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Short communication

Synthesis and pharmacological activity of new carbonyl derivatives of 1-aryl-2-iminoimidazolidine

Part 3. Synthesis and pharmacological activity of 1-aryl-5,6(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*]imidazoles

Dariusz Matosiuk[a,*], Sylwia Fidecka[b], Lucyna Antkiewicz-Michaluk[c], Izabela Dybala[d], Anna E. Koziol[d]

[a] *Department of Synthesis and Technology of Drugs, Medical University in Lublin, Staszica 6, 20-081 Lublin, Poland*
[b] *Department of Pharmacodynamics, Medical University, Staszica 6, 20-081 Lublin, Poland*
[c] *Department of Biochemistry, Institute of Pharmacology, Polish Academy of Sciences, Smętna 12, 31-343 Kraków, Poland*
[d] *Department of Crystallography, Maria Curie-Sklodowska University, Pl. Marii Curie-Sklodowskiej 3, 20-031 Lublin, Poland*

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Abstract

Synthesis and pharmacological activity of 1-aryl-5,6(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*]imidazoles (**D**) are presented. The title compounds were obtained from 1-aryl-2-iminoimidazolidines (**1**) by cyclization reaction with oxalic acid derivatives–ethyl ester (**2**) or chloride (**3**). They were tested for pharmacological activity in animal and binding assay tests. With moderate acute toxicity ($LD_{50} \sim 200$ mg kg^{-1}, i.p.), they exhibited significant analgesic and serotonergic activities as results of the 'writhing' and the 'hot plate' tests indicated, and reduced number of 'head twitch' episodes after 5-HTP (5-hydroxytryptophan) administration. Reversion of the antinociception produced in the 'writhing' test by small dose of naloxon (5 mg kg^{-1}) can suggest an opioid-like mechanism of their analgesic activity. The probable receptor inhibition mechanism of their analgesic and serotonergic activity was confirmed in the binding assay tests (by radioligand displacement) toward the opioid μ and serotonin 5-HT_2 receptors. Additionally, they exhibited affinity toward the benzodiazepine (BZD) receptor as well, although in behavioral tests compounds did not produce any clear depressive effect on the central nervous system (CNS) of mice. Simple chemical structure of the title compounds, in comparison to other carbonyl derivatives of 1-aryl-2-iminoimidazolidine presented in this series of papers, underline very important role both of a hydrophobic moiety (aromatic ring) and polar groups (hydrogen-bond acceptors) in the serotonin receptor interaction. The co-existence of opioid-like, serotonergic and BZD receptor inhibition activity can be very interesting and can lead to creation of the novel group of antidepressants.

Keywords: CNS activity; Serotonergic activity; 5-HT_2 receptor recognition pharmacophore model; 1-Aryl-5,6(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*]imidazoles; Molecular structures

1. Introduction

5-Hydroxytryptamine (5-HT, serotonin) has been recognized for almost 50 years as a compound implicated in the regulation of a number of physiological processes. 5-HT is found in high concentrations in the gastrointestinal tract, in platelets, and in specific regions of central nervous system (CNS). It is spread in the body, but the exact sites and modes of action of 5-HT are often very difficult to explain which is probably, in large part, the consequence of numerous serotonin-receptor subtypes defined both by pharmacological analyses and cDNA cloning. Besides the effects on the platelet aggregation and gastrointestinal motility, 5-HT shows a regulatory effect on the CNS. It influences sleep, cognition, sensory perception, motor activity, temperature regulation, nociception, appetite, sexual

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* Correspondence and reprints

E-mail address: darek@eskulap.am.lublin.pl (D. Matosiuk).

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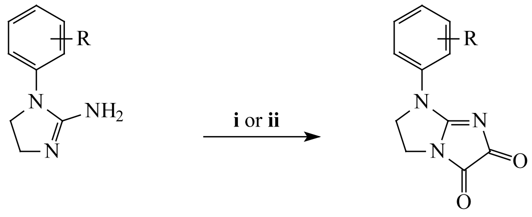
behavior and hormone secretion. Serotonin plays a very important role in the mood disorders, especially in the anxiety and depression, aggression and impulsivity, or control of the sleep-wakefulness cycle. Regulation of the mood disorders is possible either by agonistic or antagonistic action on the certain type of the serotonin receptors. The majority of recently used anxiolytics and antidepressants have affinity toward the 5-HT receptors, mainly 5-HT$_{1A}$, 5-HT$_{2A/2C}$ and 5-HT$_3$ [1], usually correlated with an effect on norepinephrine and dopamine functions. Partial agonizm of the benzodiazepine (BZD) receptor is also involved in the treatment of acute anxiety [1].

2. Chemical part

The 1-aryl-5,6(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*]imidazole derivatives (**D**) were synthesized by condensation of the 1-aryl-2-aminoimidazolines-2 [2] (**1**) and oxalic acid derivatives such as diethyl ester (**2**) or chloride (**3**) (Scheme 1). Both reagents gave similar results. Moreover, when the reaction was carried out with diethyl oxalate (**2**) under mild conditions (reaction temperature in range of 100–120 °C) it was possible to obtain chain derivatives—products of the acylation of the 1-aryl-2-aminoimidazolines-2 by one ester group of the ethyl oxalate.

The physicochemical properties of the synthesized compounds are presented in Table 1. NMR spectral characteristic of the imidazo–imidazolines (**D**) revealed in their ^{1}H-NMR spectra two double-doublet signals of the H2 and H3 at ca. 4 and 4.5 ppm, with the coupling constants of $J \sim 9$ and $J' \sim 7$ Hz. The difference between chemical shifts of both signals can suggest an important difference in the acidity of hydrogen atoms which was confirmed by the chemical shift values of respective carbon atoms in their ^{13}C-NMR spectra (ca. 38 ppm for C2 and 51 ppm for C3). The chemical shift values of C=O carbon atoms exhibit unequal character as well. The $\Delta\delta$ value is about 15 ppm and can suggest that the C6=O carbonyl group is a much better hydrogen-bond acceptor than the C5=O group. The spectral data of the compounds **D** are presented in Table 2.



i = $(COOEt)_2$, 100-120°C, 2h, 200-220°C, 0.5h;
ii = $(COCl)_2$, NEt_3, acetone, DMAP, room temp., 6h.

Scheme 1.

Table 1
Physicochemical properties of 1-aryl-5,6(1*H*)dioxo-2,3-dihydroimidazo-[1,2-*a*]imidazoles (**D**)

No.	R	R_f	M.p. (°C)	Yield (%)	
				(i)	(ii)
D1	H	0.37	280–1	53.6	30.7
D2	4-CH_3	0.34	237–8	49.7	38.2
D3	4-Cl	0.31	266–8	41.3	30.9
D4	4-CH_3O	0.39	242–4	30.8	18.3
D5	2-Cl	0.32	283–5	–	21.7

The IR spectra confirmed the structure of compounds investigated. They can be recognized by the presence of specific absorption bands of C=N moiety at 1580 and two absorption bands of C=O moieties at 1710 and 1680 cm^{-1}. The absence of any N–H characteristic bands in the shorter wave length region (ca. 3400 cm^{-1}) suggests cyclic structure. Both carbonyl groups exhibit unequal character confirmed by absorption of different wave lengths.

The X-ray analysis of the 1-(4-methylphenyl)-5,6(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*] imidazole (**D2**) crystals confirmed both the molecular structure and the preferred pattern of interactions between molecules.

3. Crystallographic part

The structural data obtained for **D2** indicated planarity both of the heterocyclic dihydroimidazo–imidazole system and the phenyl ring, and a co-planarity of these fragments in the solid state (Fig. 1). The torsion angles within the five-membered rings are less than 3°, which—as a result—introduces stress and affects the endocyclic valence angles. The respective angles at the C*sp^3* atoms are: N1–C2–C3 105.1(2) and C2–C3–N4 102.1(2)°, while angles at the carbonyl C-atoms are: N4–C5–C6 101.9(2) and C5–C6–N7 109.4(2)°. The C=O bond distances are of different length as well as the C–N distances within the guanidine unit (Fig. 1).

The only hydrogen-bond donors of **C2** are the hydrophobic C–H groups and an arrangement of molecules in the crystal lattice enables formation of the strongest possible contacts, i.e. the C–H···O hydrogen bonds. Multiple intermolecular contacts involve the carbonyl O-atoms as acceptors and the acidic C2, C3 and phenyl C-atoms as donors (Fig. 2). In the homomolecular environment, some non-equivalence of the O5 and O6 as acceptors is observed (Table 3), indicating shorter H···O6 distances and the C–H···O6 angles closer to 180° than that of the C–H···O5.

Table 2
NMR data of 1-aryl-5,6(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*]imidazoles (**D**)

No.	^{1}H-NMR			^{13}C-NMR					
	H2 (dd, *J* (Hz))	H3 (dd, *J* (Hz))	Ar (m)	C2	C	C5	C6	C7a	Ar
D1	3.97 ($J = 9$, $J' = 7.5$)	4.50 ($J = 8.9$, $J' = 7.6$)	7.30–7.80	38.2	51.5	158.4	174.7	169.8	120.8, 126.6, 129.8, 136.8
D2	3.99 ($J = 9$, $J' = 7.3$)	4.46 ($J = 8.9$, $J' = 7.6$)	7.30–7.70	37.8	51.0	157.9	174.0	168.9	120.0, 129.7, 134.1, 135.4
D3	4.00 ($J = 9$, $J' = 7.5$)	4.48 ($J = 9$, $J' = 7.7$)	7.30–7.70	38.1	51.1	158.8	174.3	169.8	120.4, 132.6, 135.2, 136.7
D4	3.96 ($J = 9$, $J' = 7.5$)	4.46 ($J = 8.9$, $J' = 7.6$)	7.25–7.65	38.3	51.4	158.3	174.4	168.7	120.2, 130.9, 133.9, 136.2
D5	3.94 ($J = 9$, $J' = 7.4$)	4.45 ($J = 9$, $J' = 7.7$)	7.20–7.70	37.1	50.9	158.9	174.1	169.9	118.1, 119.4, 122.7, 126.5, 128.9, 137.5

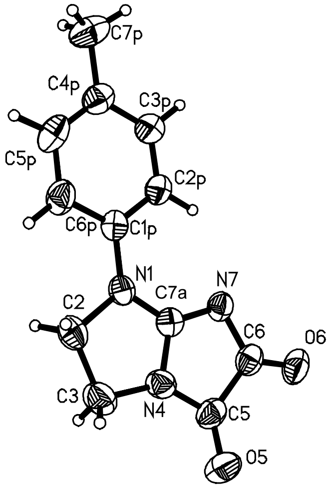


Fig. 1. View of the **D2** molecule. Bond distances within the 5,6(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*]imidazole system are: N1–C8, 1.328(2); N1–C2, 1.481(2); C2–C3, 1.527(3); C3–N4, 1.452(3); N4–C5, 1.362(2); N4–C8, 1.374(2); C5–O5, 1.204(2); C5–C6, 1.556(3); C6–O6, 1.214(2); C6–N7, 1.379(2); N7–C8, 1.318(2) Å.

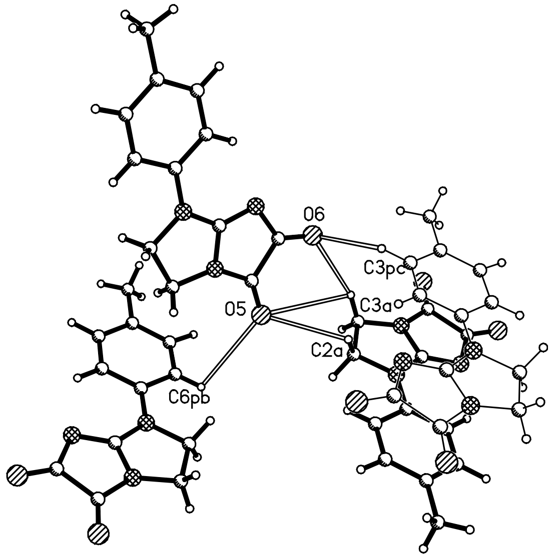


Fig. 2. The intermolecular C–H···O contacts between the neighboring **D2** molecules in the crystal.

Table 3
Geometry of the intermolecular C–H···O contacts in the crystal of **D2**

	C–H (Å)	C···O (Å)	H···O (Å)	∠ CH···O (°)
C2^{a}–H21···O5	0.99(2)	3.187(3)	2.86	100
C3^{a}–H31···O5	0.94(3)	3.424(3)	3.14	100
C3^{a}–H31···O6	0.94(3)	3.407(3)	2.50	162
C6p^{b}–H6p···O5	0.93(2)	3.342(3)	3.07	105
C3p^{c}–H3p···O6	0.98(3)	3.424(3)	2.55	147

Symmetry codes: a = $x+0.5$, $0.5-y$, $z+0.5$; b = $0.5-x$, $y-0.5$, $1.5-z$; c = $0.5-x$, $y-0.5$, $2.5-z$.

4. Pharmacological part

4.1. Behavioural tests

Two compounds, **D1** and **D2**, were tested for their pharmacological activity. They exhibited moderate acute toxicity: 180 mg kg^{-1} (133.3–243.0) for **D1** and 178 mg kg^{-1} (145.9–217.2) for **D2**, respectively. High doses of the investigated compounds initially induced sedation and then slight excitation with tremor, and finally convulsions. Rigidity, piloerection and hypersensitiveness were also observed. The depressive action on spontaneous locomotor activity in mice was observed after administration of both investigated compounds in the doses of 0.2 LD$_{50}$ only (Fig. 3). Amphetamine hyperactivity was not changed by both compounds. In other tests (hexobarbital-induced narcosis, motor coordination, body temperature, pentylenetetrazole-induced seizures) the investigated substances did not produce any clear effects. In the ‘hot plate’ test the investigated compounds, given at 0.1 LD$_{50}$, significantly prolonged the response to thermal stimuli but only in 30 min of the experiment (data not shown). The potent antinociceptive activity of both compounds was observed in the ‘writhing’ test in mice. Naloxone (5 mg kg^{-1}) significantly attenuated the antinociceptive effects of **D1** but not that of **D2** (Fig. 4). The head-twitch responses to 5-hydroxytryptophan (5-HTP) were significantly decreased by both tested compounds (Fig. 5).

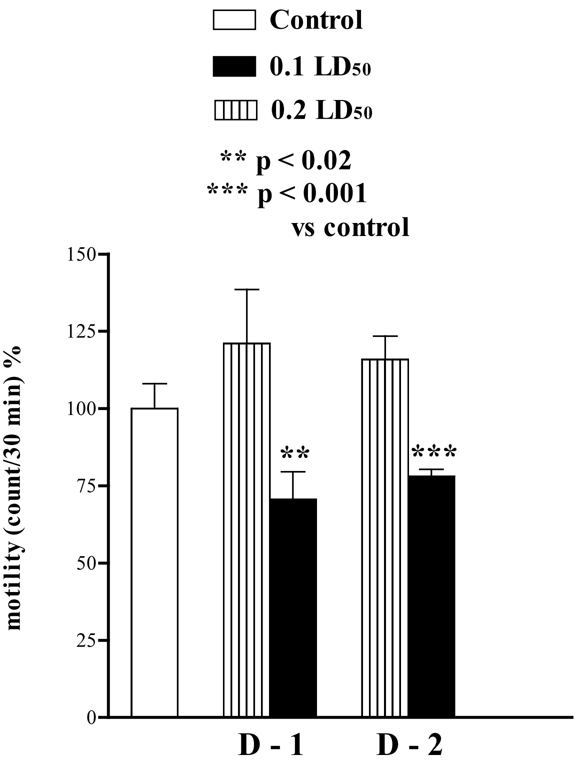


Fig. 3. The influence of the tested compounds (**D1**, **D2**) on the spontaneous locomotor activity of mice. Note: number of movements in the control groups was 163–214 (= 100%). The results are expressed as mean ± SEM for a group of 10 mice.

4.2. Receptors affinity tests

One compound, **D1**, with the highest activity in the behavioural tests was subjected to the binding affinity tests. Three types of receptors were investigated: i.e. opioid (type μ), serotonin (5-HT_2) and BZD. Radioligand displacement method was applied. Selective antagonists for each receptor were used: naloxone in the presence of sodium ions for the opioid μ receptor, ketanserin for the 5-HT_2 receptor and flunitrazepam for the BZD receptor. The affinity was calculated as EC_{50} values and the results are presented in Table 4. The specific binding, represented by displacement curves, is shown in Figs. 6–8.

5. Results and discussion

Results of the pharmacological investigation showed that the series **D** possess significant influence on the CNS of laboratory animals. The most important seems to be their antinociceptive and serotonergic effects.

The antinociceptive activity of investigated compounds in doses used was comparable with effects expressed by morphine administration in doses 0.5–2 mg kg^{-1} in the writhing test in mice [3]. This activity can be explained by structure similarities to other carbonyl derivatives of 1-aryl-2-iminoimidazolidine, presented in this series of papers [2,3], and underline the high importance of both hydrophobic and polar, e.g. hydrogen-bond, interactions between ligands and the active domain of the opioid receptors. In contrast, the serotonergic activity and affinity toward the 5-HT_2 receptor could not be unequivocally explained.

An expected analgesic/antinociceptive activity was confirmed applying the non-classical μ receptor pharmacophore [2]. Reversion of the antinociceptive effect by small dose of naloxone suggests that this group of compounds can act on the same active site in the receptor structure. The lower affinity between all four

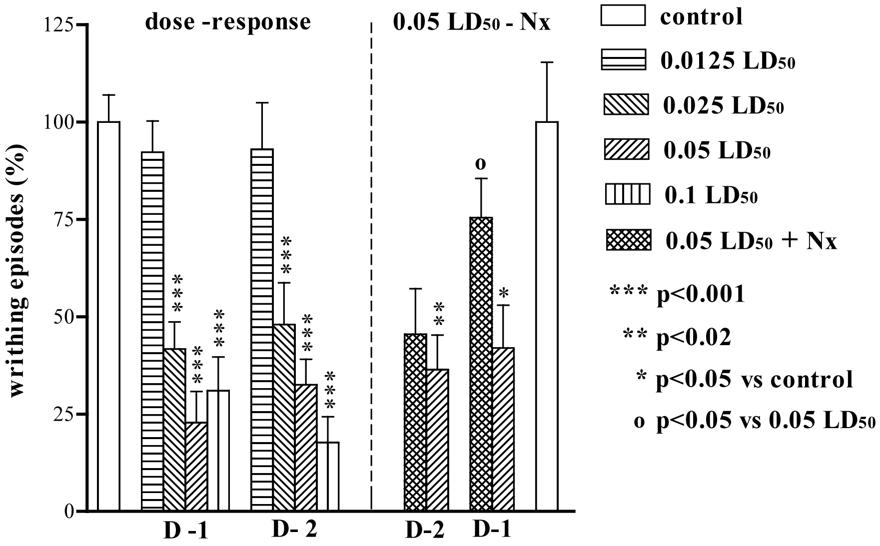


Fig. 4. The antinociceptive effects of the tested compounds (**D1**, **D2**) and the influence of naloxone (Nx), 5 mg kg^{-1}, s.c., on the activities assessed in the 'writhing' test in mice. Note: Number of writhing episodes in the control groups was 24–35 (= 100%). The results are expressed as means ± SEM of a group of eight mice.

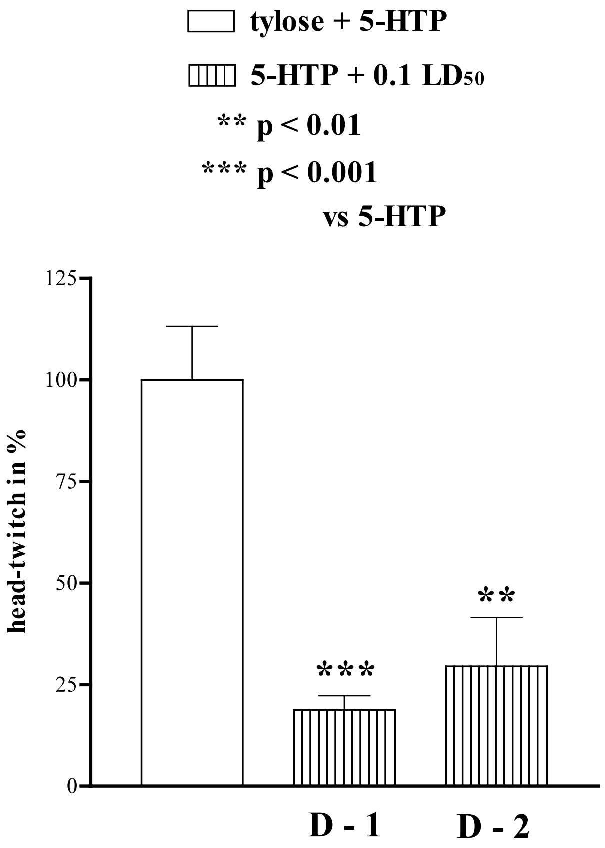


Fig. 5. The influence of the tested compounds (**D1**, **D2**) on the 'head twitch' responses evoked by 5-HTP (180 mg kg^{-1}). Note: number of 'head twitch' in the control group was 11.5 (= 100%). The results are expressed as mean ± SEM of a group of eight mice.

Table 4
EC_{50} values (μmol) in binding tests of compound **D1** on the opioid μ, serotonin 5-HT_2 and BZD receptors

	μ	5-HT_2	BZD
Naloxone	0.013	–	–
Morphine	0.20	–	–
Ketanserin	–	0.002	–
Serotonin	–	1.90	–
Clonazepam	–	–	0.001
Diazepam	–	–	0.005
D1	14.0	8.4	31.0

series investigated can be probably caused by the lack of the third point of the pharmacophore in the molecules of series **D** (lack of the second hydrophobic moiety). Despite that, the presence of only two pharmacophoric elements seems to be satisfactory for interaction with the receptor. Nevertheless, presence of one hydrophobic moiety and two polar carbonyl groups seems to be responsible for the serotonergic activity. Ketanserin, having a dioxo-heterocyclic ring, is a potent and selective antagonist of the 5-HT_2 receptor, and its structure gave rise to a large group of selective 5-HT_{2A} antagonists useful as drugs [4]. This moiety is considered responsible for formation of two of six contacts with the receptor [4]. Similar substructure can be found in the group of selective 5-HT_{1A} agonists of buspirone family [5,6]. Although, the moiety itself is not included in the 5-HT_{2A} antagonist pharmacophore model it is said that 'the additional hydrophobic substituents or a carbonyl group in the heterocyclic ring enhances the antagonistic potency' [7]. In 5-HT_{1A} pharmacophore models the presence of the carbonyl moiety as well as hydrophobic one and the basic nitrogen atom is considered crucial. These moieties, although not fully included in the model of receptors inhibition/excitation, could be important in the recognition step.

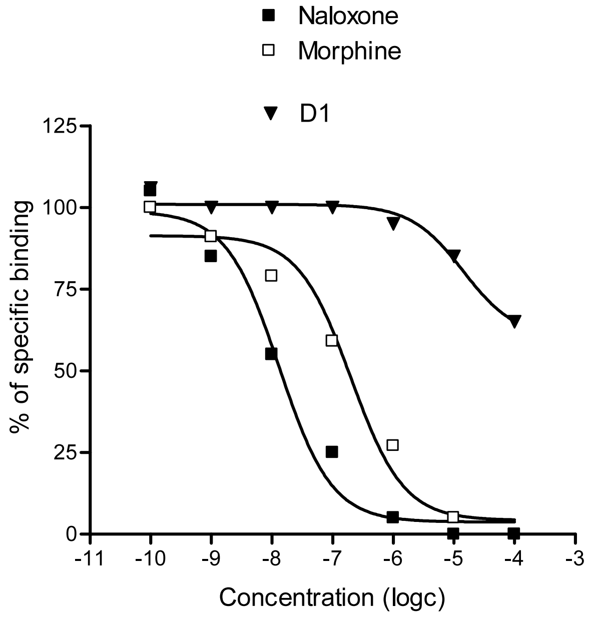


Fig. 6. Displacement of ^{3}H-naloxone from its binding sites by the investigated compound **D1** (the μ opioid receptor–limbic forebrain).

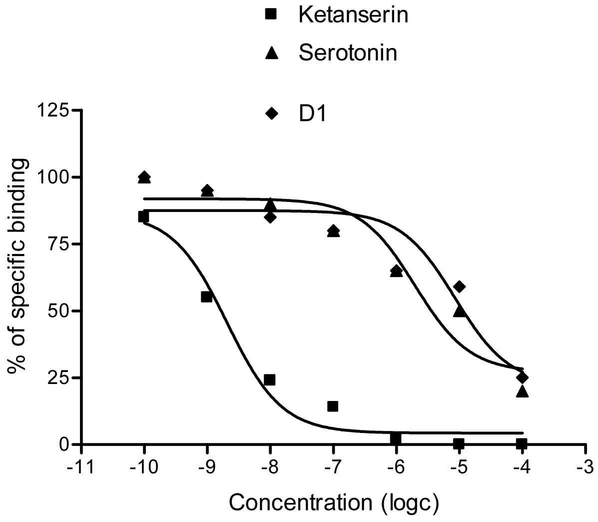


Fig. 7. Displacement of ^{3}H-ketanserin from its binding sites by the investigated compound **D1** (the 5-HT_2 receptor).

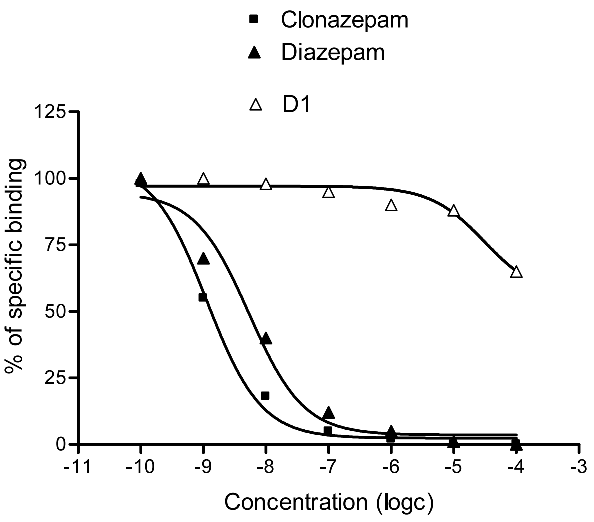


Fig. 8. Displacement of ^{3}H-flunitrazepam from its binding sites by the investigated compound **D1** (the BZD receptor).

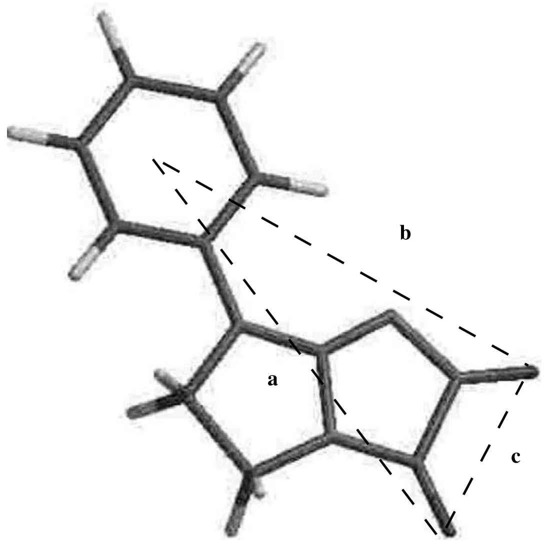


Fig. 9. Intramolecular distances and angles in the proposed pharmacophore of 5-HT$_2$ receptor recognition for the series **D** ($a = 7.1$, $b = 6.5$, $c = 2.9$ Å; $ab = 25$, $bc = 90$, $ac = 65°$) in comparison to the respective dimensions of **C2** [2] ($a = 7.1$, $b = 5.8$, $c = 4.3$ Å; $ab = 37$, $bc = 87$, $ac = 56°$).

On the basis of the molecular structure of series **D**, we would like to propose the serotonergic 5-HT$_2$ receptor recognition pharmacophore (Fig. 9). It includes one hydrophobic and two polar (carbonyl) groups. It seems that interactions with both polar groups should be direct and close to the plane of the molecule (Fig. 10). Structure analysis of previously investigated [3] 1,6-diaryl-5,7(1*H*)dioxo-2,3-dihydroimidazo[1,2-*a*][1,3,5]triazine derivatives (**C**) seems to confirm this theory.

An additional rigid substituent (aromatic ring) at N6 blocking direct access to the oxo groups is probably the main cause that those compounds expressed no affinity toward the 5-HT$_2$ receptor in the binding affinity test.

The interactions of carbonyl groups within the proposed pharmacophore can be considered as isolated or chelating. The last one seems to be more probable. It can be confirmed by the network of weak hydrogen bonds formed between **D2** molecules in the homomolecular crystal (Fig. 2, Table 3), as well as between four other literature available 4,5-dioxo-dihydroimidazo derivatives [8–10] substituted by hydrophobic groups only. Formation of the three-center hydrogen bond with the neighboring carbonyl O-atoms being acceptors of the same hydrogen atom is considered important as well in the pharmacophore model of the AMPA/Gly$_N$ receptor antagonists [11], with quinoxaline-2,3-dion template.

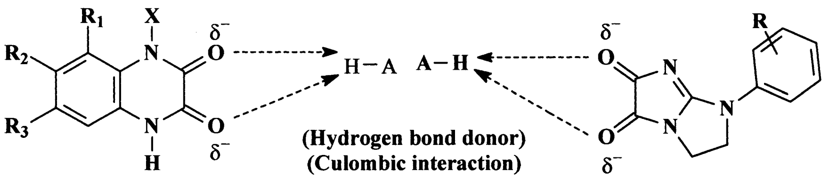
It can be expected that similar interactions, even in presence of weak acceptor-type hydrogen bonds only, are responsible for bonding of molecules of series **D** into the 5-HT$_2$ receptor.

In the firs part of this series of papers [2], bonding of the other groups of compounds (**A** and **B**), but on the lower level than that of **D**, to the 5-HT$_2$ receptor was confirmed. This weaker bonding could results from the presence of only one strong hydrogen-bond acceptor site, i.e. carbonyl moiety, in the molecules of imidazoline-urea derivatives (series **A** and **B**).

6. Experimental

6.1. Chemical analysis

Chemicals (oxalic acid derivatives) were purchased from Merck as ‘synthesis grade’ and used without further purification. Melting points (m.p.) were determined on a Boetius apparatus and are given uncor-



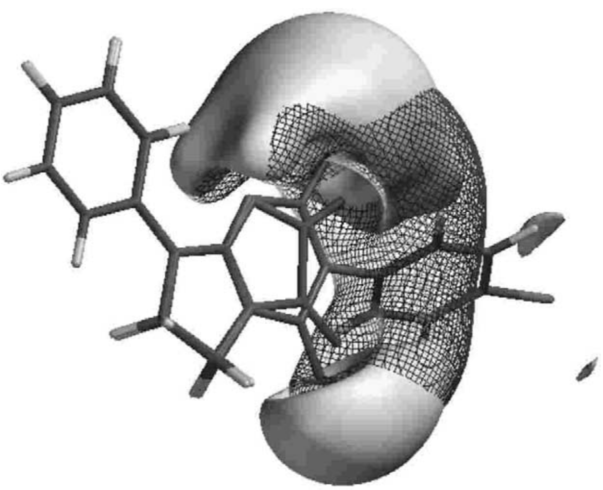


Fig. 10. Fitting of the optimized structures of the compounds **C2** and **D1** (three nitrogen atoms of guanidine moiety are fitted). EPM is presented as equipotential surface −20 kcal mol^{-1} (solid for **C2** [3], mesh for **D1**). Note: energy optimization performed by PC Spartan Pro [20], ab initio module, RHF approximation, 3-21G(*) basis set.

rected. NMR spectra (^1H and ^{13}C) were recorded on a Varian Gemini 200 MHz and a Brucker 200 MHz spectrometers in d_6-DMSO with TMS as an external standard at 295 K. TLC was performed on commercial Merck SiO_2 60 F_{254} plates with eluent systems: **(I)** chloroform–methanol (4:1) and **(II)** benzene–diethyl ether (8:1); visualization: UV light $\lambda = 254$ and 355 nm. Flash chromatography was performed on commercial silica-gel 60 mesh (Merck) in a glass column (45 × 250 mm) with eluent system **II**. Elemental analyses were performed on a Perkin–Elmer analyser and were in range of ±0.5% for each element analysed (C, H, N, Cl).

6.1.1. *Synthesis of 1-aryl-5,6(1H)dioxo-2,3-dihydroimidazo[1,2-a]imidazoles (method **i**) (general procedure)*

Free base of 1-aryl-2-iminoimidazolidine [2] (0.05 mol) was added into 100 mL of ethyl oxalate and the mixture was heated in 100–120 °C for 30 min, and 200–220 °C for next 30 min. The amount of the collected EtOH was a measure of the reaction course. The mixture was refrigerated overnight and the precipitate yielded was collected. It was purified by recrystallization from propanol-2.

6.1.2. *Synthesis of 1-aryl-5,6(1H)dioxo-2,3-dihydroimidazo[1,2-a]imidazoles (method **ii**) (general procedure)*

Free base of 1-aryl-2-iminoimidazolidine [2] (0.05 mol) was added into 100 mL of C_3H_6O containing 0.1 mol (10.5 g) of Et_3N. The 0.5 mol (6.35 g) of oxalil chloride in 50 mL of C_3H_6O was added over the time of 30 min. The mixture was stirred for 6 h at room temperature. During that time precipitation of the solid started. The crude product was separated, solved in a small amount of DMF and the crystallization process was started with addition of 50 mL of propanol-2. The precipitation yielded was collected and purified as mentioned above.

6.2. *X-ray crystal structure analysis*

Diffraction data for **D2** were measured on a Siemens P3 diffractometer at ambient temperature (295 K) using variable speed for $\theta - 2\theta$ scans and graphite monochromated molybdenum radiation Mo Kα ($\lambda = 0.71073$ Å). A single crystal of dimensions 0.15 × 0.20 × 0.45 mm was used. The crystal is monoclinic with the space group $P2_1/n$ and unit-cell parameters: a, 9.074(1); b, 11.657(2); c, 10.322(2) Å; β, 97.91(1)°; V, 1081.3(3) $Å^3$; Z, 4, d_{calc}, 1.408 g cm^{-3}.

Of 2664 reflections collected up to $\theta_{max} = 27.56°$, 2511 were independent reflections [$R_{int} = 0.0304$], and they were used in the calculations. Crystal structure was solved by direct methods using the SHELXS-86 [12] program and refined by the full-matrix least-squares on F^2 using the SHELXL-93 program [13]. The non-hydrogen atoms were refined with anisotropic displacement parameters. The H-atoms were located in a difference electron-density map and were refined with individual isotropic displacement parameters. Final discrepancy factors are $R_1 = 0.0473$, $wR_2 = 0.1018$ for $I > 2\sigma(I)$, and $R_1 = 0.1030$, $wR_2 = 0.1206$ for all data; $S = 0.943$.

6.3. *Pharmacological analysis*

6.3.1. *Behavioural tests*

6.3.1.1. Materials and methods. The experiments were performed on male Albino Swiss mice (17–30 g). The animals were kept 8–10 to a cage, at room temperature of 20 ± 1 °C, on a 12/12 h dark–light cycle. Standard food (Bacutil, Motycz, Poland) and water were available ad libitum. The investigated compounds (**D1**, **D2**) were administered intraperitoneally (i.p.) as suspensions in aqueous solution of 0.5% methylcellulose (tylose). The compounds were injected 60 min before the test. The controls received an equivalent volume of the solvent.

All tests performed, suggested by Vogel and Vogel [14] are generally accepted as basic in investigation of the central activity by behavioral methods.

The acute toxicity of the compounds was assessed in mice according to the Litchfield and Wilcoxon [15].

The activity of the compounds was assessed in the locomotor activity tests (measured in photoresistor actometer for single mouse for 30 min) as: (a) spontaneous activity; (b) amphetamine-induced hyperactivity—mice received subcutaneously (s.c.) 5 mg kg^{-1} of amphetamine 30 min before the test.

Nociceptive reactions were studied in: (a) the ‘hot plate’ test (56 °C) described by Eddy and Leimbach [16]—the test was carried out 60 min before and 30, 60, 90 and 120 min after the compounds administration; (b) the acetic acid (0.6%) induced ‘writhing’ test [17]—the number of writhing episodes was measured for 10 min starting 5 min after the i.p. administration of the acid solution.

Hexobarbital (65 mg kg^{-1})—induced narcosis was recorded as the time elapsing between the loss and recovery of the righting reflex.

Motor coordination was evaluated in the rota-rod test [18].

Body temperature in normothermic mice was measured in the rectum by means of thermistor thermometer.

Pentylenetetrazole (110 mg kg^{-1}, s.c.)—induced convulsions were evaluated as the number of mice clonic seizures, tonic convulsions and dead animals.

‘Head twitch’ responses after 5-HTP, according to Corne et al. [19]. Mice received 5-HTP (180 mg kg^{-1}, i.p.) and number of head twitches was recorded in six 2-min intervals (4–6, 14–16, 24–26, 34–36, 44–46, 54–56 min).

Influence of naloxone (5 mg kg^{-1}, s.c.) on antinociceptive effect of tested compounds was assessed in the writhing test.

The compounds were injected in doses equivalent to 0.2, 0.1, 0.05, 0.025 and 0.0125 LD_{50} (36, 18, 9, 4.5 and 2.25 mg kg^{-1}, respectively). Statistics: the obtained data were calculated by Student’s t-test and χ^2-test with Yates correction (pentylenetetrazole-induced seizures).

6.3.2. Binding assays

The affinity of compound **D1** toward the opioid μ, serotonin $5\text{-}HT_2$ and BDZ receptors was investigated by radioligand displacement method. The radioligands ^{3}H-flunitrazepam (specific activity 81 Ci $mmol^{-1}$), ^{3}H-ketanserin (specific activity 63.7 Ci $mmol^{-1}$) and ^{3}H-naloxone (specific activity 52 Ci $mmol^{-1}$) were purchased from Amersham. In all experiments samples were counted for radioactivity in a Beckman LS 3801 scintillation counter. The specific binding was defined as the difference between the total and unspecific binding. EC_{50} was estimated using a GraphPad Prism computer program.

6.3.2.1. Displacement of ^{3}H-naloxone (52 Ci $mmol^{-1}$) by investigated compounds. Tissues (cerebral cortex) from individual naive animals were processed separately. They were homogenised at 0 °C in 20 volumes of 50 mmol L^{-1} Tris–HCl buffer pH 7.6. The homogenate was centrifuged at 0 °C and 25 000 × g for 30 min, and the pellet was rehomogenised and incubated in a shaking water bath at 37 °C for 45 min (preincubation), and then recentrifuged at 0 °C and 25 000 × g for 30 min. The pellet (fraction P_1+P_2) was stored at −20 °C for no longer than 48 h. For incubation it was reconstituted in 20 volumes of the incubation buffer pH 7.6. The incubation mixture contained 100 mmol L^{-1} NaCl; final volume 550 μL consisted of 450 μL membrane suspension, 50 μL of ^{3}H-naloxone solution (2.5 nmol) and 50 μL of buffer containing seven concentrations (1 nmol–100 μmol) of the investigated compound **D1**. For measuring the unspecific binding, naloxone in a final concentration of 10 μmol was used. The incubation was carried out in duplicates, in a shaking water bath, at 30 °C for 30 min. The addition of the radioligand initiated the incubation, which was terminated by rapid filtration through GF/C Whatman fiberglass filters. The filters were then rinsed twice with 5 mL portions of ice-cold incubation buffer and were placed in plastic scintillation minivials. Scintillation fluid (3 mL) was added, and the samples were counted for radioactivity.

6.3.2.2. Displacement of ^{3}H-ketanserin (63.7 Ci $mmol^{-1}$) by investigated compounds. Tissues (structures) from individual naive animals were processed separately. They were homogenised at 0 °C in 20 volumes of 50 mmol $^{-1}$ Tris–HCl buffer, pH 7.6. The homogenate was centrifuged at 0 °C and 25 000 × g for 15 min, and then the pellet was rehomogenised and incubated in a shaking water bath at 37 °C during 15 min (preincubation), and then recentrifuged at 0 °C and 25 000 × g for 30 min. After decanting the supernatant, the pellet (fraction P_1+P_2) was stored at −20 °C for no longer than 48 h. For incubation it was reconstituted in 20 volumes the incubation buffer pH 7.6. The incubation mixture (final volume 550 μL) consisted of 450 μL membrane suspension, 50 μL of ^{3}H-ketanserin solution (0.6 nmol) and 50 μL of buffer containing seven concentrations (1 nmol–100 μmol) of the compound investigated **D1**. For measuring unspecific binding, cold ketanserin in a final concentration of 10 μmol was used. The incubation was carried out in duplicates, in a shaking water bath, at 37 °C for 20 min. The addition of the radioligand initiated the incubation, which was terminated by rapid filtration through GF/C Whatman fiberglass filters. The filters were then rinsed twice with 5 mL portions of ice-cold incubation buffer and were placed in plastic scintillation minivials. Scintillation fluid (3 mL) was added, and the samples were counted for radioactivity.

6.3.2.3. Displacement of ^{3}H-flunitrazepam (81 Ci $mmol^{-1}$) by investigated compounds. Tissues from individual naive animals were processed separately. They were homogenised at 0 °C in 20 volumes of 50 mmol L^{-1} Tris–HCl buffer, pH 7.6. The homogenate was centrifuged at 0 °C and 1000 × g for 15 min, and then recentrifuged at 0 °C and 25 000 × g for 30 min.

After decanting the supernatant, the pellet (fraction P_2) was stored at −20 °C for no longer than 48 h. For incubation it was reconstituted in 20 volumes the incubation buffer pH 7.6. The incubation mixture (final volume 550 μL) consisted of 450 μL membrane suspension, 50 μL of ^{3}H-flunitrazepam solution (2 nmol) and 50 μL of buffer containing seven concentrations (1 nmol–100 μmol) of the investigated compound **D1**. For measuring unspecific binding, cold diazepam in a final concentration of 10 μM was used. The incubation was carried out in duplicates, in a shaking water bath, at 0 °C for 60 min. The addition of the radioligand initiated the incubation, which was terminated by rapid filtration through GF/C Whatman fiberglass filters. The filters were then rinsed twice with 5 mL portions of ice-cold incubation buffer and were placed in plastic scintillation minivials. Scintillation fluid (3 mL) was added, and the samples were counted for radioactivity.

7. Supplementary material

The lists of atomic coordinates, displacement parameters and complete geometry have been deposited at the Cambridge Crystallographic Data Centre, CCDC No. 180328 for compound **D2**. Copies of this information can be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk).

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