part of LD_{50}) ip	Number of impulses and [% of control] ± SEM after time (min)	
		30	60
Control		4015.0 ± 480.2 [100% ± 12.0]	6929.3 ± 715.6 [100% ± 10.3]
3b	1/10	2375.0 ± 385.2 [a] [59.1 ± 9.6]	4121.5 ± 465.0 [b] [59.5 ± 6.7]
	1/20	3125.9 ± 410.3 [77.8 ± 10.2]	5375.5 ± 545.2 [77.6 ± 7.9]
3d	1/10	2539.3 ± 492.8 [a] [63.2 ± 12.3]	3693.8 ± 691.6 [b] [53.3 ± 10.0]
	1/20	3884.8 ± 555.6 [96.8 ± 13.8]	5545.3 ± 732.5 [80.0 ± 10.6]
3h	1/10	2725.0 ± 345.2 [a] [67.9 ± 8.6]	3623.6 ± 654.5 [b] [52.3 ± 9.4]
	1/20	3355.9 ± 276.9 [83.6 ± 6.9]	5196.1 ± 447.5 [75.0 ± 6.5]
3i	1/10	1854.3 ± 225.1 [c] [46.2 ± 5.6]	2248.0 ± 485.0 [c] [32.4 ± 12.2]
	1/20	2584.1 ± 458.0 [a] [64.3 ± 11.4]	3275.0 ± 647.5 [b] [47.3 ± 9.3]
	1/40	3743.5 ± 524.1 [93.2 ± 13.0]	5386.8 ± 597.3 [77.7 ± 8.6]
3j	1/10	3005.0 ± 397.5 [74.8 ± 9.9]	4231.5 ± 527.3 [b] [61.0 ± 7.6]
	1/20	3854.5 ± 476.9 [96.0 ± 11.9]	5964.2 ± 638.5 [86.0 ± 9.2]
4a	1/10	1448.0 ± 319.7 [c] [36.0 ± 8.0]	2013.2 ± 346.8 [c] [29.0 ± 5.0]
	1/20	2776.1 ± 250.7 [a] [56.7 ± 6.8]	3789.1 ± 492.8 [a] [54.7 ± 7.1]
	1/40	3761.0 ± 523.4 [93.7 ± 13.0]	5814.0 ± 286.7 [83.9 ± 4.1]
4c	1/10	2224.2 ± 488.0 [a] [55.4 ± 12.1]	3326.8 ± 921.9 [a] [48.0 ± 13.3]
	1/20	3966.3 ± 542.7 [98.8 ± 13.5]	6884.3 ± 833.5 [99.3 ± 12.0]

Compounds **3a**,**c**,**g**, **4b** and **5** were not active in this test.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$; Student's t-test.

3.2. Motor coordination

Compound **3b** affected the motor coordination at the dose of 1/10 of LD_{50} during 60 min after the administration. After this period the coordination on the rota-rod was still a little impaired, but the results were not significant. All others investigated compounds at the doses equivalent to 1/10 LD_{50} had no neurotoxic properties as they did not affect the motor coordination in the rota-rod test.

3.3. Locomotor activity

Compounds **3a**,**c**,**g**, **4b** and **5** did not affect spontaneous locomotor activity. Others compounds suppressed spontaneous locomotor activity during 1 h observation period. Compounds **3b**,**d**,**h**,**j** and **4c** acted at the dose of 1/10, **3i** and **4a** up to the dose of 1/20 of LD_{50} (Table 6).

3.4. Amphetamine-induced locomotor hyperactivity

Investigated compounds **3c**,**d**,**j**, **4a**,**c** and **5** administered at the dose equivalent to 1/10 of LD_{50} did not affect the excitatory action of amphetamine in mice whereas **3a**,**b**,**g**–**i** and **4b** suppressed amphetamine-induced hyperactivity at the dose of 1/10 of LD_{50}.

3.5. Pain reactivity

Some of investigated compounds possessed quite strong analgesic activity. Assayed in writhing syndrome

test compound **4b** and **4c** acted up to the dose of 1/80, **3b** and **4a** up to 1/40, **3d** and **3i** up to 1/20, compound **3c** at the dose of 1/10 of LD_{50} (Table 7). In hot plate test compounds **3a** and **4b** acted up to the dose of 1/20, **3b**,**i** and **4c** acted at the dose of 1/10 of LD_{50}. Compounds **3c**,**d**,**g**,**h**,**j**, **4a** and **5** were not active in hot plate test (Table 8); **3g**,**h**,**j** and **5** did not show analgesic activity in both test performed.

3.6. Anxiolytic action

None of the investigated compounds, administered at the doses which did not affect the spontaneous locomotor activity, increased the number of punished crossings in the four plates test in mice.

3.7. Pentetrazol induced seizures

Investigated compounds administered at the doses equivalent to 1/10 of LD_{50} had no anticonvulsive properties in pentetrazol-induced seizures test in mice.

Table 7
The influence of investigated compounds on the pain reactivity in writhing syndrome test in mice ($n = 10$)

Compound	Dose (part of LD_{50}) ip	Mean number of writhings ± SEM
Control		7.9 ± 0.62
3b	1/10	3.5 ± 0.74 [c]
	1/20	4.0 ± 0.55 [c]
	1/40	4.8 ± 0.81 [b]
	1/80	6.0 ± 0.96
3d	1/10	3.3 ± 0.56 [c]
	1/20	5.1 ± 0.58 [b]
	1/40	6.0 ± 0.77
4a	1/10	4.9 ± 0.6 [b]
	1/20	5.4 ± 0.58 [a]
	1/40	5.9 ± 0.6 [a]
	1/80	6.2 ± 0.53
4b	1/10	3.2 ± 0.62 [c]
	1/20	3.7 ± 0.76 [c]
	1/40	4.7 ± 0.9 [a]
	1/80	5.6 ± 0.42 [b]
	1/160	7.1 ± 0.83
4c	1/10	3.3 ± 0.61 [c]
	1/20	3.9 ± 0.72 [c]
	1/40	4.5 ± 1.1 [a]
	1/80	5.2 ± 0.71 [a]
	1/160	6.8 ± 0.83
Control		8.1 ± 0.52
3c	1/10	5.8 ± 0.81 [a]
	1/20	8.4 ± 0.92
3i	1/10	4.3 ± 0.6 [c]
	1/20	5.6 ± 0.7 [a]
	1/40	7.0 ± 0.7

Compounds **3a**,**g**,**h**,**j** and **5** were not active in this test.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$; Student's t-test.

Table 8
The influence of investigated compounds on the pain reactivity in hot plate test in mice ($n = 10$)

Compound	Dose (part of LD_{50}) ip	Time of reaction on pain stimulus in seconds ± SEM
Control		3.91 ± 0.3
3a	1/10	6.4 ± 0.3 [c]
	1/20	4.9 ± 0.3 [a]
	1/40	3.4 ± 0.3
Control		4.8 ± 0.59
3b	1/10	7.9 ± 0.65 [a]
	1/20	4.2 ± 0.46
3i	1/10	7.4 ± 0.61 [b]
	1/20	5.1 ± 0.73
4b	1/10	8.5 ± 0.89 [c]
	1/20	6.5 ± 0.43 [a]
	1/40	4.7 ± 0.42
4c	1/10	7.2 ± 0.64 [a]
	1/20	4.5 ± 0.34

Compounds **3c**,**d**,**g**,**h**,**j**, **4a** and **5** were not active in this test.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$; Student's t-test.

3.8. Maximal electric shock

Investigated compounds administered at the dose of 1/10 of LD_{50} showed lack of protection against tonic seizures in maximal electric shock in mice.

3.9. Head twitches

Investigated compounds administered at a dose of 1/10 of LD_{50} did not change the number of head twitches episodes induced by 5-HTP in mice.

3.10. Arterial blood pressure

Investigated compounds administered at a dose of 1/10 of LD_{50} did not affect the arterial blood pressure and pulse rate in rats.

As it was described recently, the parent compound **V** demonstrated anorectic and potential antidepressive activity as a result of stimulation of the serotoninergic system [5]. A previous study also demonstrated that replacement in **V** of the phenyl ring of the side chain by a heterocycle (pyrimidine) or introduction to the phenyl ring of *m*-chlorine atom leads to a lack of pharmacological activity [6]. As a continuation of our efforts on optimization of the structure of **V**, we prepared a series of its new piperazine-(**3**), piperidine-(**4**) and tetrahydroisoquinoline-(**5**) analogues (Table 1). The new com-

pounds were evaluated for toxicity and in commonly used CNS tests in animal models. The results of these investigations are shown in Tables 5–8.

The toxicity of the compounds ranged from 83 (**3b**) to 750 mg/kg (**3j**) (Table 5). The negative response in the head-twitch test clearly indicated that every attempt to replace the 1-phenylpiperazine fragment of the serotoninergic agent **V** by 4-substituted piperidine (**4a**–**c**), 1,2,3,4-tetrahydroisoquinoline (**5**), 4-benzylpiperazine (**3g**) or its more complex analogs **3h**,**i** led, without exception, to a lack of serotoninergic action. A similar effect was produced by introducing on the phenyl ring of the 1-phenylpiperazinyl fragment of **V** substituents (*o*-OCH_3, *o*-Cl, *o*-Me, *m*-CF_3; **3a**–**d**) or the lengthening of the central methylene chain (**3j**).

Instead, the majority of the new isothiazolopyridines **3**–**5** assessed in the CNS in vivo studies exhibited analgesic action as a predominant profile of their activity as was evidenced in writhing and/or hot plate tests. It should be noted that the parent compound **V** was inactive in the writhig test, whereas it was not investigated in the hot plate test [5].

The strength of analgesic activity of the new isothiazolopyridines **3**–**5** depends on the structure of the side chain. In the *writhing test* (Table 7) the best results were obtained especially with 4-substituted piperidine derivatives (**4**) [1/80–1/40 LD_{50} (3.9–5.5 mg/kg)] and analogues of **V** substituted at the terminal aromatic ring (**3a**–**d**). However, in compounds **3a**–**d** the analgesic activity was strongly influenced by the type and position of the substituent. Compound *o*-Cl substituted **3b** displayed more analgesic activity [1/40 LD_{50} (2.1 mg/kg)] than those which bore *o*-CH_3 (**3c**) or *m*-CF_3 (**3d**) substituent (1/10 and 1/20 LD_{50}, respectively). Introducing to the aromatic ring other substituents, namely, *m*-Cl [6], or *o*-OCH_3 (**3a**) results in these compounds becoming inactive in the writhing test. On the other hand, it can be seen from Table 5, that the good analgesic action of the *o*-Cl derivative **3b** was accompanied by high toxicity (83 mg/kg), whereas the presence of *m*-CF_3 (**3d**) substituent seems to reduce the toxicity (573 mg/kg).

An unfavorable effect on analgesic potency in the writhing test also had replacement at the side chain of **V** of the terminal aromatic ring with benzyl residue (**3g**) or its more complex analogs (**3h**,**i**). These modifications led to compounds less active (**3i**; 1/20 LD_{50}) or ineffective (**3g**,**h**) in this test (Table 7). Attempts to replace the 1-phenylpiperazine fragment of **V** by the 1,2,3,4-tetrahydroisoquinoline (**5**) or lengthening of the central methylene chain to the ethylene bridge (**3j**) gave isothiazolopyridines practically ineffective in the every test performed.

The analgesic action of the most active compounds in the writhing test (**3b**, **4b**,**c**) was also evidenced at the *hot plate test* (1/10–1/20 LD_{50}; Table 8). An exception was the *o*-OCH_3 derivative **3a**, ineffective in the writhing test, which exhibited activity at a dose of 1/20 LD_{50} in the hot plate test. These conflicting findings may suggests that compound **3a** influences a different 'kind' of pain reaction than the other isothiazolopyridines investigated.

The data reported above suggest that the pharmacophorical portion of analgesic isothiazolopyridines **3** and **4** may be represented by the 1-benzyl(1-substituted-1-phenyl)piperidine or 1-(substituted-phenyl)piperazine fragments separated from the isothiazole ring nitrogen atom by a single methylene group.

3.11. Conclusion

The pharmacological results of the evaluation of isothiazolopyridines **3**–**5** of Mannich base type were surprisingly different from expected and showed that all the accomplished structural variations of the side chain of the sertoninergic agent **V** were not tolerated. Instead, the new analogs of **V**, in general (eight of 12 compounds assessed), were active as analgesic agents, as it was evidenced in writhing and/or hot plate tests. Among them, especially the 1-phenyl(benzyl)piperidine derivatives **4** and 1-phenylpiperazine derivatives with Cl–(**3b**) or MeO–(**3a**) substituents located in the *ortho*-position of the terminal aromatic part of the side chain exhibited analgesic action. According to the X-ray data and the results predicted by the AM1 method in a water environment for **V** and **3a**, we may suggest that a quite different profiles of pharmacological action of **V** and its *o*-substitued (OCH_3, Cl, Me) analogs **3a**–**c** should result from conformational restriction incorporated by the *o*-substitution rather than effects of different lipophlicity or basicity of these compounds.

4. Chemical experimental section

Melting points are uncorrected. ^1H NMR spectra were obtained with Tesla spectrometer [80 MHz, $CDCl_3$, δ (ppm)]. IR (KBr) spectra were recorded on Specord M 80 (Carl Zeiss Jena). Elemental C, H, N analyses were run on a Carlo Erba NA-1500 analyzer. All the results of the C, H, and N determinations were within $\pm 0.4\%$ of the values calculated for the corresponding formulae. Chromatographic separations were performed on a silica gel column [Kieselgel 60 (70–230 mesh), Merck]. Analytical thin-layer chromatography (TLC; R_f) was carried on Merck silica gel—$60F_{254}$ (alufolien) and visualized by UV. The calculations of the log P_{calc} values (Table 1) were made for the free bases, using the CHEMPLUS program from Hypercube, Inc., IBM PC version, implemented in HYPERCHEM program package.

4.1. General procedure for preparation of derivatives **3d**,**g**–**i**, **4b**,**c**, **5**

To a stirred mixture of 2.1 g (0.01 mol) of 2-hydroxymethylisothiazolopyridine (**2**) [5] in 40 ml of ethanol 0.01 mol of an appropriate amine was added [1-(*m*-trifluoromethylphenyl)piperazine—for obtaining **3d**, 1-benzylpiperazine—**3g**, 1-piperonylpiperazine—**3h**, 1-(4-chlorobenzhydryl)piperazine—**3i**, 4-hydroxy-4-phenylpiperidine—**4b**, 4-benzylpiperidine—**4c**, 1,2,3,4-tetrahydroisoquinoline—**5**] and the stirring was continued for 12 h. Afterwards, the solvent was distilled off and the residue was crystallized with charcoal from the appropriate solvent (**3g**–*n*-heptane; **3i**, **4c**–*n*-hexane; **3d**, **3h**, **4b**–ethanol) to give pure product (yield 50–70%). The crude product **5** was purified by column chromatography (SiO_2, ethyl acetate, $R_f = 0.78$) and crystallization from *n*-hexane (yield 40%).

4b: ^{1}H NMR: 1.72–2.37 m[5H, 3′ and 5′-CH_2 piperidine + OH (D_2O exchangeable)], 2.58 s(3H, CH_3), 2.71 s(3H, CH_3), 3.05 m(4H, 2′- and 6′-CH_2 piperidine), 4.7 s(2H, CH_2), 6.92 s(1H, 5-H), 7.25–7.62 m(5H, ArH).

5: ^{1}H NMR: 2.57 s(3H, CH_3), 2.75 s(3H, CH_3), 2.95 m(4H, NCH_2CH_2Ar), 3.9 s(2H, NCH_2Ar), 4.83 s(2H,CH_2), 6.9 s(1H, 5-H), 7.08 s(4H, ArH).

4.2. General procedure for preparation of derivatives **3c**,**e**,**f**, **4a**

A mixture of 0.01 mol of hydrochloride of corresponding amine [1-(*o*-methylphenyl)piperazine for obtaining **3c**, 1-(*o*,*m*-dimethylphenyl)piperazine—**3e**, 1-(2-ethoxyphenyl)piperazine—**3f**, 4-acetyl-4-phenylpiperidine—**4a**] in 50 ml of alcohol solution of sodium ethoxide (prepared from 0.23 g of sodium) was stirred at room temperature for 1 h. Then 2.1 g (0.01 mol) of 2-hydroxyethylisothiazolopyridine (**2**) was added and the stirring was continued for 12 h. Afterwards the solvent was distilled off and the residue was crystallized from ethanol to give pure product (yield 50–65%).

3c: ^{1}H NMR: 2.26 s(3H, Ar–CH_3), 2.59 s(3H, CH_3), 2.74 s(3H, CH_3), 2.91 s(8H, 4 × CH_2), 4.73 s(2H, CH_2), 6.77–7.71 m(5H, 4ArH + 5-H).

3e: ^{1}H NMR: 2.18 s(3H, Ar–CH_3), 2.24 s(3H, Ar–CH_3), 2.6 s(3H, CH_3), 2.75 s(3H, CH_3), 2.9 s(8H, 4 × CH_2), 4.73 s(2H, CH_2), 6.82–7.05 m(4H, 3ArH + 5-H).

3f: ^{1}H NMR: 1.43 tr(3H, CH_3–CH_2, $J = 6.8$ Hz), 2.6 s(3H, CH_3), 2.75 s(3H, CH_3), 2.95 m(4H, 2 × CH_2), 3.12 m (4H, 2 × CH_2), 4.04 q(2H, CH_2O, $J = 6.8$ Hz), 4.72 s(2H, CH_2), 6.91 s(5H, 4ArH + 5-H).

4.3. X-ray analysis of 2H-4,6-dimethyl-2-{[4-(2-methoxyphenyl)piperazin-1-yl]methyl}-3-oxo-2,3-dihydroisothiazolo[5,4-b]pyridine (**3a**)

Crystals of **3a** suitable for X-ray diffraction analysis were grown by slow evaporation of a hexane solution. The structure was solved by direct methods using SHELXS-86 [14] and refined by full-matrix least-squares with SHELXL-93 [15]. All H atoms were located from different Fourier maps and refined with isotropic displacement parameters. The molecular graphics were

Table 9

Crystal data	
Empirical formula	$C_{20}H_{24}N_4O_2S$
Formula weight	384.49
Temperature T (K)	293(2)
Wavelength λ (Å)	1.54178
Crystal system	monoclinic
Space group	$P2_1/c$
Unit cell parameters	
a (Å)	17.227(1)
b (Å)	12.047(1)
c (Å)	9.891(1)
β (°)	103.90(1)
V (Å^3)	1992.6(3)
Molecular multiplicity Z	4
D_{calc} (mg/m^3)	1.282
Radiation	CuKα
Cell parameters from	15 reflections
θ range for lattice parameters (°)	40.97–46.65
Absorption coefficient μ (/mm)	1.623
Crystal color and shape	colorless prisms
Crystal size (mm)	0.63 × 0.28 × 0.14
Data collection	
Diffractometer type	Nonius MACH3 four-circle diffractometer
Collection method	ω–2θ scans
Absorption correction	ψ scan [18] $T_{min} = 0.803$, $T_{max} = 0.998$
θ range for data collection (°)	2.64–74.07
Index ranges	$-20 \le h \le 21$, $0 \le k \le 14$, $-12 \le l \le 0$
Three standard reflections monitored every 100 reflections	
Intensity decay (%)	0.0
No. of measured reflections	3520
No. of independent reflections	3345 [$R_{int} = 0.0196$]
No. of observed reflections [$I > 2\sigma(I)$]	3344
Refinement	
Refinement method	full-matrix least-squares on F^2
Data/parameters	3345/341
Final R indices	$R = 0.0533$, $wR(F^2) = 0.1282$
Weight scheme	$w = 1/[\sigma^2(F_o^2) + (0.0303P)^2 + 1.43P]$ where $P = (F_o^2 + 2F_c^2)/3$
Goodness-of-fit on F^2	$S = 1.321$
Extinction coefficient	0.0032(2), extinction correction: SHELXL
Largest difference peak and hole (e/Å^3)	0.236 and −0.185
$(\Delta/\sigma)_{max}$	0.000

prepared using XP [16] and PARST [17] was used for geometrical calculations. Crystal and experimental data are listed in Table 9.

4.3.1. Theoretical calculations

The molecular modeling studies were undertaken to investigate conformational preferences of isothiazolopyridine derivatives **V** and **3a**. The energy minimization and geometry optimization were performed using MNDO-AM1 approximation [19] implemented in the program package HYPERCHEM rel. 4.5 [20]. The crystallographic data of compounds **V** (the structure inverted at $-x, -y, -z$ in relation to that in [12]) and **3a** were used as staring models to calculate and compare the most energetically stable conformations for isolated molecules, N21 protonated molecules and molecules in water environment [21]. The determination of the energy effects of free-rotation between the aromatic and piperazine rings was performed using molecular mechanics methods: MMX force field [22], PCMODEL-386 package [23].

5. Pharmacological experimental section

The experiments were carried out on male and female Albino–Swiss mice (body weight of 20–25 g) and male Wistar rats (200–250 g). Investigated compounds were administered intraperitoneally (ip) as a suspension in 3% Tween 80 in the constant volume of 10 ml/kg in mice and 5 ml/kg in rats. The compounds were administered in a dose equivalent to 1/10, 1/20, 1/40, 1/80 or 1/160 of LD_{50}. Control animals received the equivalent volume of solvent. Each experimental group consisted of 8–10 animals.

5.1. Acute toxicity

Acute toxicity was assessed by the methods of Litchfield and Wilcoxon [24] and presented as LD_{50} and confidence limit calculated from the mortality of mice after 24 h.

5.2. Motor coordination

Motor coordination was measured according to the method of Gross and Tripod [25]. Mice were placed for 2 min on the rod rotating with the speed of 4 rpm. The effects were evaluated 15, 30, 45, 60, 75, 90 and 105 min after the administration of the investigated compounds.

5.3. Spontaneous locomotor activity

Spontaneous locomotor activity in mice was measured by the use of DIGISCAN Optical Animal Activity Monitoring System (Omnitech Electronics, Inc. Columbus, Ohio, USA). Thirty minutes after injection of the investigated compounds mice were placed separately in the plexiglass cages ($20 \times 20 \times 30$ cm) for 1 h. The apparatus monitors animal locomotor activity via a grid of invisible infrared light beams which in an equal number traverse the animal cage from front to back and left to right. Each crossing of the light beam was recorded automatically and subjects to rapid analysis by the Digiscan Analyzer using computer program OMNI-PRO, version 2.40. Horizontal activity (the total number of beam interruptions that occurred in the horizontal sensor during observation time) was evaluated after 30 and 60 min.

5.4. Amphetamine hyperactivity

Amphetamine hyperactivity in mice was induced by D,L-amphetamine 2.5 mg/kg s.c. Investigated compounds were injected 30 min before amphetamine. The locomotor hyperactivity was measured 30 and 60 min later in the DIGISCAN Optical Animal Activity Monitoring System (Omnitech Electronics, Inc. Columbus, Ohio, USA).

5.5. Pain reactivity

Pain reactivity was measured by the writhing syndrome test of Koster et al. [26]. The test was performed in mice by the ip injection of a 0.6% solution of acetic acid in a volume of 10 ml/kg 60 min after the administration of investigated compounds. The number of writhing episodes were counted for 30 min after the injection of 0.6% acetic acid.

Pain reactivity was also measured in the hot plate test according to the method of Eddy and Leimbach [27]. Animals were placed individually on the metal plate heated to 56 °C. The time(s) of appearance of the pain reaction (licking of the forepaws or jumping) was measured. Experiments were performed 60 min after the administration of investigated compounds.

5.6. Anxiolytic properties

Anxiolytic properties were assessed by the four plate test in mice, according to Aron et al. [28], 60 min after the administration of investigated compounds at the doses which had no effect on the spontaneous locomotor activity. Mice were placed in the cages with four plates floor (11×7 cm) with 4 mm gape between each. After 15 s of adaptation the number of crossing was counted during 1 min. Each crossing was punished with direct current (180 V, 0.5 A) but not more often than every 3 s.

5.7. Pentetrazol seizures

Pentetrazol seizures in mice were induced by the pentetrazol administration at the dose of 100 mg/kg s.c. 60 min after the investigated compounds. Animals were observed during 30 min and the number of mice devel-

oping clonic and tonic seizures as well as mortality was recorded in that period.

5.8. Maximal electric shock

Maximal electric shock was induced by means of alternating current (50 Hz, 25 mA, 0.2 s) with the use of ear clip electrodes according to the method of Swinyard et al. [29]. The criterion of the convulsive response was the tonic extension of the hind limbs. The test was performed 60 min after the administration of the investigated compounds.

5.9. Head twitches

Head twitches behavior was induced by the administration of 5-hydroxytryptophan (5-HTP) at the dose 180 mg/kg ip 30 min after the investigated compounds. Animals were observed 60 min after 5-HTP administration.

5.10. Arterial blood pressure

Arterial blood pressure was determined according to the method of Gerold and Tschirky [30] using the UGO-BASILE equipment (Blood Pressure Recorder, cat. No 8006). Systolic blood pressure on the tail artery was measured 30 min after the administration of investigated compounds.

5.11. Statistics

Results obtained were presented as means and evaluated statistically using Student's t-test or exact Fischer's test. $P < 0.05$ was the criterion of the significance.

Acknowledgements

The authors wish to express their thanks to Dr Z. Lipkowska from the Institute of Organic Chemistry, Polish Academy of Sciences in Warszawa for carrying out the single-crystal measurements and Professor J. Karolak-Wojciechowska from Institute of General and Ecological Chemistry, Technical University in Lodz for her kind consent to use the PCMODEL-386 package for molecular mechanics calculations and the XP program for creating drafts.

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