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Synthesis and pharmacological assessment of derivatives of isoxazolo[4,5-*d*]pyrimidine

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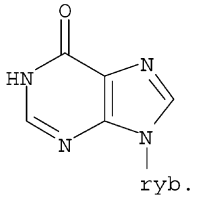
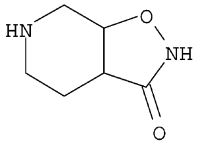
Abstract—A series of new 5-alkyl and 5-arylisoxazolo[4,5-*d*]pyrimidinones (**5a**–**g**, **6**–**8**) were prepared from 4-amino-3-oxo-isoxazolidine-5-carboxylic acid amide. Some of the aryl derivatives of isoxazolo[4,5-*d*]pyrimidine were tested pharmacologically in comparison with Diazepam. Compounds **5b**–**d** and **7** demonstrated interesting anxiolytic activity.[†]

1. Introduction

Benzodiazepine receptor (BzR) ligands are structurally diverse compounds that bind to the $GABA_A$/BzR complex. They include β-carbolines[1] imidazobenzodiazepines,[2] imidazopyridines,[3] pyrazoloquinolines,[4] pyridiindoles,[5] triazolopyridazines such as CL218872,[6] as well as some others.[7–9] Molecular biological studies have demonstrated that several receptor subunits (α_1–α_6, β_1–β_3, γ_1–γ_3, δ) combine to form the $GABA_A$ receptor complex in the mammalian central nervous system.[10,11] One from two central benzodiazepine receptors are probably formed by combinations of the subunits $\alpha_1\beta_2\gamma_2$, show many of the pharmacological effects associated with the type I BzR, and a mixture of the subunits α_2–α_3 and $\alpha_5\beta_2\gamma_2$ characterize the biological effects of the so-called type II BzR.[12] Of the chemical classes which have binding sites on this macromolecular ionophore, the benzodiazepines are the most widely studied and used. However, these compounds often produce undesirable side effects, including sedation, physical dependence, amnesia, and ethanol potentation. When the benzodiazepines are used only as antianxiety drugs, the sedative effect makes normal life most difficult for patients. For this reason, many laboratories have been searching for anxiolytics that are devoid of or exhibit markedly reduced side effects. A rational design of new benzodiazepine receptor (BzR) ligands based on reliable pharmacophore models has been proposed on the basis of binding data displayed at multiple BzR subtypes by various structural families of BzR ligands, but it is also good to take into consideration the structure of the endogenous ligand of this receptor. Therefore, in our study we have taken the structure of inosine (hypoxantine) and that of THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*e*]pyridin-3(2H)-one as the basis for the synthesis of a new non-benzodiazepine series of compounds with potential anxiolitic activity. Inosine (hypoxantine) is an endogenous ligand for the brain benzodiazepine receptor.[13,14] The THIP mentioned above is a highly selective ligand of the $GABA_A$ receptor.[15]



Inosine



THIP

We have concentrated on the synthesis of derivatives of isoxazolo[4,5-*d*]pyrimidine — a structure created from a compilation of the structure of THIP and hypoxantine, where the nitrogen atom in position 7 (hypoxantine) is replaced by an oxygen atom. Inserting appropriately substituted aryl groups in position 5 should also have profitable influence on the activity of these derivatives and to bring the structure closer to one more selective for the classical type I BzR — CL-218872.[6]

Keywords: Isoxazolo[4,5-*d*]pyrimidinones; Anxiolytic compounds; Benzodiazepine receptors; Anxiety.
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† E. Wagner, L. Becan, E. Nowakowska, Pol. P. 328352, 1998. E. Wagner, L. Becan, E. Nowakowska, Pol. P. 328382.

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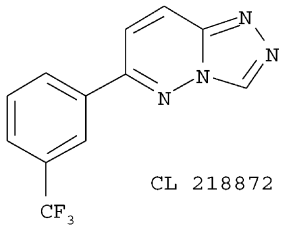
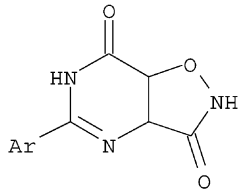


CL 218872



isoxazolo[4,5-d]pyrimidine-3,7-dione

(a general formula)

These modifications have been made for the purpose of obtaining compounds with the expected anxiolytic activity, but with possibly decreased side effects.

2. Results and discussion

2.1. Synthesis

Of the four structural isomers of isoxazolopyrimidines, only the isomer 4,5-*d* has not been thoroughly investigated. There have been only three[16–18] foreign papers published concerning the synthesis of derivatives of this isomer till now. We obtained the derivatives **5a–g**, **7**, **8** of this isomer from aminoamide **4** and the corresponding aldehydes (Scheme 1). The aminoamid **4** was prepared earlier by Stammer[19] but in low total yield. In contrast to previous work,[19], we obtained compound **4** using another method. We used a more active dimethyl ester **2** which was obtained from reaction between treo-β-hydroxy-DL-aspartic acid dimethyl ester[20] and PCl_5. Compound **2** (obtained in a very good yield) has the identical mp and IR as the one obtained by Antolini[21] from the appropriate aziridine. The intermediate **2** was converted into the hydroxamic acid **3** (by reaction with anhydrous hydroxylamine in dry methanol) and was cyclized in the presence of lithium metoxide. Compound **3** is identical to the compound obtained by Stammer.[19] It is a white amorphous solid which produces positive ninhydrin and ferric chloride tests and a positive nitroprusside test, which is specific to the isoxazole ring.[22] We converted compound **3** into aminoamide **4** with perfect efficiency by applying the Mattingly and Miller method of reduction of hydroxamic acids to amides by means of titanium trichloride.[23] Compound **4** was the same as the compound obtained by Stammer,[19] but was prepared without the two low-productivity stages (methanolysis and aminolysis).

Compound **4** was subjected to reaction with the suitable aldehydes. We obtained a number of new derivatives of isoxazolo[4,5-*d*]pyrimidine **5a–g**. The result of reaction of compound **4** with *p*-chlorobenzoyl chloride was compound **6** which, after cyclizing, gave a new 5-(4-chloro-phenyl)-3a,7a-dihydro-6H-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (**7**). Compounds **5d** and **7** were subjected to oxidation with $KMnO_4$ in acetone and the result was identical for both compounds: 5-(4-chlorophenyl)-6H-isoxazolo[4,5-*d*]-pyrimidine-3,7-dione (**8**). The oxidation also confirmed the structure of the new compounds. Compounds **5b–e** and **7** were subjected to pharmacological study.

2.2. Influence of the compounds on locomotor activity

The aim of the study was to find out if compounds of differing chemical structure, that is, **5b–e** and **7**, show anxiolytic activity comparable with the effects of diazepam.

Each of the supplied compounds was investigated in a locomotor activity test in doses of 0.1–10 mg/kg bw, 30 min after ip injection. The aim of the experiments was to estimate the highest dose not causing sedation.

It was found that the following doses of the compounds were free from sedative action: compounds **5b**, **5c**, **7** — 1 mg/kg ; compounds **5d**, **5e** — 0.1 mg/kg.

The classical benzodiazepine diazepam tested at the dose level of 2.5 mg/kg showed no sedative activity.

Table 1. The influence of the compounds **5b–e** and **7** on locomotor activity in rats, in comparison with diazepam

Substances tested	*n*	Doses (mg/kg)	Locomotor activity ±SEM
Control 0.5% CMC	8	0.4 mL	92.0±10.3
Control H_2O inj.	8	0.4 mL	90.2±9.5
Compound **5b**	8	1.0 mg/kg	88.0±8.6
Compound **5c**	8	1.9 mg/kg	101.0±10.1
Compound **5d**	8	0.1 mg/kg	92.9±9.4
Compound **5e**	8	0.1 mg/kg	103.0±12.3
Compound **5e**	8	1.0 mg/kg	114.0±14.1
Compound **7**	8	1.0 mg/kg	92.0±8.2
DIAZEPAM	8	2.5 mg/kg	87.0±6.9

Diazepam and the tested compounds were administered 30 min before the test. *n*, number of animals in the group.

Table 2. Investigation of anxiolytic activity of compounds **5b–e** and **7** in comparison with placebo, in the two compartment exploratory test in rats

Substance (mg/kg)	*n*	WSE ± SEM	BWT ± SEM	BSE ± SEM
Control 0.5% CMC	8	1.2±0.8	2.9±0.1	6.0±0.5
Control H_2O inj.	8	1.1±0.8	2.1±0.1	6.7±0.5
Compound **5b** 1.0 mg/kg	8	18.5±2.9*	6.2±1.1*	11.2±1.8*
Compound **5c** 1.0 mg/kg	8	13.4±2.3*	3.9±1.4	9.6±2.8
Compound **5d** 0.1 mg/kg	8	4.9±1.1*	4.1±1.5	7.4±2.9
Compound **5e** 0.1 mg/kg	8	1.3±0.7	1.6±0.9	4.1±2.4
Compound **5e** 1.0 mg/kg	8	1.5±0.4	1.5±0.8	3.9±1.9
Compound **7** 1.0 mg/kg	8	9.9±1.1*	4.8±0.7*	9.7±0.3*
DIAZEPAM 2.5 mg/kg	8	19.5±2.4*	12.8±3.1*	9.9±0.9*

WSE, white square entrance; BWT, black–white transition; BSE, black square entrance. The experiments were carried out 30 min after administration of the compound. *Significant differences against control group ($P<0.05$). *n*, number of rats in the group.

As shown in Table 1, the compounds investigated did not change the locomotor activity of the rats. The proper experiments were carried out after a period of 3 days, during which the rats became accustomed to the apparatus through daily exposure to it.

2.3. Anxiolytic activity of the compounds in the 'two-compartment exploratory test'

An event was recorded when a rat entered a square with all four paws (white square entrance WSE or black square entrance BSE). If a rat crossed from the white square area to the black one, or from the black to the white, a black–white transmission (BWT) was recorded. The control animals, receiving only H_2O, CMC or H_2O with Tween injections, showed a marked preference for the dark area (BSE = 6.9 ± 0.5 vs WSE < 2.0 and BWT = 2.9 ± 0.1).

Diazepam administered in the dose of 2.5 mg/kg bw showed a significant rise of all parameters: WSE, BWT and BSE (Table 2), 30 min after administration (the peak value). The pyrimidine compounds were tested for anxiolytic activity in doses free from any sedative effect (see Table 1). It was found that 1 mg/kg of compound **5b**, 1 mg/kg of compound **5c**, 0.1 mg/kg of compound **5d**, and 1 mg/kg of compound **7** showed anxiolytic activity.

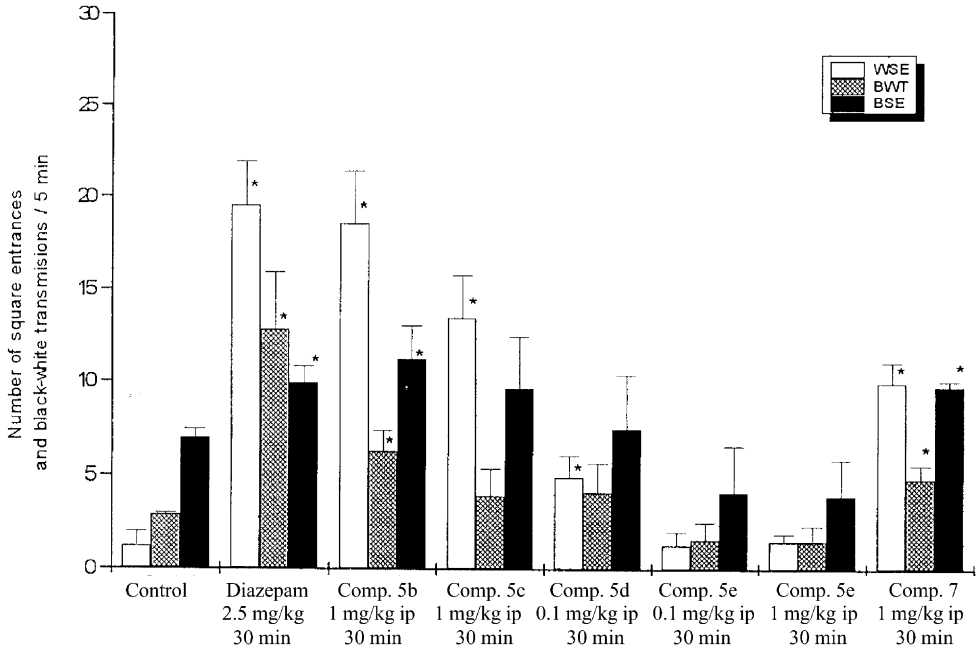


Figure 1. Investigation of anxiolytic activity of the tested compounds, in comparison with diazepam. The experiments were carried out 30 min after compound administration. WSE, white square entrance, BSE, black square entrance, BWT, black–white transition. *Significant differences against control group ($p < 0.05$).

Table 3. The effects of Ro-15-1788 on anxiolytic action of diazepam and substances **5b** and **7** in two compartment exploratory test

No	Group	*n*	WSE $X \pm$SEM	BWT $X \pm$SEM	BSE $X \pm$SEM
1.	Control	6	2.25 ± 0.25	1.25 ± −0.25	7.75 ± 0.75
2.	Ro-15-1788 (10 mg/kg ip) 30 min before the test	6	2.30 ± 0.24	1.00 ± 0.00	7.25 ± 1.10
3.	Diazepam (2.5 mg/kg ip) 30 min before the test	6	24.40 ± 1.36*	8.60 ± 0.60*	10.60 ± 0.68*
4.	Ro-15-1788 + Diazepam 30 min before the test	6	5.60 ± 0.68*,**	1.40 ± 0.24*,†	12.00 ± 1.45*
5.	Substance **5b** (1 mg/kg ip) 30 min before the test	6	18.17 ± 0.60*	5.40 ± −0.50*	10.17 ± 0.54
6.	Substance **5b** (1 mg/kg) + Ro-15-1788 (10 mg/kg) 30 min before the test	6	13.83 ± 1.57*,‡	3.33 ± 0.61*,†	7.83 ± 0.65†
7.	Substance **7** (1 mg/kg ip) 30 min before the test	6	8.83 ± 0.60*	3.60 ± 0.40*	7.17 ± 0.70
8.	Substance **7** (1 mg/kg) + Ro-15-1788 (10 mg/kg) 30 min before the test	6	14.00 ± 2.05*,‡	3.50 ± 0.56*	5.83 ± 0.70

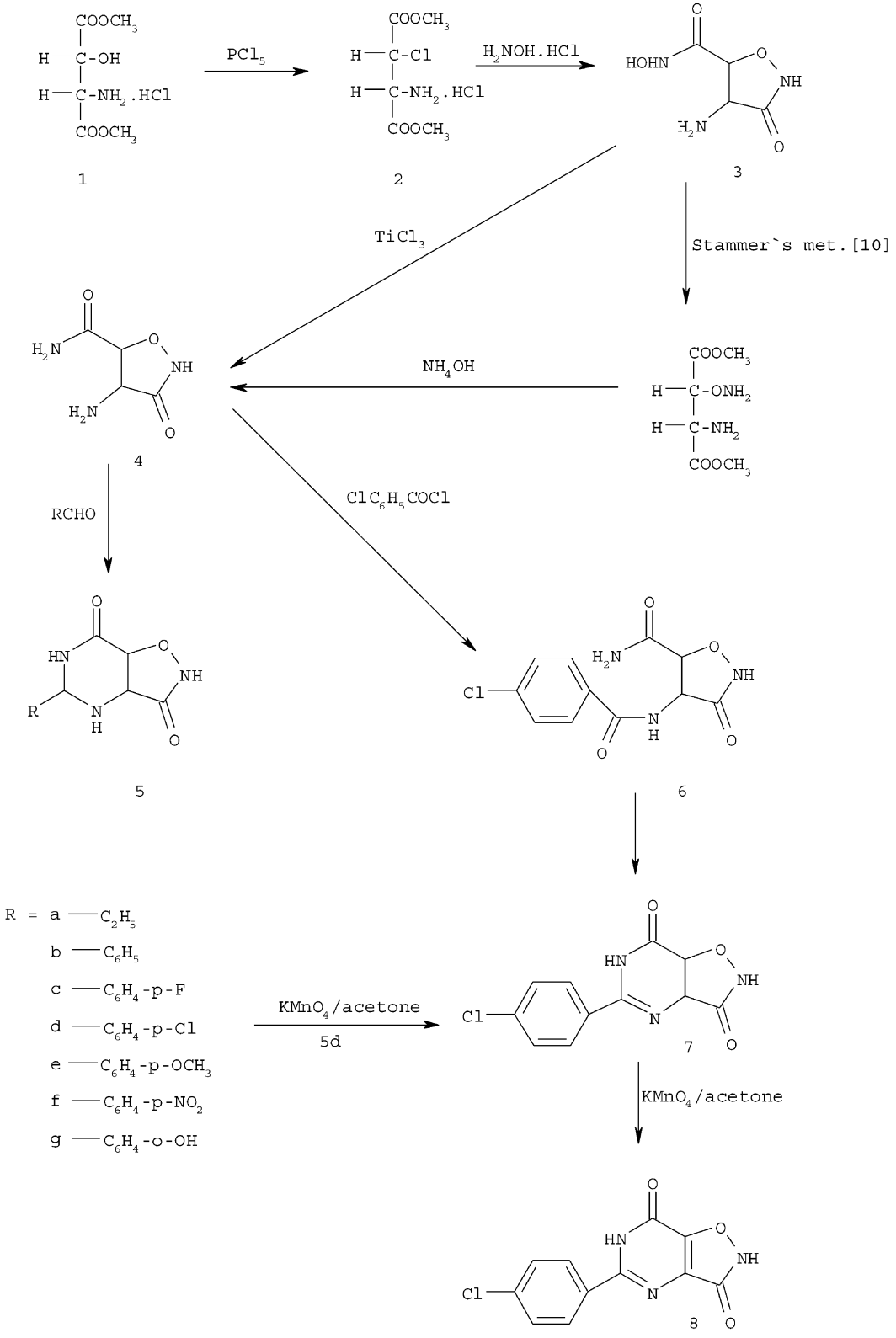
Statistically significant against the control group (1); $p < 0.05$. **Statistically significant against diazepam group (3); $p < 0.05$. †Statistically significant against group (5); $p < 0.05$. ‡Statistically significant against group (7); $p < 0.05$. Ro-15-1788 was administered in the intraperitoneal dose of 10 mg/kg 30 min before the anxiolytic test.

After compounds **5b** and **7**, a rise in all parameters was observed, while after compounds **5c** and **5d** only a rise in WSE occurred (Table 2, Fig. 1).

Compound **5e** showed no anxiolytic activity at doses of 0.1 and 1.0 mg/kg bw.

2.4. Assay in association with Flumazenil

An event was recorded whenever a rat entered a square with all four paws (white square entrance WSE or black square entrance BSE). If a rat crossed from a white square to a black one or from a black to a white, a BWT was



Scheme 1.

recorded. The control animals, which received only H_2O, CMC or H_2O with Tween injections, showed a marked preference for the dark area (BSE = 7.75 ± 0.75 vs WSE < 2.25 and BWT 1.25 ± 0.25).

Diazepam administered in the doses of 2.5 mg/kg significantly increased the value of all parameters, that is WSE, BWT and BSE (Table 3) 30 min after its administration (peak value).

Compounds **5b** and **7** produced statistically significant increases in the WSE and BWT values, which confirms the anxiolytic potential of both compounds.

Ro-15-1788 did not exhibit primary anxiolytic potential, as the WSE, BWT and BSE parameters were not different from there of the control group. Joint administration of Ro-15-1788 and diazepam resulted in antagonizing the anxiolytic effects of diazepam (decrease in WSE and BWT values), and similar results could be observed by joint administration of Ro-15-1788 and **5b**. However, no R-15-1788-related antagonism could be observed for joint administration of flumazenil and compound **7**.

3. Conclusions

The results of the biological study confirm our assumption that the derivatives of isoxazolo[4,5-*d*]pyrimidine, which are structurally similar to hypoxantine can have anxiolytic activity comparable to diazepam. All doses of the compounds investigated (including diazepam) were lower than those causing the sedative effect. The results of our study, depicted in Figure 1, show that compounds **5b**, **5c** and **7**, even at 2.5 times smaller doses, have approximately the same anxiolytic activity as diazepam, whereas compound **5d** has weaker anxiolytic activity, but at a 25 times smaller dose. Only compound **5e** lacks this activity. Derivatives of isoxazolo[4,5-*d*]-pyrimidine may constitute a new chemical tool for further research work on the efficiency of anxiolytic activity.

If Ro 15-1788 antagonizes the anxiolytic effects of classic benzodiazepines (diazepam), which would confirm the well-established hypothesis relating anxiolytic effects of benzodiazepines to their action on benzodiazepine receptors in the brain, it may be postulated that the mechanism of anxiolytic action of **5b** may be similar to that of classic benzodiazepines.

4. Experimental

4.1. General

Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. The progress of the reaction and purity of compounds were monitored by TLC analytical silica gel plates (Merck F_{254}). IR spectra were recorded on a Specord M 80 spectrometer for KBr discs. ^{1}HNMR spectra were recorded with a Bruker Avance DRX-300 instrument; chemical shifts are reported in ppm downfield from the internal tetramethysilane and coupling constants in Hz. Mass spectra were recorded on a Finnigan MAT 95. Two solvent systems, butanol–acetic acid–water (3:1:1, BAW) and CH_2Cl_2–metanol (7:1) were used. Elemental analyses were performed on a Carbo Erba NA 1500 analyzer in our faculty laboratories.

4.1.1. Dimethyl β-chloroaspartate hydrochloride (2). To the stirred suspension of 213.5 g (1 mol) threo-β-hydroxy-DL-aspartic acid dimethyl ester hydrochloride in 1 L of absolute CH_2Cl_2 was added 228.5 g (1.1 mol) PCl_5 portion-wise over a period of 1 h. The reaction mixture was stirred at room temperature for 3 h and left standing at room temperature for 12 h. The excess PCl_5 was filtered and the solution was evaporated to ca. 400 mL. The remainder was cooled to 0 °C, and the precipitate was filtered and washed successively with absolute CH_2Cl_2 and dry ether. The solid was dried over $CaCl_2$ at room temperature in vacuo, giving 187.9 g (81%) of crude product (**2**). A sample was recrystallized three times from methanol–ether: mp 89–90 °C (lit.[11] mp 88–90 °C).

4.1.2. 4-Amino-3-oxo-isoxazolidine-5-carboxylic acid hydroxyamide (3). To a solution of sodium methoxide, prepared from 27.6 g (1.2 mol) sodium in 650 mL anhydrous methanol, was added 83.4 g (1.2 mol) hydroxylamine hydrochloride and stirred at room temperature for 90 min. The mixture was cooled to 0 °C, the sodium chloride was filtered and a solution of 116 g (0.5 mol) dimethyl β-chloroaspartate hydrochloride (**2**) in 350 mL absolute methanol was added to the filtrate. This solution was cooled to −10 °C and a solution of lithium methoxide (prepared from 600 mL anhydrous methanol and 12 g LiH) was added. The mixture was cooled to −5 °C in a refrigerator over night, the reaction mixture was acidified (pH 4.5–5) with concentrated HCl at 5 °C. The suspension was evaporated to ca. 300 mL in vacuo. The remainder was cooled to 5 °C, and the precipitate was filtered and washed with absolute ethanol and ether. The solid was dissolved in a minimum amount of water and the solution stirred into 3 L of absolute EtOH. The precipitate was filtered and dried over $CaCl_2$ at room temperature in vacuo, giving 155.4 g (96.4%) of amorphous powder. The IR and TLC were identical with 4-amino-5-carbohydroxamido-3-isoxazolidone.[10]

4.1.3. 4-Amino-3-oxo-isoxazolidine-5-carboxylic acid amide (4). To a stirred solution of 16.1 g (0.1 mol) of (**3**) in 60 mL of water was added drop-wise a 20% aqueous solution of $TiCl_3$.[14] The mixture was stirred under N_2 and the pH maintained at 7 with 10% LiOH. After completion addition of the $TiCl_3$, the blue color persisted and a light yellow suspension of $Ti(OH)_4$ formed. The solid $Ti(OH)_4$ was removed by centrifugation and the solution was evaporated in vacuo to ca. 30 mL and diluted with 100 mL of absolute ethanol. After cooling in a refrigerator for the night, the precipitate was filtered, washed with 95% EtOH, and dried, giving 12.15 g of crude product (**4**). Recrystallization from NH_4OH–AcOH/EtOH gave an analytically pure compound (**4**): 11.3 g (78%); mp 203–4 °C (lit. ca. 200 °C[10]).

4.2. General procedure for the preparation of derivatives of tetrahydro-isoxazolo-[4,5-*d*]pyrimidine-3,7-dione (5a–g)

To the suspension of compound **4** (4.35 g, 0.03 mol) in 100 mL of dry methanol was added 0.033 mol of the corresponding aldehyde. The reaction mixture was stirred under reflux for 4 h. The mixture was concentrated to ca. 30 mL. After cooling, the remainder was placed in a refrigerator for 6 h, and the precipitate was filtered and washed with methanol. Solids was purified by recrystallization.

4.2.1. 5-Ethyl-tetrahydro-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (5a). From **4** and propionyl aldehyde and recrystallized from ethanol; yield 58% of white crystals; mp 189–190 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.25 (t, 3H, CH_3), 3.15 (m, 2H, CH_2), 3.16 (br s, 1H, 4-NH), 3.70 (d, J = 4 Hz, 1H, 3a-CH), 4.07 (d, J = 4 Hz, 1H, 7a-CH), 5.57 (s, 1H, 5-CH), 7.28 (s, 1H, 6-NH), 8.32 (s, 1H, 2-NH). EJ MS m/z 185 (M^+) Anal. calcd ($C_7H_{11}N_3O_3$): C, 45.40; H, 5.99; N, 22.69. Found: C, 45.55; H, 6.05; N, 22.75.

4.2.2. 5-Phenyl-tetrahydro-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (5b). Colorless needles obtained from **4** and benzaldehyde and recrystallized from methanol; yield 79%; mp 168–169 °C; ^{1}H NMR (DMSO-d_6) δ 3.18 (br s, 1H, 4-NH), 4.08 (d, J + 4 Hz, 1H, 3a-CH), 4.38 (d, J = 4 Hz, 1H, 7a-CH), 5.63 (s, 1H, 5-CH), 7.34 (s, 1H, 6-NH), 7.44–7.51 (m, 3H, 3′/4′/5′-PhH), 7.81–7.84 (m, 2H, 2′/6′-PhH), 8.22 (s, 1H, 2-NH). EJ MS m/z 233 (M^+). Anal. calcd ($C_{11}H_{11}N_3O_3$): C, 56.65; H, 4.75; N, 18.02. Found: C, 56.85; H, 4.90; N, 17.90.

4.2.3. 5-(4-Fluoro-phenyl)-tetrahydro-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (5c). Colorless needles obtained from **4** and *p*-fluorobenzaldehyde and recrystallized from methanol; yield 83%; mp 165–166 °C; ^{1}HNMR (DMSO-d_6) δ 3.75 (br s, 1H, 4-NH), 4.08 (d, J = 4 Hz, 1H, 3a-CH), 4.39 (d, J = 4 Hz, 1H, 7a-CH), 5.72 (s, 1H, 5-CH), 7.41 (s, 1H, 6-NH), 7.52–7.54 (d, J = 7 Hz, 2′/6′-ArH), 7.87–7.92 (d, J = 7 Hz, 3′/5′-ArH), 8.21 (s, 1H, 2-NH). EJ MS m/z 251 (M^+) Anal. calcd ($C_{11}H_{10}N_3O_3F$): C, 52.59; H, 4.02; N, 16.73. Found: C, 52.73; H, 4.14; N, 16.51.

4.2.4. 5-(4-Chloro-phenyl)-tetrahydro-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (5d). Colorless needles obtained from **4** and *p*-chlorobenzaldehyde and recrystallized from ethanol; yield 92%; mp 176–177 °C; ^{1}H NMR (DMSO-d_6) δ 3.81 (br s, 1H, 4-NH), 4.12 (d, J = 4 Hz, 1H, 3a-CH), 4.45 (d, J = 4 Hz, 1H, 7a-CH), 5.80 (s, 1H, 5-CH), 7.40 (s, 1H, 6-NH), 7.51–7.53 (d, J = 8 Hz, 2H, 2′/6′-ArH), 7.81–8.09 (d, J = 8 Hz, 2H, 3′/5′-ArH), 8.30 (s, 1H, 2-NH). EJ MS m/z 267 (M^+) Anal. calcd ($C_{11}H_{10}N_3O_3Cl$): C, 49.35; H, 3.76; N, 15.70. Found: C, 49.56; H, 3.88; N, 15.63.

4.2.5. 5-(4-Methoxy-phenyl)-tetrahydro-isoxazolo[4,5-*d*]pyrimidine-3,7-dione(5e). Colorless needles obtained from **4** and *p*-methoxybenzaldehyde and recrystallized from ethanol; yield 88%; mp. 179–180 °C; ^{1}H NMR (DMSO-d_6) δ 3.76 (br s, 1H, 4-NH), 3.86 (s, 3H, OCH_3), 4.07 (d, J = 4 Hz, 1H, 3a-CH), 4.37 (d, J = 4 Hz, 1H, 7a-CH), 5.69 (s, 1H, 5-CH), 7.26 (s, 1H, 6-NH), 7.36–7.39 (d, J = 8.5 Hz, 2H, 3′/5′-ArH), 7.76–7.80 (d, J = 8.5 Hz, 2H, 2′/6′-ArH), 8.25 (s, 1H, 2-NH). Anal. calcd ($C_{12}H_{13}N_3O_4$): C, 54.75; H, 4.98; N, 15.96. Found: C, 54.61; H, 5.06; N, 16.12.

4.2.6. 5-(4-Nitro-phenyl)-tetrahydro-isoxaxolo[4,5-*d*]pyrimidine-3,7-dione (5f). Ivory crystals obtained from **4** and *p*-nitrobenzaldehyde and recrystallized from ethanol; yield 72%; mp 206–207 °C; ^{1}H NMR (DMSO-d_6) δ 3.60 (br s, 1H, 4-NH), 4.19 (d, J = 4 Hz, 1H, 3a-CH), 4.45 (d, J = 4 Hz, 1H, 7a-CH), 5.90 (s, 1H, 5-CH), 7.71 (s, 1H, 6-NH), 8.07 (d, J = 9 Hz, 2′/6′-ArH), 8.27–8.30 (m, 3H, 3′/5′-ArH and 2-NH). Anal. calcd ($C_{11}H_{10}N_4O_5$): C, 47.49; H, 3.62; N, 20.14. Found: C, 47.65; H, 3.51; N, 19.97.

4.2.7. 5-(2-Hydroxy-phenyl)-tetrahydro-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (5g). Colorless crystals obtained from **4** and salicylaldehyde and recrystallized from methanol; yield 61%; mp 165–166 °C; ^{1}H NMR (DMSO-d_6) δ 4.03 (br s, 1H, 4-NH), 4.24 (d, J = 4 Hz, 1H, 3a-CH), 4.53 (d, J = 4 Hz, 1H, 7a-CH), 5.96 (s, 1H, 5-CH), 7.29–7.40 (m, 3H, 4′/5′/6′-ArH), 7.47 (s, 1H, 6-NH), 7.58–7.61 (d, 1H, J = 6 Hz, 6′-ArH), 7.94 (br s, 1H, OH), 8.39 (s, 1H, 2-NH). EJ MS m/z 249 (M^+). Anal. calcd($C_{11}H_{11}N_3O_4$): C, 53.01;H, 4.45; N, 16.86. Found: C, 53.18; H, 4.48: N, 16.72.

4.2.8. 4-(4-Chloro-benzoylamino)-3-oxo-isoxazolidine-5-carboxylic acid amide (6). To a suspension of 5.8 g (0.04 mol) compound **4** in 80 mL anhydrous DMF and 4.5 g (0.045 mol) anhydrous TEA was added 7.87 g (0.045 mol) *p*-chlorobenzoyl chloride at −5 to 0 °C portion-wise over a period of 30 min. The mixture was stirred at 0 °C for 2 h and then at room temperature for 12 h. The precipitate of TEA·HCl was filtered and washed with anhydrous DMF. The filtrate was evaporated in vacuo to dryness. The residue was crushed with 30 mL of water and mixed with a solution of $NaHCO_3$ (5%). The precipitate was filtered and washed water and dried, giving 7.1 g of crude product (**6**). The product was recrystallized from methanol giving a pure (TLC) compound (**6**), 6.24 g (55% yield); mp 215–216 °C; ^{1}H NMR (DMSO-d_6) δ 4.40 (d, J = 4 Hz, 1H, 3a-CH), 4.76 (d, J = 4 Hz, 1H, 7a-CH), 7.19 (br s, 1H, NH), 7.38 (br s, 1H, NH), 7.43 (s, 1H, NH), 7.54 (d, J = 9 Hz, 2H, 2′/6′-ArH), 7.87(d, J = 9 Hz, 2H, 3′/5′-ArH), 8.10 (s, 1H, 2-NH). EJ MS m/z 283 (M^+). Anal. calcd ($C_{11}H_{10}N_3O_4Cl$): C, 46.57; H, 3.55; N, 14.81. Found: C, 46.71; H, 3.61; N, 14.92.

4.2.9. 5-(4-Chloro-phenyl)-3a,7a-dihydro-6H-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (7). To a suspension of 4.2 g (0.014 mol) **6** in 100 mL anhydrous toluene was added 20 mL of thionyl chloride, and refluxed for 8 h. After cooling, the solvent and excess thionyl chloride were distilled off under reduced pressure to dryness. The residue was mixed with 50 mL of water and neutralized with $NaHCO_3$. The precipitate was filtered, washed with water and dried. Two recrystallizations of the product from methanol gave a pure compound (**7**), 2.2 g (59% yield); mp 203–204 °C; ^{1}H NMR (300 MHz, DMSO-d_6)

δ 4.47 (d, $J=4$ Hz, 3a-CH), 4.89 (d, $J=4$ Hz, 1H, 7a-CH), 7.56 (d, $J=9$ Hz, 2H, 2′/6′-ArH), 7.92 (d, $J=9$ Hz, 2H, 3′/5′-ArH), 9.19 (s, 1H, 2-NH), 11.45 (br s, 1H, NH). EJ MS m/z 265 (M^+). Anal. calcd ($C_{11}H_8N_3O_3Cl$): C, 49.73; H, 3.03; N, 15.81. Found: C, 49.91; H, 3.05; N, 15.92.

4.2.10. 5-(4-Chloro-phenyl)- 6H-isoxazolo[4,5-*d*]pyrimidine-3,7-dione (8). To a solution of compound **5d** or 7 (0.001 mol) in acetone (250 mL) was added 2 g potassium permanganate to compound **5d** and 1 g to compound 7 and the mixture was refluxed in a water bath. The pink color of potassium permanganate slowly disappeared. Addition of potassium permanganate (about 0.3 g each time) and refluxing continued until the pink color persisted. The hot suspension was filtered and the excess acetone was distilled off to dryness. The residue was mixed with 20 mL of water and excess potassium permanganate destroyed with aq sodium sulphite. The white precipitate was filtered washed with water and dried. Two recrystallizations of the products from ethanol gave identical pure colorless compound **8** crystals, mp 215–216 °C, from compound (**5d**) with a yield of 55%, and from compound **7** with a yield of 62%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.55 (d, $J=9$ Hz, 2H, 2′/6′-ArH), 7.96 (d, 9 Hz, 2H, 3′/5′-ArH), 9.20 (br s, 1H, 2-NH), 11.35 (br s, 1H, NH). EJ MS m/z 263 (M^+). Anal. calcd ($C_{11}H_6N_3O_3Cl$): C, 50.11; H, 2.29; N, 15.94. Found: C, 50.21; H, 2.36; N, 16.06.

4.3. General methods for the biological activity evaluation

4.3.1. Material. Animals: male Wistar rats, 180–200 g, purchased from a breeder (licencsed by the Ministry of Agriculture, Warsaw, Poland), were used in this study.

The animals were housed under standard laboratory conditions with a 12 h light/dark cycle, light on at 6 a.m., in a temperature-controlled room at 21±2 °C, humidity 60% with free access to granulated standard food and tap water. The rats were kept four per cage (30×30×20 cm). Each experimental and control group consisted of eight animals.

The study was approved by the Governmental Committee for Scientific Research, grant No. 8.364/98.

Drugs: Sodium carboxymethyl cellulose pure B.P.C. — Koch Light Laboratories Ltd London, UK (CMC).

1. H_2O injection
2. compound **5b**, **5c**, **5d**, **5e**, **7**.

Compounds **5b**, **5c** and **5e** were administered as water solutions intraperitoneally (ip) 30 min before the test. Compounds **5d** and **7** in a CMC suspension were also injected intraperitoneally (ip) 30 min before the tests (peak value time).

4.3.2. Method used. The anxiolytic effects were determined according to the 'Two-compartment exploratory test' of Crawley,[24] in the modification of Merlo-Pich and Samanin.[25]

Procedure: The apparatus employed to test 'approach-avoidance behaviour' was a conventional open field (100×100 cm), the floor consisting of a sheet of white plastic painted with a black grid dividing the field into 25 (5×5) equal squares. This surface was divided into two compartments, one consisting of a square area (40×40 cm) (four squares) in one corner of the open field with all the surfaces blackened and a roof fitted 35 cm from the floor, to prevent light entering from above. The second compartment was the remaining part of the open field (21 squares), uniformly lit by a fluorescent lamp. No partitions were provided between the two compartments, the transition lines consisting of the internal half perimeter of the black area in the corner. At the beginning of the test, all rats were placed gently in the same peripheral, lighted white square near to the black compartment. The number of transitions between the two-compartments (BWT), square entries in the black compartment (BSE), and square entries in the white compartment (WSE) were recorded for 5 min by an observer sitting quietly 2 m away, unaware of the treatment.

Whenever an animal crossed the transition line with all four paws, an event was recorded. On removal of the rat the floor was thoroughly cleaned. Each rat was assessed in the novelty test only once, always between 10.00 a.m. and 2.00 p.m.

Locomotor activity was measured in the treated and control groups using eight 20.5×28×21 cm wire-grid cages each with two horizontal infrared photocell beams along the long axis 3 cm above the floor. Photocell interruptions were recorded by electromechanical counters in an adjacent room. After 30 min of habituation to the novel cage, rats were injected with the moclobemide and the photocell activity was recorded at 10-min intervals for 1 h. This test provided an index of basal locomotor activity of animals in a familiar environment, necessary to indicate the presence of the central stimulant effects of the compounds used in the novelty test.

4.3.3. Statistics. The statistical significance of the results of the anxiolytic trials was analysed according to the two-tailed Student t-test.[26] Statistical analysis for locomotor activity test was done by Dunnett's t-test.

4.4. The effect of Ro-15-1788 on the anxiolytic efficacy of diazepam and compounds 5b and 7 in the two-compartment exploratory test

4.4.1. Materials and methods. Animals: male Wistar rats, 180–200 g, purchased from a breeder (licencsed of the Ministry of Agriculture, Warsaw, Poland, were used in this study.

The animals were housed under standard laboratory conditions with a 12-h light/dark cycle, light on at 6 a.m., in a temperature controlled room at 21±2 °C, humidity 60%, with free access to granulated standard food and tap water. The rats were kept four per cage (30×30×20 cm). Each experimental and control group consisted of six animals.

The study was approved by the Governmental Committee for Scientific Research, grant No. 8.364/98.

4.5. Compounds

1. 3,7-Dioxo-5-phenyl-2,3,4,5,6,7,3a,7a-octahydroisoxazol[4,5-*d*]pyrimidine (**5b**) was administered at 1 mg/kg ip 30 min before the anxiolytic test.
2. 3,7-Dioxo-5-(*p*-chlorophenyl)-2,3,6,7,3a,7a-hexahydroisoxazol[4,5-*d*]pyrimidine (**7**) was administered at 1 mg/kg ip 30 min before the anxiolytic test.
3. Diazepam was administered at 2.5 mg/kg 30 min before the test.
4. Ro-15-1788 was administered ip 30 min before the test.

4.6. Anxiolytic effects

Anxiolytic effects were determined based on the two-compartment exploratory test of Crawley[24] in the modification of Merlo-Pich and Samanin.[25]

4.6.1. Procedure. The apparatus for testing the approach-avoidance behavior was a conventional open field (100×100 cm), the floor consisting of a sheet of white plastic painted with a black grid dividing the field into 25 (5×5) equal squares. This surface was divided into two compartments, one consisting of a square area (40×40 cm) (four squares) in one corner of the open field with all the surfaces blackened and a roof fitted 35 cm from the floor, to prevent light entering from above. The second compartment was the remaining part of the open field (21 squares), uniformly lit by a fluorescent lamp. No partitions were provided between the two compartments, the transition lines consisting of the internal half perimeter of the black area in the corner. At the beginning of the test all rats were placed gently in the same peripheral, illuminated white square, near to the black compartment. The number of transitions between the two compartments (BWT), square entries in the black compartment (BSE) and square entries in the white compartment (WSE) were recorded for 5 min by an observer sitting quietly 2 m away, unaware of the treatment.

An event was recorded whenever the animal crossed the transition line with all four paws. On removal of the rat, the floor was thoroughly cleaned. Each rat was assessed in the novelty test only once daily, always between 10.00 a.m. and 2.00 p.m.

4.7. Statistics

The data are shown as mean values ± SEM. The allocation of the animals to the experimental groups was randomized: (I) the distribution of the experimental data was normal; (II) the variances were homogenous. Statistical analysis for the anxiolytic test was done by the nonparametric Kruskal–Wallis's test for unpaired data and by the Friedman test for paired data. Statistical significance was then tested by the post-hoc Man test.

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