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Preliminary communication

Synthesis, brain antihypoxic activity and cell neuroprotection of 1-substituted-3,7-dimethylxanthines

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Abstract – Five newly synthesised original compounds were investigated for acute toxicity, influence on hexobarbital sleeping time, effect on the locomotor activity, and brain antihypoxic activity. Two of the compounds were tested in a model of glutamate induced neurotoxicity in the brain cell culture using a cell viability test. Our studies indicate that compounds **2a–c** and **4** prolonged the survival time of mice in the model of anoxic hypoxia. Only compound **3** expressed antihypoxic activity in the model of circulatory hypoxia, evaluated with a statistical significant increase of the survival time. Compound **4** (1-[3-(2,3-dihydro-3-oxobenzisosulfonazol-2-yl)-propyl]-3,7-dimethylxanthine) in concentration range 0.3–3 μM statistically significantly antagonised the glutamate induced neurotoxicity. Compound **4** is important for further investigations on in vivo models of brain dementia. © 2000 Éditions scientifiques et médicales Elsevier SAS

3,7-dimethylxanthines / acute toxicity / brain antihypoxic activity / glutamate induced neurotoxicity

1. Introduction

Xanthine derivatives possess different pharmacological activity. Some of the asymmetrically substituted xanthines are useful for treating CNS diseases such as Alzheimer's type dementia [1]. For example, a new xanthine derivative, Propentophylline, was recently introduced as a drug with neuroprotective properties for treatment of brain dementia [2], boosting the search for new xantine derivatives with neuroprotective actions.

We report here the preparation of five newly synthesised 3,7-dimethylxanthine derivatives, and describe their pharmacological and toxicological properties: acute toxicity, effect on the locomotor activity, influence on hexobarbital sleeping time and brain antihypoxic activity. Since glutamate is the most important neurotoxic amino acid in the CNS [3, 4], we have also studied the effects of two 3,7-dimethylxanthine derivatives on the glutamate induced neurotoxicity in brain cell cultures.

2. Results and discussion

2.1. Chemistry

The 1-substituted 3,7-dimethylxanthine derivatives (**2a–c, 3** and **4**) indicated in *figure 1* were prepared by reaction between 1-(ω-haloalkyl)-3,7-dimethylxanthines (**1a–c**) and previously prepared sodium salts of imidazole derivatives and sodium 2,3-dihydro-3-oxobenzisosulfonazole. The 1-(ω-haloalkyl)-3,7-dimethylxanthines used were obtained by known methods [5, 6]. The progress of the reaction was followed on TLC.

When 1-(3-chloropropyl)-3,7-dimethylxanthine **1a** was used as starting material to obtain **2a,c**, **3** and **4** their yield was found to be remarkably lower and reaction time about two times longer. This was probably due to low reactivity of this compound in comparison with **1b**. We also

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found that if there is no protection from the atmospheric moisture whilst obtaining **2a,c, 3** and **4** from both of **1a** and **1b**, the hydrolysis took place and the yield of the new compounds decreased. The hydrolysis product is 1-(3-hydroxypropyl)-3,7-dimethylxanthine [5].

The second approach to obtain the title compounds was illustrated with the synthesis of **2b** as outlined in *figure 2*. Starting from 1-(2-bromoethyl)-2-methyl-5-nitroimidazole (**6**) and 3,7-dimethylxanthine (**5**) via its sodium salt (**5a**), **2b** was prepared in a fairly good yield without the presence of contaminating by-products.

Compounds **2a–c** and **4** are partly soluble in water and alcohols, but soluble in dimethylformamide and dimethylsulfoxide. Compound **3** is soluble in water, dimethylformamide, dimethylsulfoxide and sparingly in alcohols.

The new compounds were characterised by microanalyses, FTIR and NMR spectrometry and results were consistent with the assigned structures.

^{1}H- and ^{13}C-NMR spectra of **3** are of basic interest. The proton signals between 5 and 7 ppm are characteristic for substituted urea, due to to *N*-(2,5-dioxo-4-imidazolidinyl)-ureido side chain. Thus, the signal at 5.82 ppm as singlet corresponds to amino group protons. Each of the signals at 5.27 and 6.91 ppm, as doublets, correspond to one proton – first of allantoine ring and second from the ureido side chain. The signal corresponding to NH-proton at position 3 of allantoine ring, as singlet, appears at 8.33 ppm. In unsubstituted *N*-(2,5-dioxo-4-imidazolidinyl)-urea there is one more signal at about 10 ppm, which corresponds to NH-proton at position 1 [7]. In the spectrum of **3** this signal is not present. The rest of the signals correspond to the protons of the substituted xanthine ring and propyl side chain. The values of the chemical shifts of the carbon atoms registered by ^{13}C-NMR spectra were compared with simulated values [8]. These data confirm the struc-

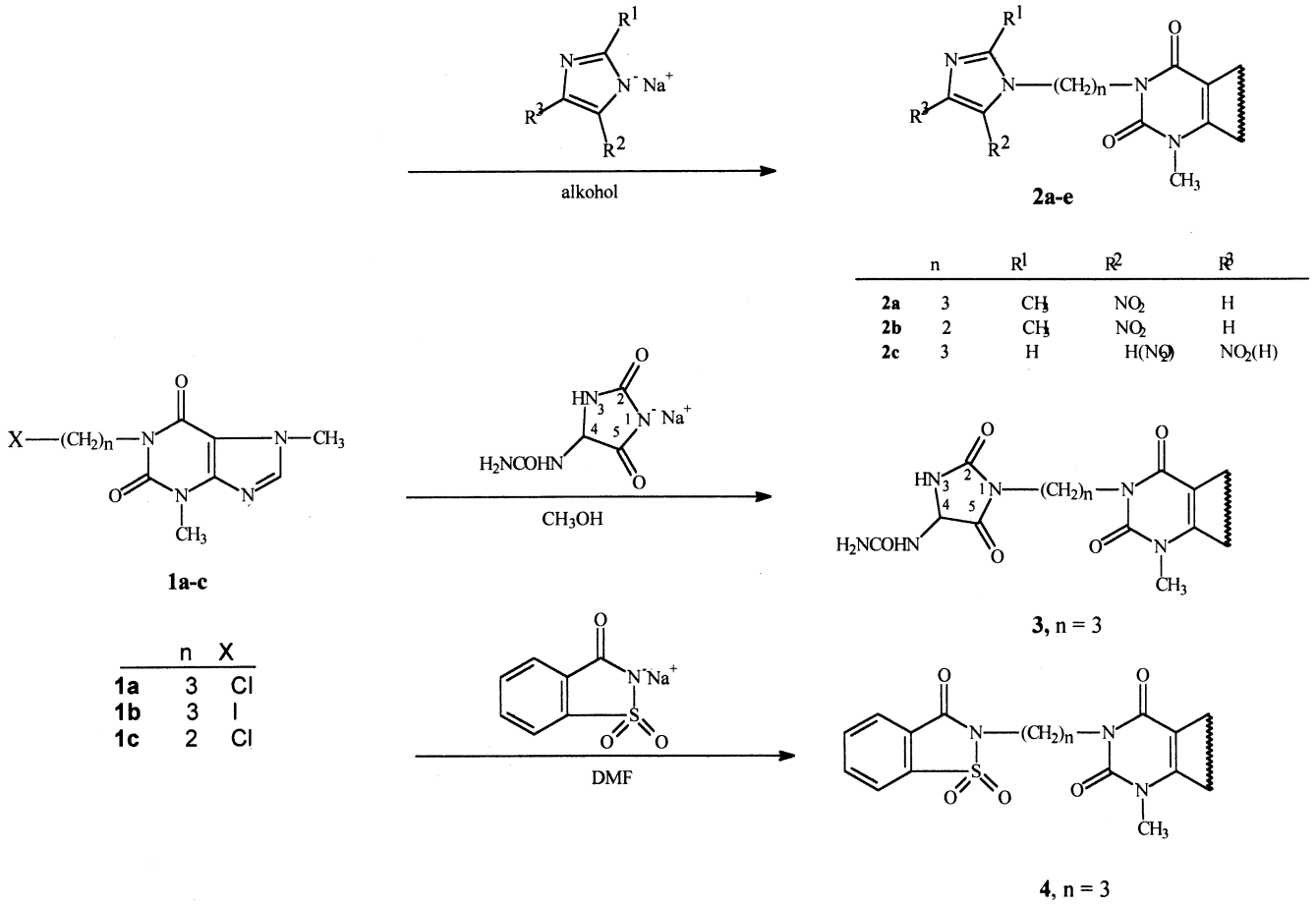

Figure 1. Reaction pathway for the synthesis of 1-substituted-3,7-dimethylxanthines (**2a–c, 3** and **4**).

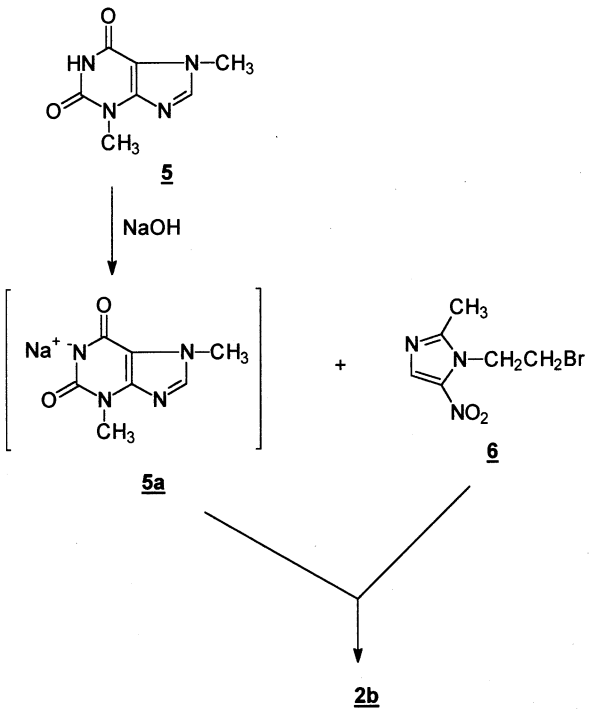

Figure 2. Second synthetic approach for obtaining compound **2b**.

ture of **3** and prove that 2,5-dioxo-4-ureidoimidazolidine was bound to the propyl side chain at position 1, and not at position 3.

2.2. Pharmacology

Newly synthesised compounds **2a–c**, **3**, **4** were investigated for acute toxicity, influence on Hexobarbital sleeping time and locomotor activity of mice. Assessment of the compounds' effect in three different models of provoked brain hypoxia is also reported. Two compounds were studied for the antagonism of the glutamate induced neurotoxicity.

Analysis of the obtained experimental data on the acute toxicity (LD_{50}) of **2a–c**, **3**, **4**, as compared to 3,7-dimethylxanthine, showed that these compounds caused an acute toxicity (LD_{50}) which was significantly ($P \leq 0.05$) lower than when 3,7-dimethylxanthine was used (*table I*).

Compounds **2a** and **2c** ($LD_{50} > 5000$ mg/kg b.w.) were practically nontoxic. This effect is probably due to the influence of the substituent in the side chain of the structures.

All tested compounds, when administered in a dose 1/10 and 1/20 ratio of $LD_{50,}$ significantly ($P \leq 0.05$) increased the hexobarbital sleeping time in comparison with 3,7-dimethylxanthine, which at similar doses does not affect the hexobarbital sleeping time (*table II*).

The locomotor activity investigations show that all tested compounds, except **2b,** decreased the spontaneous locomotor activity in comparison with the control group at doses $^{1}/_{10}$ and $^{1}/_{20}$ of LD_{50} (*figures 3* and *4*).

Accordingly with data from Ishii et al. [9] medial temporal oxygen metabolism was markedly affected in patients with mild-to-moderate Alzheimer's disease. In this respect, we wanted to analyse the effect of the xantine derivatives in different models of provoked brain hypoxia. The results showed that compounds **2a–c** and **4** prolonged the survival time of mice in the model of anoxic hypoxia, whereas only compound **3** had an antihypoxic activity in the model of circulatory hypoxia (*table III*).

Table I. Acute toxicity (LD_{50}) of compounds **2a–c**, **3**, **4** and theobromine in mice after intraperitoneal administration.

Compound	LD_{50} (mg/kg) b.w.	Range of values
2a	>5000	–
2b	740.08	588.66/930.44
2c	5000	–
3	>2000	–
4	>3000	–
Theobromine	552.4	472.7/645.6

Table II. Influence of compounds **2a–c**, **3**, **4** and theobromine on hexobarbital sleeping time of mice.

Compound	Doses (mg/kg) b.w., i.p.	Sleeping time (X/SD) min
Control (HB)	80	17.6/4.0
2a	250	25.0/16.5[a]
2a	500	41.5/12.0[a]
2b	37	20.0/9.9
2b	74	24.1/8.3[b]
2c	250	57.9/4.4[a]
2c	500	58.3/2.0[a]
3	100	24.4/2.2[a]
4	150	21.8/1.7[a]
4	300	26.5/2.5[a]
Theobromine	55	14.7/6.16

[a] $P \leq 0.05$ statistically significance in comparison with control group.
[b] $P \leq 0.1$ statistically significance in comparison with control group.

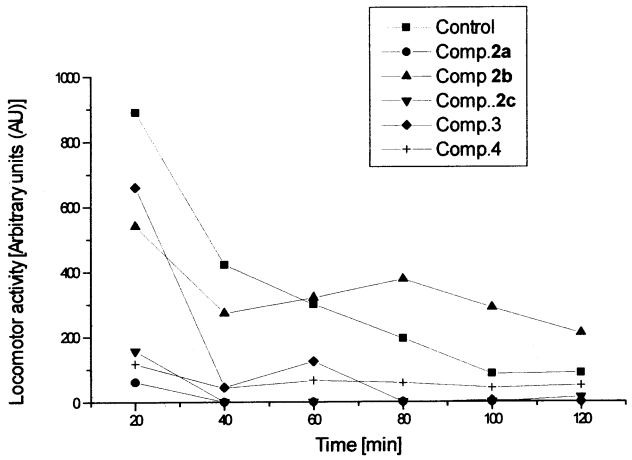

Figure 3. Effects of compounds **2a–c**, **3**, **4** and theobromine (**Theobr**.) given i.p. in $^{1}/_{10}$ ratio of LD_{50} on locomotor activity of mice at 20 min intervals up to 120 min with $P \leq 0.05$ (indicated by asterisk) in comparison with control group.

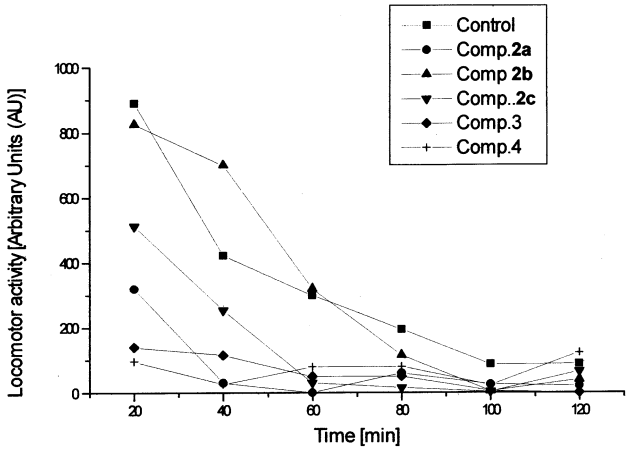

Figure 4. Effects of compounds **2a–c**, **3**, **4** and theobromine (**Theobr**.) given i.p. in $^{1}/_{20}$ ratio of LD_{50} on locomotor activity of mice at 20 min intervals up to 120 min with $P \leq 0.05$ (indicated by asterisk) in comparison with control group.

Several data in the literature suggest that excitotoxicity plays an important role in the Alzheimer's disease. For example, Jansen et al. [10] observed reduction of about 40–50 % of the NMDA receptor binding sites (including glutamate) in different brain regions, assessed post-mortem in patients with Alzheimer's disease and Malcolm et al. [3] have showed that transient exposure to 100 μM glutamate results in neurotoxicity in primary cultures of mouse cerebellar granule cells. In this respect we have evaluated the effect of compounds **2c** and **4** on the brain hypocampal neuronal toxicity induced by glutamate (see *figure 5*). The results indicated that compound **4** significantly antagonises the glutamate induced toxicity in concentration range 0.3–3 μM.

The fact that compound **4** antagonises the glutamate induced toxicity in neuronal cultures together with its lower toxicity suggest that compound **4** could be a good candidate to be used in the treatment of neurodegenerative diseases. Obviously, further and extensive research should be done using in vivo models of brain dementia.

3. Experimental protocols

3.1. Chemistry

Melting points were measured in °C and corrected (Büchi 535). FTIR spectra were recorded on a Shimadzu FTIR 8101M. ^{1}H- and ^{13}C-NMR spectra were recorded at ambient temperature on a Bruker-250 WM (250 MHz) spectrometer. Chemical shifts are given in parts per million (δ-ppm) downfield from tetramethylsilane as internal standard. Splitting patterns are designed as follows: s, singlet; d, doublet; t, triplet; and m, multiplet. The following abbreviations are used: Im – imidazole ring, Xanth – xanthine ring, Allan – alantoine ring, Ur – ureido side chain and Ar – aromatic protons. Preparative thin-layer chromatography was performed on DC-Alufolien Kieselgel 60 F_{254} (Merck) (0.20 mm) sheets with solvents (vol. parts): system I: 25 % ammonium hydroxide–chloroform–acetone–ethanol (1:3:3:4), system II: (chloroform–ethanol (1:1), detected at UV 254 nm. Elemental analyses were performed by the microanalytical laboratory of Faculty of Pharmacy (Medical University-Sofia). Microanalytical results were within $\pm$ 0.4 % of the theoretical values. All solutions were dried over anhydrous sodium sulfate and evaporated on a Büchi rotary evaporator at reduced pressure. Non-commercially available intermediates required for the synthesis of novel derivatives of 3,7-dimethylxanthine were obtained as referenced: 1-(3-chloropropyl)-3,7-dimethylxanthine (**1a**) [5]; 1-(3-iodopropyl)-3,7-dimethylxanthine (**1b**) [5]; 1-(2-chloroethyl)-3,7-dimethylxanthine (**1c**) [6]; 1-(2-bromoethyl)-2-methyl-5-nitroimidazole (**6**) [11].

The given yields are those of analytically pure product. No efforts were made to optimise the yields.

Table III. Brain antihypoxic activity of compounds **2a–c**, **3**, **4** and theobromine on mice.

Compound	Doses (mg/kg), b.w., i.p.	Antihypoxic activity		
		Anoxic hypoxia (X ± SD) min	Haemic hypoxia (X ± SD) min	Circulatory hypoxia (X ± SD) min
Control	–	14.6 ± 0.8	14.6 ± 2.1	19.8 ± 5.3
2a	250	21.1 ± 6.4[a]	12.2 ± 1.5[a]	16.8 ± 4.8
2a	500	24.4 ± 6.6[a]	9.5 ± 1.3[c]	15.3 ± 8.1
2b	37	15.9 ± 2.9	7.1 ± 0.6[c]	16.6 ± 4.0
2b	74	18.3 ± 2.2[a]	10.0 ± 1.8[c]	18.2 ± 5.6
2c	250	18.4 ± 2.2[b]	9.5 ± 1.6[c]	16.1 ± 3.9
2c	500	21.1 ± 3.0[a]	7.9 ± 1.9[c]	16.9 ± 6.8
3	100			19.2 ± 4.9
3	200	14.4	13.3	40.4 ± 2.5[a]
4	150	25.4[a]		
4	300	30.2[a]	11.4	19.9 ± 1.3
Theobromine	55	17.5 ± 1.8	13.2 ± 2.6	39.9 ± 19.4[a]

[a] $P \leq 0.05$ statistically significance in comparison with control group.
[b] $P \leq 0.1$ statistically significance in comparison with control group.
[c] $P \leq 0.001$ statistically significance in comparison with control group.

*3.1.1. 1-(3-Chloropropyl)-3,7-dimethyl-2,3,6,7-tetrahydro-1H-2,6-purinedione (**1a**)*

Sodium methoxide 30% in absolute methanol (2.7 g, 0.05 mol) was added to a 3,7-dimethylxanthine (9 g, 0.05 mol) dissolved in 250 mL absolute methanol. The reaction mixture was stirred under reflux for 1 h and 1-bromo-3-chloropropane (47.2 g, 0.3 mol) was added. The reaction mixture was stirred under reflux for 17 h. The pH of the solution was maintained at 9 by addition of sodium methoxide. The progress of the reaction was followed on TLC (II). After removing the solvent, chloroform was added and separated sodium bromide was filtered. Chloroform was removed under reduced pressure and the crude product was recrystallised from absolute methanol. Yield: 78 %. M.p.: 142–144 [5].

*3.1.2. 1-(3-Iodopropyl)-3,7-dimethyl-2,3,6,7-tetrahydro-1H-2,6-purinedione (**1b**)*

Sodium iodide (7.5 g, 0.05 mol) was added to a solution of **1a** (10.3 g, 0.04 mol) in 250 mL anhydrous acetone. The reaction mixture was stirred under reflux for 56 h. The progress of the reaction was followed on TLC (II). After removing the solvent, chloroform was added and separated sodium chloride was filtered. Chloroform was removed under reduced pressure and the crude product was recrystallised from absolute methanol. Yield: 77 %. M.p.: 120–122 [5].

*3.1.3. 1-(2-Chloroethyl)-3,7-dimethyl-2,3,6,7-tetrahydro-1H-2,6-purinedione (**1c**)*

Sodium methoxide 30% in absolute methanol (2.7 g, 0.05 mol) was added to a 3,7-dimethylxanthine (9 g, 0.05 mol) dissolved in 250 mL absolute methanol, and after that the solvent was removed. The sodium salt of 3,7-dimethylxanthine was dissolved in 70 mL water, and 1,2-dichloroethane (130 g, 1.5 mol) and Linopharm

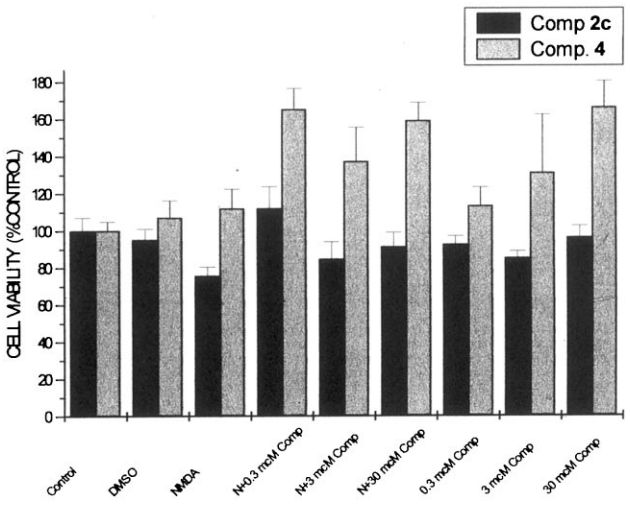

Figure 5. Effects of compounds **2c**, and **4** on glutamate induced neurotoxicity.

(0.35 g, 5 % of water amount) were added. The reaction mixture was stirred under reflux for 70 h. The progress of the reaction was followed on TLC (I). After cooling, the two layers were separated by filtration. After drying with anhydrous sodium sulfate and filtration, the organic layer was distilled. The residual solid was recrystallised from ethanol. The product was dried at 90–95 °C. Yield: 85 %. M.p.: 149–151 [6].

3.1.4. General procedure for the preparation of sodium salts of imidazole derivatives

Sodium methoxide (0.012 mol, 2.17 g 30 % methanolic solution in 10 mL absolute methanol) was added to the corresponding imidazole derivative (0.01 mol) dissolved in 30 mL absolute methanol. The mixture was heated under reflux for 1.5 h. Then this solution was used for obtaining the novel compounds. If necessary the solvent was removed and imidazole derivative sodium salt was dissolved in an appropriate solvent to prepare the novel products.

*3.1.5. General procedure for the preparation of compounds **2a–c** and **3***

To a solution of fresh prepared imidazole derivative sodium salt in appropriate anhydrous solvent was added **1b** (3.48 g, 0.01 mol) or **1c** (2.42 g, 0.01 mol) dissolved in 20 mL of the same solvent. The reaction mixture was stirred under reflux. The reaction time was established by TLC monitoring. Separated sodium halide was filtered when hot, the filtrate was kept for 12 h at 4 °C. The crude product was filtered and washed three fold with methanol to give corresponding compound. If necessary the product was recrystallised from methanol or ethanol.

*3.1.6. 3,7-dimethyl-1-[3-(2-methyl-5-nitro-1H-imidazol-1-yl)-propyl]-2,3,6,7-tetrahydro-1H-2,6-purinedione (**2a**)*

Compound **2a** was prepared from 2-methyl-5-nitroimidazole sodium salt and **1b** in anhydrous 2-ethoxyethanol as described above for 6 h. Yield: 2.6 g (75%). $R_f = 0.73$ (I). M.p.: 230–233 (ethanol). IR (cm^{-1}) Nujol: 1710 (νC=O), 1695 (νC=O), 1651, 1610, 1550 (νC=C, νC=N), 1344 (νNO_2); ^{1}H-NMR (DMSO-d_6): 2.38 (s, 3H, $-CH_3$); 2.46–2.52 (m, 2H, CH_2); 3.21 (s, 3H, CH_3); 3.37 (s, 3H, CH_3); 4.14 (t, 2H, $J = 6.5$ Hz, CH_2); 4.62 (t, 2H, $J = 7$ Hz, CH_2); 7.59 (s, 1H, C_8–H); 8.29 (s, 1H, C–H, Im). Anal. $C_{14}H_{16}N_7O_4$ (N).

*3.1.7. 3,7-dimethyl-1-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)-ethyl]-2,3,6,7-tetrahydro-1H-2,6-purinedione (**2b**)*

3.1.7.1. Method A

Compound **2b** was synthesised in the same manner in methanol from 2-methyl-5-nitroimidazole sodium salt and **1c** for 20 h in yield 1.95 g (59 %).

3.1.7.2. Method B

Sodium methoxide (0.012 mol, 1.94 g 33 % methanolic solution in 10 mL absolute methanol) was added to 3,7-dimethylxanthine (0.01 mol, 1.8 g) dissolved in 140 mL absolute methanol and the mixture was refluxed for 3 h. Then the volume of the solution was reduced to 60 mL and 1-(2-bromoethyl)-2-methyl-5-nitroimidazole (**6**) (0.01 mol, 2.34 g) dissolved in 20 mL methanol was added. The reaction mixture was heated under reflux for 15 h. The isolation of **2b** was carried out as described in General procedure. Yield: 2.25 g (68%). $R_f = 0.47$ (I). M.p.: > 300 °C. IR (cm^{-1}) Nujol: 1694 (νC=O), 1668 (νC=O); 1605, 1592, 1545, (νC=C, νC=N), 1345 (νNO_2); ^{1}H-NMR (DMSO-d_6): 3.35 (s, 3H, CH_3); 3.57 (s, 3H, CH_3); 4.11 (t, 2H, $J = 4.2$ Hz, CH_2); 4.35 (t, 2H, $J = 5.4$ Hz, CH_2); 7.95 (s, 1H, $C8_8$–H), 8.20 (s, 1H, C–H–Im). Anal. $C_{13}H_{15}N_7O_40$ (N).

*3.1.8. 3,7-dimethyl-1-[3-(4(5)-nitro-1H-imidazol-1-yl)-propyl]-2,3,6,7-tetrahydro-1H-2,6-purinedione (**2c**)*

2c was obtained as described in general method from 4(5)-nitroimidazole sodium salt and **1b** for 21 h in absolute methanol to give 2.0 g (56%). $R_f = 0.68$ (I). M.p.: 214–217, IR (cm^{-1}) Nujol: 1698 (νC=O), 1685 (νC=O); 1600, 1590, 1543, (νC=C, νC=N), 1350 (νNO_2); ^{1}H-NMR (DMSO-d_6): 2.04–2.15 (m, 2H, CH_2); 3.33 (s, 3H, CH_3); 3.41 (s, 3H, CH_3); 3.91 (t, 2H, $J = 6.2$ Hz, CH_2); 4.11 (t, 2H, $J = 7$ Hz, CH_2); 8.01 (s, 1H, C_8–H); 8.30 (s, 1H, C–H, Im); 8.43 (s, 1H, C–H, Im). Anal. $C_{13}H_{15}N_7O_4$ (N).

*3.1.9. N-[1-[3-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-1-purinyl)propyl]-2,5-dioxo-4-imidazolidinyl]-urea (**3**)*

Compound **3** was obtained as described above from *N*-(2,5-dioxo-4-imidazolidinyl)-urea (allantoine) sodium salt and **1b** for 36 h in absolute methanol. After concentration of the filtrate to minimal volume the separated solid was filtered and the combined precipitate was washed with cold methanol. Yield: 1.5 g (40%); $R_f =$ 0.22 (I). M.p.: 194–196 (methanol). IR (cm^{-1}) Nujol:

3565–3370 (νN–H), 1774–1651 (νC = O); 1617, 1553 (νC=C, νC=N). ^{1}H-NMR (DMSO-d_6): 1.79 (t, 2H, J = 6.4 Hz, CH_2); 3.33–3.42 (m, 5H, CH_3, CH_2); 3.82–3.86 (m, 5H, CH_3, CH_2); 5.27 (d, 1H, J = 8 Hz, Im); 5.82 (s, 2H, NH_2); 6.91 (d, 1H, J = 8 Hz, Im); 7.97 (s, 1H, C8–H); 8.33 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6): 172 (CO-5-Allan), 157.5 (CO-Ur), 156.4 (CO-2-Allan), 154.4 (CO-6-Xanth), 150.9 (CO-2-Xanth), 148.4 (C-4-Xanth), 143 (C-8-Xanth), 106.7 (C-5-Xanth), 61.5 (C-4-Allan), 38.4 ($-CH_2$-N-1-Xanth), 35.9 (CH_3-N-7-Xanth), 33.3 (CH_2-N-1-Allan), 29.5 (CH_3-N-3-Xanth), 26.5 (CH_2). Anal. $C_{14}H_{18}N_8O_5$ (N).

*3.1.10. 2-[3-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-1-purinyl)propyl]-2,3-dihydro-1H-1λ^6-benzo[d]-isothiazole-1,1,3-trione (**4**)*

A solution of sodium 2,3-dihydro-1H-1λ^6-benzo[d]isothiazole-1,1,3-trione (saccharin, 0.01 mol, 2.05 g) and added **1b** (3.48 g, 0.01 mol) in 40 mL DMF was heated under reflux for 25 h. After cooling to room temperature 160 mL water were added. A waxy product was separated and after 24 h it became a yellow solid. The crude product was recrystallised from abs. ethanol to give 3.2 g (80%). R_f = 0.84 (I). M.p.: 191–193; IR (cm^{-1}) Nujol: 1732 (νC=O), 1703 (νC = O), 1659, 1551 (νC=C, νC=N), 1326 (ν^{as} SO_2), 1182 (ν^{s} SO_2). ^{1}H-NMR (DMSO-d_6): 2.11 (t, 2H, J = 7 Hz, CH_2), 3.46 (s, 3H, CH_3), 3.76–3.81 (m, 5H, CH_2, CH_3), 3.86–3.96 (m, 2H, CH_2), 8.00 (s, 1H, C_8–H), 8.07–8.24 (m, 4H, Ar). Anal. $C_{17}H_{17}N_5O_5S$ (N).

3.2. Biological evaluation

The experiments were conducted on eight male white rats (8-days-old) of the Wistar line, and 410 male white mice, weighing 18/22 g.

Acute toxicity (LD_{50}) of the studied compounds was assessed by dissolving in saline (0.9% NaCl) with 1–2 drops of Tween 80, and administering to mice via intraperitoneal (i.p.) route. LD_{50} was evaluated at four or five different doses, each on the six animals and calculated by the method of Litchfield–Wilcoxon [12].

3.2.1. Influence on hexobarbital sleeping time (HBST)

The studied compounds were administered to male mice i.p. at doses $^1/_{10}$ of LD_{50}. The same volume 0.1/10 g b.w., of solvent (0.9% NaCl) was administered to the controls. The solution of Hexobarbital sodium at a dose of 80 mg/kg b.w. was administered i.p. to the animals 30 min after administration of the compounds' solutions under study. Sleeping time was measured in minutes by observing the righting reflex recovery.

3.2.2. Influence on locomotor activity

A group of six animals was put on in actometer (Activity Cage, Ugo Basile, Italy) and the locomotor activity in arbitrary units was determined at 20 min intervals for 120 min. The tested compounds at a dose $^1/_{10}$ of LD_{50} were administered to the animals and they were tested in the apparatus for 120 min under analogical conditions, and the results were compared with those in the control vehicle-treated group.

3.2.3. Antihypoxic activity

The tested compounds were given in doses $^1/_{20}$ and $^1/_{10}$ of LD_{50}. Three models of provoked brain hypoxia were used:

Anoxic hypoxia — 30 min after the i.p. administration of the tested compounds to the mice (8 per group), they were placed into hermetic bottles of 200 mL volume. The survival time (in min) was determined and compared with control vehicle treated group [13].

Haemic hypoxia — the animals (8 per group) were injected with sodium nitrite in dose 360 mg/kg b.w. i.p. 30 min after the i.p. administration of compounds. The survival time (in min) for each animal is defined as time, measured from the induction of the hypoxia (caused by the introduction of the hypoxant) until death [14].

Circulatory hypoxia was induced 30 min after the i.p. administration of compounds by sodium fluoride in dose 150 mg/kg b.w. i.p. to the mice (10 per group). The survival time (in min) for each animal is defined as time, measured from the induction of the hypoxia by the hypoxic agent until death [15].

Excitotoxicity studies. The effect on the brain neuronal toxicity was studied according the method of Malcolm [3] using the neuronal cell culture vialibility method. The studied compounds were added preliminary in the cell culture in concentrations 0.3, 3 and 30 μM and preincubated for 2 h.

All the pharmacological and biochemical results underwent statistical processing by the Student–Fischer t-test at $P \leq 0.05$.

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