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SYNTHESIS AND PHARMACOLOGICAL SCREENING OF PYRAZOLO[3,4–*c*]PYRIDAZINE DERIVATIVES

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Abstract: Ethyl 3–chloro–6–(4–chlorophenyl)–pyridazine–4–carboxylate [**VII**] was cyclized with some nucleophilic reagents (hydrazine hydrate or *N*–monosubstituted hydrazines) to the new derivatives of pyrazolo[3,4–*c*]pyridazine [**IXa–d**]. The structures of the novel compounds were confirmed by elemental and spectral analyses. The effect of several synthesized derivatives on the central nervous system was studied.

Keywords: derivatives of pyridazine and pyrazolo[3,4–*c*]pyridazine, synthesis, structures, pharmacological screening.

Continuing our studies on the synthesis and pharmacological properties of fused systems derivatives (1), in this study we concentrate on a fused [6+5] system, *i.e.* pyrazolo[3,4–*c*]pyridazine. The physicochemical and pharmacological properties of pyrazolo[3,4–*c*]pyridazine are hardly known (2–6). In this paper, we report on the synthesis and pharmacological screening of a series of new 5–(4–chlorophenyl)–3–oxo–*1H*–pyrazolo[3,4–*c*]pyridazine derivatives.

INVESTIGATIONS, RESULTS AND DISCUSSION

Chemistry

Results of chemical experiments are shown in Schemes 1 and 2. α–Cyanoacid [**I**] was prepared as previously reported (7). Its hydrolysis and esterification yielded the respective diethyl ester [**II**]. In the reaction of [**II**] with hydrazine hydrate in ethanol, at room temperature, two products were invariably separated: dihydrazide [**III**] and 4–ethoxycarbonylpyridazinone [**IV**]. The result of this reaction depends on the amount of the reagents used (see Experimental part). The mixture was separated by a column chromatography. The chemical structure of compound [**III**] was confirmed by elemental and spectral analyses and also by a chemical route. The same product was also obtained in the reaction of diester [**II**] with hydrazine hydrate (method B). Next, compound [**IV**] was aromatized with bromine in acetic acid to yield acid [**V**] or ester [**VI**], respectively. This result depended on the temperature and time of reaction (see experimental part). Reaction of ester [**VI**] with phosphorus oxychloride at 75–80°C for 4 hrs gave chloroester [**VII**] in good to excellent yields (Scheme 1).

Chloroester [**VII**] was easily converted into 5–(4–chlorophenyl)–3–oxo–1*H*–pyrazolo[3,4–*c*]pyridazine [**IXa**] in reaction with excess hydrazine hydrate. However, the reaction of chloroester [**VII**] with excess methylhydrazine gave always two compounds: bicyclic derivative [**IXb**] and 6–(4–chlorophenyl)–3–methylhydrazinopyridazine [**VIII**]. Pyrazolo[3,4–*c*]pyridazine derivatives [**IXb–d**] were also obtained in good yields by the cyclization of chloroester [**VII**] with appropriate hydrazines in refluxed propan–1–ol (Scheme 2).

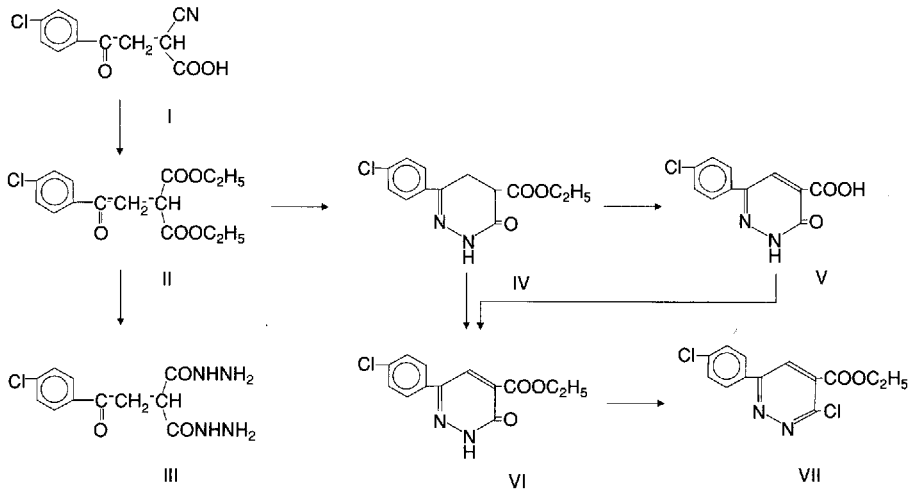
Pharmacology

Compounds [**VII**] and [**IXb**] were subjected to preliminary pharmacological studies. The two compounds investigated showed a similar biological activity. They had a low acute toxicity, with LD_{50}>1000 mg/kg (Table 2). Both compounds impaired the spontaneous and amphetamine–induced locomotor activity at their highest doses, equivalent to 1/10 of LD_{50} (Table 3 and 4). These compounds were not active in any others tests used.

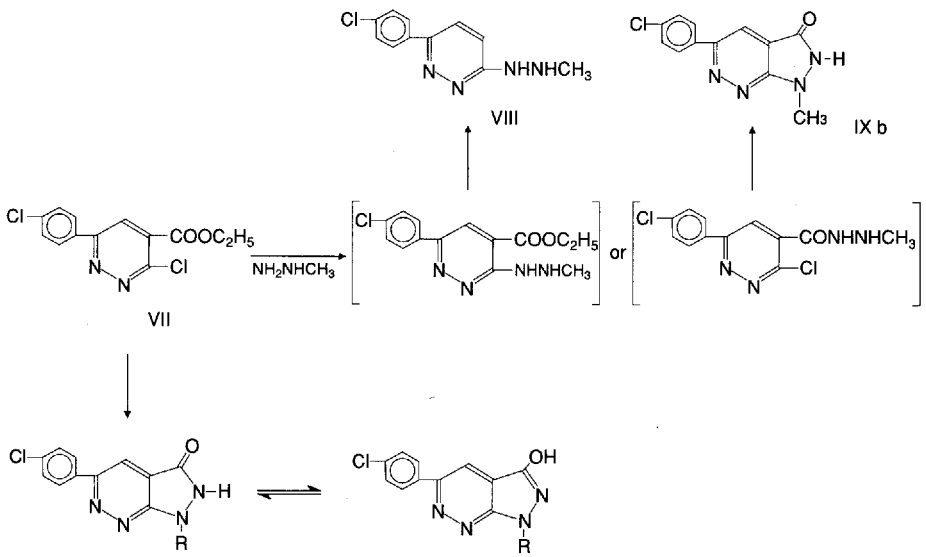
EXPERIMENTAL

Chemistry

All melting points are uncorrected. IR spectra were recorded on a Unicam SP–1000 spectrophotometer. ^{1}H NMR spectra were measured with a Tesla BS–587 (80 MHz) spectrometr. Results of



Scheme 1.



IX a R = -H
IX b R = -CH_3
IX c R = -CH_2CH_2OH
IX d R = -C_6H_5

Scheme 2.

elemental analyses agreed to within ±0.4% with the theory. The progress of the reaction and purity of each the compound were checked by TLC (Silica gel 60F 254 plates; Merck). The chromatograms were developed with: a) (7:3:1, v/v) benzene : ethyl ether : methanol; b) (1:1, v/v) methanol : chloroform; c) methanol : glacial acetic acid (9:1, v/v), d) (4:5:2, v/v) benzene : ethyl ether : methanol.

Spots were visualized in the iodine vapors. Single spots were obtained at all the plates.

α–Cyano–β–(p–chlorobenzoyl)propionic acid [**I**] was obtained according to the procedure described previously (7).

Diethyl 4–chlorophenacylmalonate [**II**]

A mixture of the α–cyanoacid [**I**] (4.8 g, 20 mmols) and 3 cm^3 of conc. H_2SO_4 in absolute ethanol (20 cm^3) was refluxed for 9 h. After cooling, water was added in excess, the mixture was extracted three times with diethyl ether and the ethereal layers were combined and shaken with a aqueous 3% Na_2CO_3, then washed with water, dried over Na_2SO_4, and evaporated in vacuo. The oily residue was used in the subsequent step without any further purification.

Yield 5.4 g (86%), b.p. decomp.

IR (film) : ν=1720, 1675 (C=O).

^{1}H NMR($CDCl_3$): δ=1.2 (t, 6H, $2CH_3$), 2.68 (m, 1H, CH), 3.53 (d, 2H, CH_2), 4.13 (q, 4H, $2CH_2$), 7.34 and 7.86 (2d, 4H, phenyl).
Formula: $C_{15}H_{17}O_5Cl$ (312.7)–calcd/found: 57.6/57.8% C; 5.5/5.4% H.

Ethyl 6–(4–chlorophenyl)–4,5–dihydro–3–okso–2*H*–pyridazin–4–carboxylate [**IV**]

To a cold solution of crude ester [**II**] (5.5 g, 17 mmols) in absolute ethanol (7 cm^3) 20.8% ethanolic solution of hydrazine (1.4 cm^3) was slowly added. The mixture was stirred for 2 h at 5–10°C, then left at room temperature for 3–5 days. The crude product (m.p. 137–158°C) was collected by filtration. The reaction mixture was purified by a column chromatography on silica gel eluted with (1:9, v/v) ethanol–chloroform to give [**IV**], which was recrystallized from ethanol. The elution was further continued with (1:1, v/v) methanol–chloroform to give [**III**] (21%), m.p. 199–200°C.
IV. Yield 54%, m.p. 168–170°C (ethanol), R_f (A)=0.78
IR (KBr) : ν=3240 (NH), 1740, 1680 (C=O).

Dihydrazide of 4–chlorophenacylmalonic acid [**III**]

Method A. The compound [**III**] was isolated according to the method described for compounds [**IV**].

Method B. To a cold solution of crude ester [**II**] (5.4 g, 17 mmols) in absolute ethanol (10 cm^3) a 20.8% ethanolic solution of hydrazine (6.65 cm^3) was added. The mixture was stirred for 2h at 5–10°C, then left at room temperature for 24 h. The precipitate was collected by filtration and recrystallized.

The compounds [**III**] obtained by methods A and B had identical properties; the m.p. of their mixture was not lowered.
Yield 75–80%, m.p. 199–200°C
R_f (B)=0.79, R_f (C) = 0.78
IR (KBr): ν=3380, 3320 (NH, NH_2), 1725, 1685 (C=O).
Formula: $C_{11}H_{13}O_3N_4Cl$ (284.7) – calcd/found: 46.4/46.8% C; 4.6/4.4% H; 19.7/19.4% N.

6–(4–Chlorophenyl)–3–oxo–2*H*–pyridazine–4–carboxylic acid [**V**]

To a stirred solution of ethyl ester [**IV**] (1.0 g, 3.6 mmols) in glacial acetic acid (10 cm^3) at 55°C 2 cm^3 of a solution of bromine (0.35 cm^3, 6.8 mmols in glacial acetic acid) was added dropwise. After 4.5–7.0 h stirring at 55–60°C, the mixture was left at room temperature for 24 h. The precipitate was collected by filtration, washed with diethyl ether and dried.
IR (KBr): ν=3260–2900 (NH, OH, CH, CH_2), 1760, 1670 (C=O).

Ethyl 6–(4–chlorophenyl)–3–oxo–2*H*–pyridazine–4–carboxylate [**VI**]

Method A (from ester [**IV**]). To a stirred solution of ethyl ester [**IV**] (1.0 g, 3.6 mmols) in glacial acetic acid (18 cm^3) at 40°C, 2 cm^3 of a solution of bromine (0.35 cm^3, 6.8 mmole in glacial acetic acid) was added dropwise. After 4 h stirring at room temperature, an excess of water was added, and the crystalline ester [**VI**] was collected by filtration.

Method B (from acid [**V**]). A mixture of acid [**V**] (0.6 g, 2.4 mmols) and 0.45 cm^3 of conc. H_2SO_4 in absolute ethanol (15 cm^3) was refluxed for 14 h. The mixture was cooled and crude ester [**VI**] was collected by filtration.

The physico–chemical properties of compound [**VI**] obtained by methods A and B were the same.
R_f (A) = 0.39
IR (KBr): ν=3260, 3180 (NH), 1720, 1690 (C=O).

Ethyl 3–chloro–6–(4–chlorophenyl)–pyridazine–4–carboxylate [**VII**]

Ester [**VI**] (0.6 g, 2.2 mmols) was heated in $POCl_3$ (2.3 cm^3) at 75–80°C for 4 h. The excess of $POCl_3$ was distilled off, and the residue treated with ice water and alkalized with a 3% Na_2CO_3. The product was extracted several times with diethyl ether and, after evaporation of the solvent, recrystallized from either *n*–heptane or ethanol.
R_f (A) = 0.53, R_f (D) = 0.79
IR (KBr): ν=1755 (C=O).

5–(4–Chlorophenyl)–3–oxo–*1H*–pyrazolo[3,4–*c*] pyridazine [**IXa**]

Chloroester [**VII**] (0.6 g, 2.0 mmols) was refluxed in 80% hydrazine hydrate (2 cm^3) on a water bath for 15 min. The excess of hydrazine was distilled off *in vacuo*, and the residue dissolved in water and acidified with glacial acetic acid. The precipitated product was collected by filtration, washed with water and recrystallized from ethanol.
R_f (A)=0.39
IR (KBr): ν=3180 (NH), 1650 (C=O).

5–(4–chlorophenyl)–1–methyl–3–oxo–pyrazolo[3,4–*c*]pyridazine [**IXb**] and 6–(4–chlorophenyl)–3–methylhydrazinopyridazine [**VIII**]

Chloroester [**VII**] (1.0 g, 3.3 mmols) was refluxed in methylhydrazine (8.95 cm^3) on a water

bath for 15 min. The mixture was cooled, water was added in excess, and compound [**VIII**] was collected by filtration. The aqueous filtrate, was acidified with glacial acetic acid, and precypitated the bicyclic compound [**IXb**] was collected by filtration.

VIII. IR (KBr): ν=3380 (NH), 1610 (C=C, C=N).

IXb. IR (KBr): ν=3260 – 3100 (NH, OH).

1–Alkyl/aryl–5–(4–chlorophenyl)–3–oxo–pyrazolo[3,4–*c*]pyridazine [**IXb–d**]

General method. Chloroester [**VII**] (0.5 g, 1.7 mmols) and appropriate hydrazine (3.4 mmols) in propan–1–ol (5 cm^3) was refluxed for 4.5 –5.5 h. The resulting crystalline product was collected by filtration, washed with water, and recrystallized from an appropriate solvent (see Table 1).

IXc. R_f (D)=0.57, (KBr): ν=3280–3100 (NH, OH).

IXd. R_f (D)=0.74, (KBr): ν=3300 (NH), 1700 (C=O).

Pharmacology

Experiments were carried out on male and female Albino–Swiss mice (body weight 18–25 g) and male Wistar rats (body weight 180–250 g). The compounds examined were administered intraperitoneally (*i.p.*) as suspensions in 3% Tween 80 at in constant volume of 10 ml/kg (mice) and 5 ml/kg (rats). The compounds were administered in doses equivalent to 1/10, 1/20, 1/40, 1/80 or 1/160 of LD_{50}. The control animals received the equivalent volume of the solvent. Each experimental group consisted of 8 animals.

The following pharmacological tests were performed:

1. Acute toxicity in mice.
2. Motor coordination in the rota–rod test in mice.
3. Spontaneous locomotor activity in mice.
4. Body temperature in normothermic mice.
5. Pain reactivity in the „writhing syndrome'' test in mice.
6. Anxiolytic properties in the „four plates'' test in mice.
7. Pentetrazol–induced seizures in mice.
8. Maximal electric shock in mice.
9. Amphetamine–induced locomotor hyperactivity in mice.
10. Hypothermic effect of reserpine in mice.
11. Arterial blood pressure in rats.

Acute toxicity was assessed by the method of

Table 1. Physical and analytical data of compounds [**IV–IXd**]

Comp. No.	Formula Molecular weight	M.p. (°C) Solvent	Yield %	Analyses Calculated/found			^{1}H NMR δ (ppm)[a]
				%C	%H	%N	
IV	$C_{13}H_{13}O_3N_2Cl$ 280.7	168-170 ethanol	54	55.6 55.7	4.7 4.6	10.0 10.2	1.2 (t, 3H, CH_3, J=8Hz), 3.2 (m, 2H, CH_2), 3.5 (t, 1H, CH), 4.16 (q, 2H, CH_2, J=8Hz), 7.5 (m, 4H, phenyl), 10.86 (s, 1H, NH).
V	$C_{11}H_7O_3N_2Cl$ 250.6	296-297 ethanol	83	52.7 53.1	2.8 2.8	11.2 11.4	
VI	$C_{13}H_{11}O_3N_2Cl$ 278.7	169-171 propan-2-ol	A.85 B.75	56.0 56.3	4.0 3.9	10.1 10.3	1.43 (t, 3H, CH_3, J=8Hz), 4.46 (q, 2H, CH_2, J=8Hz), 7.26 (s, 1H, H-5), 7.43 nad 7.77 (2d, 4H, phenyl), 8.28 (s, 1H, NH).
VII	$C_{13}H_{10}O_2N_2Cl_2$ 297.1	128-130 ethanol	95	52.5 52.8	3.4 3.5	9.4 9.3	
VIII	$C_{11}H_{11}N_4Cl$ 234.7	168-169 propan-2-ol	40	56.3 56.5	4.7 4.9	23.9 23.8	3.43 (s, 3H, CH_3), 3.8 (m, 2H, 2NH), 7.36 (m, 4H, phenyl), 7.9 (d, 2H, H-4, H-5, J=9Hz)
IXa	$C_{11}H_7ON_4Cl$ 246.7	338-340 ethanol	65	53.6 53.8	2.9 3.0	22.7 22.8	7.56 (m, 3H, 2H of phenyl and NH), 8.18 (d, 2H of phenyl), 8.19 (s, 1H, H-4), 8.48 (s, 1H, NHCO)
IXb	$C_{12}H_9ON_4Cl$ 260.7	303-305 ethanol	60	55.3 55.0	3.5 3.5	21.5 21.6	4.04 (s, 3H, CH_3), 7.57 and 8.16 (2d, 4H, phenyl), 7.99 (s, 1H, H-4), 8.47 (s, 1H, NH)
IXc	$C_{13}H_{11}O_2N_4Cl$ 290.7	219-221 propan-1-ol	55	53.7 54.0	3.8 3.9	19.3 18.9	
IXd	$C_{17}H_{11}ON_4Cl$ 322.8	164-166 ethanol	54	63.3 62.9	3.4 3.3	17.4 17.0	

A – from chloroester [**VIII**], B – from chloroacid [**V**].

[a] Spectra were run in $CDCl_3$ for [**IV**], [**VI**], [**VIII**], and in DMSO–d_6 for [**IXa**], [**IXb**].

Litchfield and Wilcoxon (8) and presented as LD_{50} calculated from the mortality of mice after 24 hours.

Motor coordination was measured according to the method of Gross and Tripod (9). The mice were placed for 2 min on the rod rotating at 4 rmp. The effects were evaluated 15, 30, 45, 60, 75, 90 and 105 min after administration of the compounds investigated.

Spontaneous locomotor activity in mice was measured in circular photoresistor actometers (32 cm in diameter). After the injection of the compounds tested, the animals were placed in the actometers for 1 h. Each crossing of the light beam was recorded automatically. The number of impulses was recorded after 30 and 60 min.

Body temperature in normothermic mice was determined by measuring the rectal temperature with a thermistor probe at a constant ambient temperature of 22°C ± 1°C. Measurements were taken twice before and 30, 60, 90, 120, 150 and 180 min after the administration of the compounds tested.

Pain reactivity was measured by the „writhing syndrome'' test of Witkin *et al.* (10). The test was performed in mice by an *i.p.* injection of 3% solution of acetic acid in a volume of 10 ml/kg 60 min after administration of the compounds tested. The number of writhing episodes was evaluated for 30 min after injection of 3% acetic acid.

Anxiolytic properties were assessed by the „four plates'' test in mice according to Aron *et al.* (11) 60 min after administration of the compounds investigated at doses not affecting spontaneous locomotor activity. The mice were placed in the cage with a 4 plates floor (11 x 7 cm) having a 4 mm gap between each. After 15 sec 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 sec.

Pentetrazol seizures were induced in mice by the pentetrazol administration at a dose of 100 mg/kg s.c. 30 min after treatment with the compounds tested. The animals were observed for 30 min and the number of mice developing clonic and tonic seizures, and deaths were recorded over that period.

Maximal electric shock was induced by means of alternating current (50 Hz, 25 mA, 0.2 sec) by the use of ear clip electrodes according to the method of Swinyard *et al.* (12). The criterion to indicate the convulsive response was the tonic extension of the hind limbs. The test was performed 60 min. after the administration of the compounds tested.

Amphetamine hyperactivity in mice was induced by *d*-amphetamine 2.5 mg/kg *s.c.* The compounds investigated were injected 30 min before the amphetamine administration. The locomotor hyperactivity was measured 30 and 60 min later in the photoresistor actometers.

Reserpine hypothermia in mice was induced by reserpine, 2.5 mg/kg *s.c.*, administered 18 h before the first temperature measurement in the rectum with a thermistor thermometer. The initial temperature was the mean of two measurements

Table 2. The influence of the compounds investigated on acute toxicity in mice

Comp. No.	LD_{50} (mg/kg)	95% confidence limit
VII	1800.0	(1723.0–1894.0)
IXb	1050.0	(937.5–1176.0)

Table 3. The influence of the compounds investigated on spontaneous locomotor activity in mice (n=8)

Comp. No.	Dose (part of LD_{50})	Number o impulses ± SEM after: 30 min	60 min
Control	–	210.4±10.0	325.3±16.3
VII	1/10	132.7±22.3[a)]	176.3±33.4[a)]
	1/20	219.4±30.0	303.1±27.2
IXb	1/10	143.9±19.2[a)]	191.3±27.0[b)]
	1/20	215.9±27.0	323.4±51.1

[a)] - $p<0.01$; [b)] - $p<0.001$ *vs.* control, Student's *t*-test

Table 4. The influence of the compounds investigated on the amphetamine-induced (2.5 mg/kg) locomotor hyperactivity in mice (n=8).

Comp. No.	Dose (part of LD_{50})	% of control ± SEM after: 30 min	60 min
Control	–	100.0±12.6 (383.3±47.0)	100.0± 7.0 (573.5±40.0)
VII	1/10	52.4±16.4[a)]	53.9± 7.5[b)]
	1/20	74.2±12.1	58.6±11.9
IXb	1/10	31.2± 8.1[b)]	35.4±12.1[b)]
	1/20	68.6±13.3	72.7±11.6

[a)] – $p<0.5$; [b)] – $p<0.001$ *vs.* control, Student's *t*-test

carried out at 30 min intervals before the administration of the compounds tested. Then, the temperature was measured 30, 60, 90,120,150 and 180 min later.

Arterial blood pressure was determined according to the method of Gerold and Tschirky (13) by using the UGO–BASILE equipment (Blood Pressure Recorder, cat. No. 8006). Systolic blood pressure on the tail artery was measured 30 min after administration of the compounds investigated.

Statistics

Results are presented as the means, and vere evaluated statistically by using either Student's t test or exact Fischer's test.

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