Synthesis,Toxicological, Pharmacological, and Bronchodilating Activity *in vitro* of some Xanthineacetic Acid Derivatives

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Summary

The possibility of the preparation of some ester derivatives of dimethylxanthines from 1-theobromine- and 7-theophylline acetic acids and 7-(2-hydroxyethyl)-theophylline by DCC/DMAP-mediated esterification under mild conditions was studied. The structures of the compounds synthesized and by products isolated were demonstrated by microanalyses, UV-, IR-, and ^{1}H NMR data. Acute toxicity assessment of the compounds on mice showed that compounds **4**, **5**, **6**, and **7** are less toxic than aminophylline. A pharmacological study of the *in vitro* broncholytic effect (IC_{50} and pD_2 values) of the derivatives and aminophylline showed that the new compound **4** (1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7*H*-purine-7-acetic acid 2-(1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7*H*-purin-7-yl)ethyl ester) has a strong bronchodilating effect on serotonine- and acetylcholine-induced spasm in guinea pig trachea. The same compound does not influence barbiturate – induced hypnosis and locomotor activity, unlike to the effect of the aminophylline, used as a reference substance.

Introduction

Theophylline and aminophylline, a soluble complex formed between theophylline and ethylendiamine[1], are two of the most effective drugs rather frequently used in the treatment of asthma. However, the adverse reactions of theophylline, such as tachycardia, ventricular arrhythmia, central nervous system convulsions[2, 3, 4], and the severe allergic reactions and cardiotoxicity[5] of aminophylline are well known, and interfere with their usefulness. Moreover, the mechanism of bronchodilating action of theophylline is not well enough clarified. It is a well established fact that the ester moiety affects the hydrophylic-lipophylic balance (HLB) and has an essential bearing on both the availability of a "prodrug" and molecular modification of chemical structures with biological activity[6]. A variety of acyloxyalkyl-type prodrugs of theophylline were prepared in order to enhance topical delivery of theophylline. These prodrugs[7] include the simple 7-acyloxyalkyl derivatives, which are stable, bioreversible theophylline prodrugs with enhanced membrane transport (i.e. dermal) properties. It is possible to obtain new compounds with biological activity comparable to that of aminophylline, but with lower toxicity, or no cardiotoxic effects by producing esters of 7-theophylline- and 1-theobromine-acetic acid.

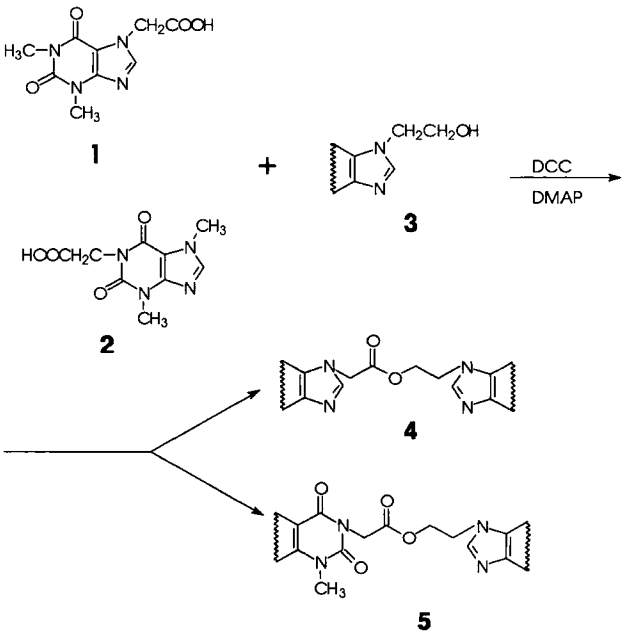
We report here the preparation of two ester derivatives of 7-theophylline- and 1-theobromine-acetic acid, the isolation of the by products of the reaction, and describe their toxicological and pharmacological effects.

Results and Discussion

Synthesis

7-Theophylline- and 1-theobromine-acetic acids (**1**, **2**) were obtained from theophylline-sodium (theobromine-sodium) and the sodium salt of chloroacetic acid by a method reported in the literature [8, 9].

The acyloxyalkyl derivatives of **1** and **2** were obtained by stepwise esterification of the acids with etofylline (7-(2-hydroxyethyl)-theophylline (**3**)) in the presence of *N*,*N*-dicyclohexylcarbodiimide (DCC) and 4-*N*,*N*-dimethylamino)-pyridine (DMAP) as catalyst. The reaction was carried out in anhydrous DMF. The molar ratio between **1**(**2**),**3**, DCC, and



Scheme 1

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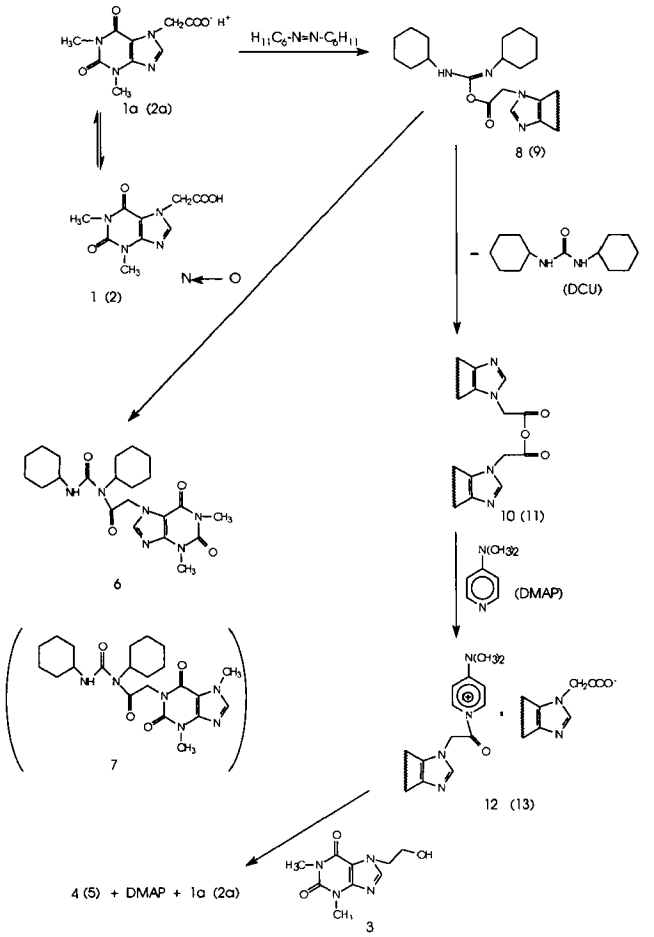
DMAP was respectively 1.1:1:1.3:3:0.25 mmol. The progress of reaction was monitored by TLC (until exhaustion of **1** and **2**) with two systems as a mobile phase.

The main side reaction according to the proposed mechanism of the process[10] (Scheme 2) is the formation of *N*-acyl ureas (**6** and **7**) by an intramolecular rearrangement of the *O*-acyl urea (**8**). The by-products were recorded by TLC in the course of reaction, and they were acyclic urea derivatives. Some compounds assigned to this group possess biological activity, and are widely used in the practice of therapy (i.e. Carbromal, Ethylphenacetamide).



Scheme 2

Pharmacology

The newly synthesized compounds **4**, **5**, **6**, and **7** were assayed for acute toxicity, influence on locomotor activity, hexobarbital sleeping time. Compounds **4** and **5** were tested for *in vitro* antagonism to acetylcholine-, serotonine-, and histamine-induced tracheal contractions. Aminophylline was used as a reference compound.

Analysis of the obtained experimental data on the acute toxicity (LD_{50}) of **4**, **5**, **6**, and **7**, as compared to aminophylline, showed that the compounds had acute toxicity statistically significantly (p 0.05) lower than the standard substance aminophylline (Table 1).

Table 1. Acute toxicity (LD_{50}) of compounds **4**, **5**, **6**, **7**, and aminophylline in mice after intraperitoneal administration.

Compound	LD_{50} (mmol/kg)	Range of values (mmol/kg)
4	2.50*	2.21 – 3.21
5	2.05*	1.92 – 2.19
6	> 7.88*	–
7	2.32*	2.02 – 2.66
aminophylline	0.77	0.68 – 0.88

* $p \leq 0.05$ statistically significant in comparison with aminophylline.

As shown by *in vivo* experiments on the influence of the compounds on locomotor activity of mice the compounds **4** and **6** did not influence locomotor activity throughout the observation period. Compound **5** caused a statistically significant decrease of locomotion between 10 – 70 min of test (Fig. 1). Compound **7** decreased locomotion between 40 – 90 min of test. Aminophylline increased locomotion between 30 – 90 min following intraperitoneal administration.

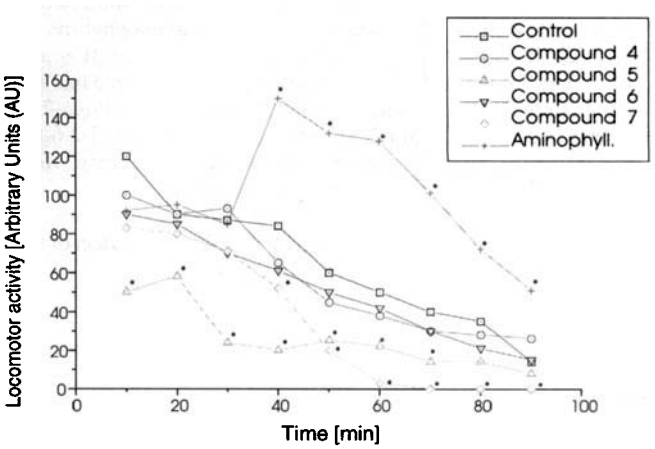


Figure 1. Effects of compounds **4**, **5**, **6**, **7**, and aminophylline (**Aminophyll.**) given i.p. in 1/10 ratio of LD_{50} on locomotor activity of mice at 10 min. intervals up to 90 min. with $p \leq 0.05$ (indicated by asterisk) in comparison with control group.

The effects of the compounds in doses of $^1/_{10}$ of LD_{50} on hexobarbital induced sleeping time are shown in Table 2. Compound **4** and **6** did not influence hexobarbital sleeping time. Compound **5** increased, and compound 7 decreased statistically significantly hexobarbital sleeping time.

In vitro experiments on isolated guinea pig trachea showed that the bronchodilating effect on acetylcholine-induced spasm, exerted by compound **4** ($pD_2 = 4.35$), was statistically significantly stronger than that exerted by aminophylline ($pD_2 = 4.15$) (Table 3). Compound **5** did not influence acetylcholine spasm in guinea pig trachea, with relaxation being observed only at 3×10^{-4} mol/L concentration.

A high bronchodilating effect *in vitro* on serotonine-induced spasm was shown by compound **4** ($pD_2 = 5.80$), followed by aminophylline ($pD_2 = 5.25$). A statistical significance between these two compounds was observed in con-

Table 2. Influence of compounds **4**,**5**, **6**, and **7** on hexobarbital sleeping time of mice.

Compound	Doses (1/10 of LD_{50}) mmol/kg i. p.	Sleeping time ($X + SD$) min
Control	–	18.9 ± 4.3
4	0.25	19.2 ± 3.5
5	0.20	32.9 ± 4.8*
6	0.79	21.3 ± 4.5
7	0.23	10.1 ± 5.4*
aminophylline	0.08	18.1 ± 3.8

* $p \leq 0.05$ statistically significance in comparison with control group.

Table 3. Influence of **4** , **5**, and aminophylline on acetylcholine-induced guinea pig trachea contraction *in vitro*.

Compound	Concentration [mol /L]	Relaxation [%]	IC_{50} pD_2
4	1×10^{-6}	10.0 ± 3.3 *	
	3×10^{-6}	19.6 ± 4.7 *	
	1×10^{-5}	27.4 ± 7.2 *	$IC_{50} = 4.49 \times 10^{-5}$M
	3×10^{-5}	49.3 ± 9.1 *	$pD_2 = 4.35$
	1×10^{-4}	76.1 ± 4.1 *	
	3×10^{-4}	100	
5	1×10^{-4}	0 *	–
	3×10^{-4}	13.2 ± 4.5 *	–
aminophylline	1×10^{-6}	0	
	3×10^{-6}	4.9 ± 1.9	
	1×10^{-5}	12.2 ± 3.4	$IC_{50} = 7.15 \times 10^{-5}$M
	3×10^{-5}	37.1 ± 7.0	$pD_2 = 4.15$
	1×10^{-4}	63.1 ± 6.2	
	3×10^{-4}	100	

The data were presented as $M \pm SD$ ($n = 6$). * – $p \leq 0.05$ statistically significant differences in comparison with aminophylline.

Table 4. Influence of **4** , **5**, and aminophylline on serotonine-induced guinea pig trachea contraction *in vitro*.

Compound	Concentration [mol /L]	Relaxation [%]	IC_{50} pD_2
4	1×10^{-6}	56.4 ± 4.9 *	
	3×10^{-6}	78.9 ± 5.1 *	$IC_{50} = 1.16 \times 10^{-6}$M
	1×10^{-5}	100 *	$pD_2 = 5.80$
5	1×10^{-4}	0 *	–
	3×10^{-4}	11.4 ± 3.2 *	–
aminophylline	1×10^{-6}	36.3 ± 5.1	
	3×10^{-6}	53.8 ± 7.3	$IC_{50} = 5.62 \times 10^{-6}$M
	1×10^{-5}	73.6 ± 5.6	$pD_2 = 5.25$
	3×10^{-5}	100	

The data were presented as $M \pm SD$ ($n = 6$). * – $p \leq 0.05$ statistically significant differences in comparison with aminophylline.

centrations all tested. Compound **5** did not affect serotonine-induced spasm in guinea pig trachea (Table 4).

Experiments with histamine-induced spasm on guinea pig trachea showed that the effect of aminophylline on this spasm ($pD_2 = 5.27$) was stronger. The effect of compound **4** ($pD_2 = 4.44$) was statistically significantly less pronounced than that of aminophylline. Compound **5** did not influence this spasm, with a relaxation (9.2%) recorded only at concentration 3×10^{-4} mol/L.

The results of the *in vitro* pharmacological screening experiments on guinea pig avascular (bronchial) smooth muscles show that compound **4** has an appreciable bronchodilating effect. This compound has significantly higher effect *in vitro* on acetylcholine and serotonine-induced spasm in guinea pig trachea, and significantly lower effect (histamine-induced spasm), as compared to aminophylline.

Table 5. Influence of **4** , **5**, and aminophylline on histamine-induced guinea pig trachea contraction *in vitro*.

Compound	Concentration [mol /L]	Relaxation [%]	IC_{50} pD_2
4	1×10^{-6}	4.4 ± 1.2 *	
	3×10^{-6}	16.4 ± 5.1 *	
	1×10^{-5}	49.7 ± 7.5 *	$IC_{50} = 7.30 \times 10^{-5}$M
	3×10^{-5}	81.1 ± 5.2 *	$pD_2 = 4.14$
	1×10^{-4}	100	
5	1×10^{-4}	0 ± 1.4	–
	3×10^{-4}	9.2 ± 2.8 *	–
aminophylline	1×10^{-6}	21.1 ± 4.5	
	3×10^{-6}	48.2 ± 3.4	
	1×10^{-5}	78.4 ± 6.8	$IC_{50} = 5.4 \times 10^{-6}$ M
	3×10^{-5}	95.5 ± 7.2	$pD_2 = 5.27$
	1×10^{-4}	100	

The data were presented as $M \pm SD$ ($n = 6$). * – $p \leq 0.05$ statistically significant differences in comparison with aminophylline.

The stability of compounds **4** and **5** in solution at pH = 7.4 (without products of hydrolysis, confirmed by TLC and UV spectral determination) for 4 h suggest that their effect is produced by whole molecule action.

Comparative pharmacological and toxicological experiments on compounds **4** and **5** indicated that **4** has higher broncholytic effect *in vitro* (except for histamine-induced spasm). The acute toxicity of compound **4** is more than three times lower than that of the standard preparation of aminophylline. On the other hand, this compound fails to influence significantly barbiturate induced hypnosis and locomotion. This fact is an advantage in comparison with aminophylline because it suggests that the compound is without CNS side effects and thus appearts highly very promising for further detailed pharmacological and toxicological experiments.

Experimental

Chemistry

Melting points were measured in °C and corrected (Büchi 535). UV spectra were recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer. IR spectra were recorded on a Pye Unicam SP3-200. ^{1}H NMR spectra were recorded at ambient temperature on a Bruker-250 Wm. (250 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to TMS as internal standard. TLC was performed on DC-Alufolien Kieselgel 60 F_{254} (Merck) (0.20 mm) sheets with solvents: ethanol–acetone–chloroform (4:3:3 *v/v/v*) – system 1 and formic acid–chloroform–acetone–ethanol (1:3:3:4 *v/v/v/v*) – system 2. Detected at UV 254 nm. The novel structures were supported by microanalyses and characteristic UV, IR, and NMR data quoted.

1,2,3,6-Tetrahydro -1,3-dimethyl-2,6-dioxo-7H-purine-7-acetic acid 2-(1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7H-purin-7-yl)ethyl ester (**4**)

7-Theophylline-acetic acid 2.62 g (11 mmol), etofylline 2.2 g (10 mmol), and DMAP 0.3 g (2.5 mmol) were dissolved in 50 ml DMF at room temperature. After complete dissolution, DCC 2.7 g (13 mmol) dissolved in 10 ml DMF was added. After a few seconds, a white precipitate of *N,N*-dicyclohexylurea (DCU) was formed. The reaction mixture was stirred for 6 h and filtered. After concentration to minimal volume of the filtrate on rotary evaporator (Büchi Rotavapor R-114), anhydrous ethanol was added and the solution was kept for 24 h at –5 °C. The separated crystals were filtered and recrystallized from anhydrous ethanol to yield 2.35 g (52.9%) of **4**; mp 240 – 242 °C; R_f = 0.75 (system 1).– IR (KBr): ν = 3110 – 2910 cm^{-1} (CH_2, CH_3), 1750 (C=O ester), 1690, 1650 (C=O), 1600 – 1540 with max. at 1575 (C=C, C=N), 1275 (C–O–C ester); δ = 1450 cm^{-1} (CH_2), 1420 (CH_3), 1380 (CH_3).– UV (EtOH/$CHCl_3$, 5:1): $\lambda_{max.}$ (log ε) = 234 nm (0.852), 274 (1.911).– ^{1}H NMR (DMSO-d_6): δ = 7.99 (s, 1H, 8-H), 7.89 (s, 1H, 8-H), 5.11 (s, 2H, CH_2), 4.48–4.28 (m, 2H, CH_2), 3.46–3.41 (m, 2H, CH_2), 3.31 (s, 6H, 2 × CH_3), 3.21 (s, 6H, 2 × CH_3). Anal. ($C_{18}H_{20}N_8O_6$) C, H, N.

2,3,6,7-Tetrahydro-3,7-dimetyl-2,6-dioxo-1H-purine-1-acetic acid 2-(1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7H-purin-7-yl)ethyl ester (**5**)

The product was obtained by a process analogous to **4**. Yield – 1.82 g (41 %); mp 161 – 163 °C; R_f = 0.86 (system 2).– IR (KBr): ν = 3100 – 2910 cm^{-1} (CH_2 ,CH_3), 1700 (C=O ester), 1660, 1605 (C=O), 1535 – 1580 with max. at 1550 (C=C, C=N), 1285 (C–O–C ester). – UV (EtOH/$CHCl_3$, 5:1): $\lambda_{max.}$ (log ε) = 234 nm (1.001), 274 (1.879).– ^{1}H NMR (DMSO-d_6): δ = 7.99 (s, 2H, 2 × 8–H), 4.93 (t, *J* = 5.16 Hz, 2H, CH_2), 4.28 (t, *J* = 5.32 Hz, 2H, CH_2), 3.73 – 3.67 (m, 2H, CH_2), 3.42 (s, 3H, CH_3), 3.33 (s, 3H, CH_3), 3.22 (s, 6H, 2 × CH_3). Anal. ($C_{18}H_{20}N_8O_6$) C, H, N.

N-(7-Acetyl-1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7H-purine)-N,N′-dicyclohexylurea (**6**)

After filtration of **4** the solution (DMF/EtOH) was removed on rotary evaporator and the residual oil was dissolved in minimal volume ethylacetate when hot. After cooling the separated crystals were filtered and recrystallized from acetone to yield 0.33 g (7.5 %); mp 237 – 238 °C ; R_f = 0.81 (system 1).– IR (KBr): ν = 3280 cm^{-1}(NH), 3100 – 2900 (CH_2 ,CH_3), 1710 (C=O amide I), 1660 (C=O from urea), 1555 (C=C, C=N); δ = 1600 cm^{-1} (NH amide II).– UV (EtOH/$CHCl_3$, 5:1): $\lambda_{max.}$ (log ε) = 244 nm (0.607), 278 (1.559).– ^{1}H NMR (DMSO-d_6): δ = 11.07 (s, 1H, NH), 8.31 (s, 1H, 8-H), 5.58 – 5.61 (d, 2H, CH_2), 3.31 (s, 6H, 2 × CH_3), 1.73 – 1.01 (m, 22H, cyclohexyl H). Anal. ($C_{22}H_{32}N_6O_4$) C, H, N.

N-(1-acetyl-2,3,6,7-terahydro-3,7-dimethyl-2,6-dioxo-1H-purine)-N,N′-dicyclohexylurea (**7**)

The product was obtained by a procedure analogious to that for to **6**. Yield – 0.29 g (6.5%); mp 213 – 215 °C; R_f =0.89 (system 2).– IR (KBr): ν = 3330 cm^{-1} (NH), 3105 – 2900 (CH_2 ,CH_3), 1710 (C=O amide I), 1680 (C=O from urea), 1575 (C=C, C=N), δ = 1635 cm^{-1} (NH amide II).– UV (EtOH/$CHCl_3$, 5:1): $\lambda_{max.}$ (log ε) = 234 nm (0.791), 274 (1.755).– ^{1}H NMR (DMSO-d_6): δ = 10.92 (s, 1H, NH), 8.00 (s, 1H, 8-H), 5.11 (s, 2H, CH_2), 3.32 (s, 3H, CH_3), 3.34 (s, 3H, CH_3), 1.72 – 1.00 (m, 22H, cyclohexyl H). Anal. ($C_{22}H_{32}N_6O_4$) C, H, N.

Pharmacology
Materials and Methods

The experiments were conducted on forty guinea pigs weighing 500 – 600 g, and 200 male white mice with weight 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 intraperitoneally (i.p.) route. LD_{50} was evaluated from 4 or 5 different doses, each on the 6 animals and calculated by the method of Litchfield-Wilcoxon [11] using a personal computer.

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 dose 80 mg/kg b.w. was administered i.p. to the animals 30 min after administration of solutions of the compounds under study. Sleeping time was measured in minutes by observing the righting reflex recovery.

Influence on locomotor activity. Group of 6 animals was put on in actometer (Activity Cage, Ugo Basile, Italy) and the locomotor activity in arbitrary units was determined at 10-min intervals for 90 min. The tested compounds at dose $^1/_{10}$ of LD_{50} were administered to the animals and they were tested in the apparatus for 90 min under analogical conditions, and the results were compared with those in the control vehicle-treated group.

The broncholytic effect of the compounds was likewise evaluated during *in vitro* experiments on isolated guinea pig trachea according to Castillo & de Beer[12]. For this purpose the organ isolated was immersed in an organic bath (cup) of volume 30 ml with Krebs-Henseleit solution, at temperature 37 C and carbogen aeration. Tracheal contractions induced by acetylcholine – 1×10^{-6} M, serotonine 1×10^{-6} M and histamine 1×10^{-6} M (all compounds from Sigma, USA analytically graded) – were recorded by means of isotonic transducer and writing device Unirecord, obtained from Ugo Basile Co (Italy). Cumulative concentration-effect curves of the tested compounds were plotted, and the mean effective concentrations EC_{50} and pD_2 were calculated using the regression analysis method[13]. The results underwent statistical processing by the Student-Fischer t-test at $p \leq 0.05$.

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