Novel 1,2,5-oxadiazine derivatives - synthesis and in vitro biological studies

Barbarić, Monika; Kraljević, Sandra; Grce, Magdalena; Zorc, Branka

Source / Izvornik: **Acta Pharmaceutica, 2003, 53, 175 - 186**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:163:776738

Rights / Prava: In copyright / Zaštićeno autorskim pravom.

Download date / Datum preuzimanja: **2024-03-28**

Repository / Repozitorij:

Repository of Faculty of Pharmacy and Biochemistry University of Zagreb

Acta Pharm. 53 (2003) 175–186 Original research paper

Novel 1,2,5-oxadiazine derivatives – Synthesis and *in vitro* biological studies

MONIKA BARBARIĆ[1]*
SANDRA KRALJEVIĆ[2]
MAGDALENA GRCE[2]
BRANKA ZORC[1]

[1] *Faculty of Pharmacy and Biochemistry University of Zagreb, Zagreb, Croatia*

[2] *Rudjer Bošković Institute Zagreb, Croatia*

A new synthetic approach to the 1,2,5-oxadiazine ring system is described. 2-Substituted or 2,4-disubstituted 2*H*-1,2,5-oxadiazine-3,6(4*H*,5*H*)-dione derivatives **4** were prepared by cyclisation of hydroxamic acids **3** derived from *N*-(1-benzotriazolylcarbonyl)-amino acids **1**. The structures of the synthesised compounds were fully characterised by IR, ^{1}H and ^{13}C NMR spectroscopy and elemental analysis. The aim of this study was to evaluate biological activity of the newly synthesised oxadiazine derivatives. Cytotoxic and cytostatic activities were tested on two cell lines (HeLa and GMK) and evaluated by MTT-test. Two human DNA viruses (adenovirus 7 and herpesvirus 1) and two human RNA viruses (coxsackievirus B5 and echovirus 7) were used in the antiviral test. Selected biological studies indicated that 2-phenyl-2*H*-1,2,5-oxadiazine-3,6(4*H*,5*H*)-dione (**4a**) and 4-benzyl-2-phenyl-2*H*-1,2,5-oxadiazine-3,6(4*H*,5*H*)-dione (**4c**) statistically significantly inhibited cell growth. A minor antiviral effect was observed upon adenovirus, herpesvirus and enteroviruses.

Keywords: 1,2,5-oxadiazine derivatives, synthesis, cytotoxic effect, cytostatic effect, antiviral activity, cytopathic effect

Received June 12, 2003
Accepted August 25, 2003

Oxadiazines are interesting and promising heterocyclic compounds. A diversity of biological effects is associated with oxadiazines bearing heteroatoms at 1,2,4 or 1,3,4 positions. 6*H*-1,2,4-oxadiazine-3,5-(2*H*,4*H*)-dione, the 6-oxa analogue of uracil, has been shown to significantly inhibit growth in several bacterial strains while not being highly inhibitory to mammalian cells (1). 1,3,4-Oxadiazine derivatives exhibit cardiovascular, antibacterial, plant growth regulating, miticidal and nematocidal, acricidal, insecticidal and anticonvulsive activities (2, 3). In addition, oxadiazines are useful intermediates in the synthesis of tenidap prodrugs or β-lactam antibiotics, in particular in the synthesis of carbapenems and penems (4, 5).

* Correspondence, e-mail: mlovrek@yahoo.com

On the other hand, 1,2,5-oxadiazines are not very common heterocyclic systems. A comprehensive review of their synthesis and reactivity has been reported by Smalley (6). The promising therapeutic potential of this class of compounds prompted us to synthesise and biologically screen several 1,2,5-oxadiazine derivatives. Our synthetic approach involves hydroxamic acids as the starting compounds, which themselves have been of multifold biological and pharmacological interest (7–9).

Herein we present a new synthetic approach to 1,2,5-oxadiazines derivates and their biological activity on mammalian cells. Furthermore, their antiviral activity against adenoviruses, herpes viruses and enteroviruses was investigated.

EXPERIMENTAL

Materials and analytical methods

Melting points were determined on a Boëtius Microheating Stage (Franz Küstner Nachf. KG, Germany) and were uncorrected. IR spectra were recorded on a FTIR Perkin Elmer Paragon 500 spectrometer (Perkin Elmer, UK). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 spectrometer (Varian, USA), operating at 300 and 75.5 MHz for the ^{1}H and ^{13}C nuclei, respectively. Samples were measured in DMSO-d_6 solutions at 20 °C in 5 mm NMR tubes. Chemical shifts (δ) in ppm were referred to TMS. Coupling constants (J) in Hz, were observed through three bonds.

For TLC, silica gel plates Kieselgel 60 F_{254} (Merck, Germany) and the following solvent mixtures were used: cyclohexane/ethyl acetate/methanol (3:1:0.3), cyclohexane/ethyl acetate (1:1), chloroform/methanol (9:1). Spots were visualised by short-wave UV light and iodine vapour. Column chromatography was performed on silica gel (Kemika, Croatia), 0.063–0.200 mm, with dichloromethane/methanol (9:1) or cyclohexane/ethyl acetate (1:1) as eluent.

Amino acids were purchased from Kemika (Croatia). *N*-Phenylhydroxylamine was prepared by reduction of nitrosobenzene with L-ascorbate (11). All solvents were of analytical grade purity and were dried prior to use.

Syntheses

N-(1-benzotriazolylcarbonyl)-amino acids (N-Btc-amino acids, 1a-c). – *N*-Btc-glycine (**1a**), *N*-Btc-L-alanine (**1b**) and *N*-Btc-L-phenylalanine (**1c**) were synthesised following the procedure published previously (12).

N-(1-benzotriazolylcarbonyl)-amino acid chlorides (2a-c). – Chlorides **2** were prepared from *N*-Btc-amino acids **1a-c** and thionyl chloride (13).

Hydroxamic acids 3a-c. – To a suspension (**2a**) or solution (**2b,c**) of 3–5 mmol chloride **2** in 55–80 mL cold toluene (0 °C), a solution of 3–5 mmol *N*-phenylhydroxylamine (PHA) in 45–75 mL toluene and a solution of 5.5 mmol *N*-methylmorpholine (MM) in 30 mL toluene were added simultaneously and dropwise. The reaction mixture was stirred at room temperature for 3–24 h.

Product **3a** partially precipitated from the reaction mixture, together with MM hydrochloride. The precipitate was filtered off and dissolved in a water/ethyl acetate mix-

ture. The organic layer was extracted with water, twice with cold 1% HCl solution and twice with water, then dried over anhydrous sodium sulphate, filtered and evaporated to give 0.525 g (35%) of **3a**. An additional amount (0.315 g, 21%) of **3a** was isolated from toluene mother liquor by an analogous extraction procedure.

Products **3b** and **3c** were isolated as follows. The reaction mixture was extracted with water, three times with 1% HCl solution and two times with water. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. The analytically pure **3b** was obtained by purification on silica gel column (eluent: dichloromethane/methanol 9:1) and additional trituration with ethyl acetate and cyclohexane. Pure product **3c** was obtained by recrystallisation with ether/cyclohexane mixture.

1,2,5-Oxadiazine-3,6-dione derivatives (4a,c). – To a solution of 1.6 mmol of hydroxamic acid **3a** or **3c** in 130 mL acetone, 5 mL of 10% solution of sodium carbonate was added. The reaction mixture was stirred at room temperature for 2.5 h. Precipitated inorganic salt was filtered off and the mother liquor was evaporated. The residue was dissolved in dichloromethane and extracted twice with cold 1% HCl solution and twice with water. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. Products **4a** and **4c** were purified by column chromatography (eluent: cyclohexane/ethyl acetate 1:1).

Cell lines

Green monkey kidney cells (GMK; ATCC CCL-26, USA) and human cervical carcinoma cells (HeLa; ATCC CCL-2, USA) were used. Monolayer cell cultures were grown in a modified eagle medium (DMEM; Dulbecco minimum essential medium, Difco, USA) supplemented with 10% inactivated foetal calf serum (FS; Gibco BRL, UK), 2 mmol L^{-1} L-glutamine, 10 mmol L^{-1} Hepes (pH 7.4) and antibiotics (100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin), and were incubated at 37 °C and 5% of CO_2.

MTT-test

MTT-test was used to measure the mitochondrial activity in cells (14). When added, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) formed formazane crystals in live cells and cells in an early apoptosis cycle (programmed cell death). Formazane crystals dissolved when dimetyl sulphoxide (DMSO) was added. This enabled the spectrophotometric reading at 570 nm. According to the MTT-test results, it was possible to determine if a tested compound acted as a cytotoxic agent, cytostatic agent or if it stimulated cell growth. The tested compound had a cytotoxic effect on cells (CT) if spectrophotometric values after 72 h of incubation were below the initial control values measured after 24 h of incubation. Values at 72 h of incubation below the control values measured after 72 h of incubation indicated the inhibitory effect (*IC*) of cell growth, *i.e.*, cytostatic effect, whereas values at 72 h of incubation higher than control values measured after 72 h of incubation meant that the tested compound stimulated cell growth (proliferative effect).

Acyclovir (Ac) was used as a positive control compound at the same concentrations as **4a** and **4c**. Each compound was dissolved in DMSO to produce 0.1 mol L^{-1} solution

and further diluted in DMEM to concentrations of 10^{-4} mol L^{-1} (c_1), 10^{-5} mol L^{-1} (c_2) and 10^{-6} mol L^{-1} (c_3). The final concentration of DMSO in different dilutions of compounds was less than 0.1% and at that concentrations it did not influence the cell growth.

Antiviral assay

Five different viral titres (V-1 to V-5, relative titres) obtained by serial dilution (1:2 for adenovirus and herpesvirus, and 1:10 for enteroviruses) were used to test the inhibition of the cytopathic effect (CPE) (15). HeLa and GMK cells were seeded at 10^5 cells *per* mL on 24-well microtitre plates (Becton Dickinson, USA). Viral infection was performed on one-day-old confluent cell monolayers with addition of the tested compounds at different concentrations. The CPE was followed by an optical microscope 24 h after infection for enteroviruses (coxsackievirus and echovirus) and 48 h for adenovirus and herpesvirus. The CPE of enteroviruses was observed on GMK cells, while CPE of adenovirus and herpesvirus was observed on HeLa cells (10).

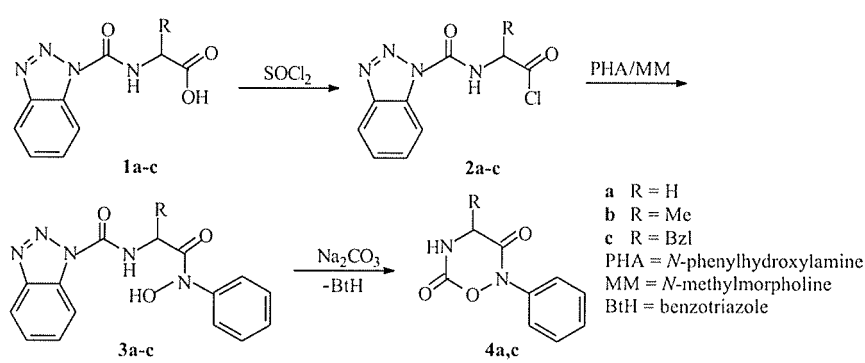
Statistics

The results are shown as the percentage of CPE inhibition compared to CPE without compounds on each plate. The results were statistically analysed on a personal computer by ANOVA test and shown graphically. The criteria for statistical significance was $p < 0.05$. The concentration values that inhibit 50% of cell growth (IC_{50}) were calculated using the linear regression model.

RESULTS AND DISCUSSION

Syntheses

The starting compounds for the synthesis of hydroxamic acids **3** were *N*-Btc-amino acids (**1a-c**). They were prepared from the appropriate amino acids and 1-benzotriazole carboxylic acid chloride and converted into the corresponding chlorides **2** by means of



Scheme 1

Table I. Reaction conditions and analytical data of hydroxamic acids **3a-c** *and 1,2,5-oxadiazine-3,6-dione derivatives* **4a,c**

Compound No.	R	Solvent	Time (h)	Yield (%)	M.p. (°C)	Molecular formula (M_r)	CHN analysis (%) Calcd./found C	H	N	IR (KBr) ν_{max} (cm^{-1})
3a	H	Toluene	24[a]	56	147–149	$C_{15}H_{13}N_5O_3$ (311.29)	57.88 57.18	4.21 4.49	22.50 22.09	3342, 3186, 2876, 1731, 1687, 1659, 1594, 1530, 1496, 1447, 1396, 1301, 1286, 1236, 1090, 1004, 753, 691, 609
3b	CH_3	Toluene	3[a]	15	132–135	$C_{16}H_{15}N_5O_3$ (325.32)	59.07 58.43	4.65 4.98	21.53 21.09	3252, 2930, 1734, 1712, 1675, 1593, 1529, 1450, 1380, 1285, 1235, 1136, 1072, 1034, 936, 840, 755, 695
3c	$CH_2C_6H_5$	Toluene	22[a]	31	130–133	$C_{22}H_{19}N_5O_3$ (401.42)	65.83 64.84	4.77 5.04	17.45 17.14	3241, 3030, 2925, 1740, 1658, 1594, 1522, 1496, 1448, 1388, 1287, 1233, 1087, 1043, 928, 834, 750, 696, 615, 573
4a	H	Acetone	2.5[b]	13	115–117	$C_9H_8N_2O_3$ (192.17)	56.25 55.78	4.19 4.43	14.58 14.59	3280, 3163, 1747, 1692, 1590, 1495, 1445, 1407, 1365, 1137, 1104, 1006, 763, 691, 539
4c	$CH_2C_6H_5$	Acetone	2.5[b]	28	96–98	$C_{16}H_{14}N_2O_3$ (282.29)	68.08 67.70	5.00 5.23	9.92 9.84	3265, 3169, 1772, 1688, 1594, 1490, 1365, 1130, 988, 729, 692

[a] 1 h at 0 °C, the remaining period at room temperature.
[b] Room temperature.

thionyl chloride (12, 13). In the next reaction step, acyl chlorides **2** reacted with *N*-phenylhydroxylamine and gave the following hydroxamic acids: *N'*-hydroxy-*N'*-phenyl--*N*-Btc-glycine amide (**3a**), *N'*-hydroxy-*N'*-phenyl-*N*-Btc-L-alanine amide (**3b**), and *N'*--hydroxy-*N'*-phenyl-*N*-Btc-L-phenylalanine amide (**3c**) (Scheme 1). It is worth pointing out that the analogous reactions of *N*-Btc-amino acid chlorides with hydroxylamine hydrochloride in our hands failed to give the corresponding hydroxamic acids (toluene or acetonitrile were used as solvents and triethylamine or *N*-methylmorpholine as hydrogen chloride acceptors).

Under basic conditions, hydroxamic acids **3** readily underwent cyclisation to afford 1,2,5-oxadiazine-3,6-diones (**4**) (Scheme 1). The substituent on C-4 depends on the starting amino acid, while the substituent on C-2 atom depends on the hydroxylamine used in the previous reaction step. Two oxadiazine derivatives, *viz.* 2-phenyl-2*H*-1,2,5-oxadiazine--3,6(4*H*,5*H*)-dione (**4a**) and 4-benzyl-2-phenyl-2*H*-1,2,5-oxadiazine-3,6(4*H*,5*H*)-dione (**4c**), were isolated and analysed, while the corresponding L-alanine derivative (**4b**) was only detected by TLC.

Spectral assignment and CHN analysis of the synthesised new compounds **3** and **4** confirmed their structures. IR spectra of **3** showed the absorption maxima of carbonyl group bound to benzotriazole at 1731–1739 and the hydroxamic acid carbonyl group at 1658–1687 cm^{-1}. In the IR spectra of oxadiazine derivatives, two carbonyl absorptions were visible: carbonyl at position 3 at 1747–1772 cm^{-1} and carbonyl at position 6 at 1688–1692 (amide I) and 1590–1594 cm^{-1} (amide II).

^{1}H NMR spectra of products **1a-c** confirmed presence of carboxylic acid group at 13.15–12.00, NH group at 9.42–9.34, aromatic protons at 8.15–7.50 and C-2 atom at 4.50–4.04 ppm. ^{13}C spectra confirmed presence of two carbonyl groups: carboxylic acid carbonyl at 173.28–172.18 and carbonyl bound to benzotriazole at 149.44–148.87 and, finally, chiral C-2 atom at 54.98–41.84 ppm.

^{1}H NMR spectra of products **3a-c** revealed presence of *N*-hydroxyl group at 11 ppm, instead of carboxy group. Hydroxamic acid carbonyl was shifted to lower ppm values (167.80–165.25 ppm) than carboxylic acid carbonyl, while carbonyl bound to benzotriazole, aromatic C-atoms and C-2 had practically the same values as in compounds **1a-c**.

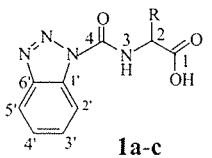
^{1}H NMR spectra of products **4a,c** confirmed presence of NH group at 8.84–8.72, aromatic protons at 7.56–7.27 and C-2 atom at 4.49–4.10 ppm. ^{13}C spectra confirmed presence of two carbonyl groups at 164.85–164.56 and 152.83–152.69, aromatic C-atoms at 137.39–122.41 and C-2 atom at 55.48–44.20 ppm.

Reaction conditions, yields and analytical data of the newly synthesised compounds are presented in Table I and NMR spectral data are given in Table II.

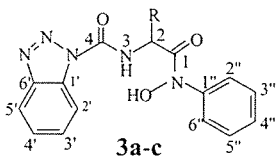
Biological studies

The synthesized oxadiazines were used in biological studies. Values obtained by the MTT-test were all below control values 72 h following incubation (Figs. 1 and 2). No growth inhibition of GMK cells was observed for compounds **4a** and **4c** at concentration c_3, but at concentrations c_1 and c_2 a statistically significant growth inhibition of GMK cells was observed, ranging from 40% to 60% (Fig. 1). Similarly, a statistically significant growth inhibition of 75% of HeLa cells was observed for **4a** at concentration c_1 (Fig. 2),

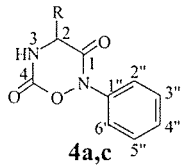
Table II. ^{1}H *NMR and* ^{13}C *NMR data of Btc-amino acids* ***1a-c****[a], hydroxamic acids* ***3a-c*** *and 1,2,5-oxadiazine-3,6-dione derivatives* ***4a,c****[b]*



1a-c



3a-c



4a,c

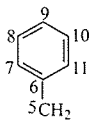
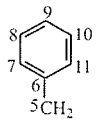
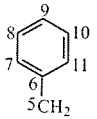
Compound No.	R	^{1}H NMR	^{13}C NMR
1a	H	12.00 (s, 1H, OH), 9.42 (t, 1H, 3, *J* = 5.7 Hz), 8.14 (dd, 2H, 2', 5', *J* = 8.2 Hz), 7.67 (t, 1H, 3', *J* = 7.7 Hz), 7.50 (t, 1H, 4', *J* = 7.5 Hz), 4.04 (d, 2H, 2, *J* = 5.6 Hz)	172.62 (1), 149.44 (4), 145.54 (1'), 131.28 (6'), 130.14 (5'), 125.67 (4'), 119.89 (3'), 113.49 (2'), 41.84 (2)
1b	$^{5}CH_3$	12.85 (s, 1H, OH), 9.34 (d, 1H, 3, *J* = 7.7 Hz), 8.14 (dd, 2H, 2', 5', *J* = 9.00 Hz), 7.67 (t, 1H, 3', *J* = 7.3 Hz), 7.50 (t, 1H, 4', *J* = 7.3 Hz), 4.50-4.43 (m, 1H, 2), 1.50 (d, 3H, 5, *J* = 7.3 Hz)	173.28 (1), 148.87 (4), 145.50 (1'), 131.35 (6'), 130.01 (5'), 125.67 (4'), 119.88 (3'), 113.54 (2'), 49.12 (2), 16.73 (5)
1c		13.15 (s, 1H, OH), 9.34 (d, 1H, 3, *J* = 8.3 Hz), 8.15 (d, 1H, 2', *J* = 8.3 Hz), 8.06 (d, 1H, 5', *J* = 8.3 Hz), 7.65 (t, 1H, 3', *J* = 7.7 Hz), 7.49 (t, 1H, 4', *J* = 7.7 Hz), 7.32 (d, 2H, 7, 11, *J* = 7.3 Hz), 7.23 (t, 2H, 8, 10, *J* = 7.5 Hz), 7.14 (t, 1H, 9, *J* = 7.2 Hz), 4.72-4.65 (m, 1H, 2), 3.29 (s, 2H, 5)	172.18 (1), 148.98 (4), 145.48 (1'), 137.85 (6), 131.23 (6'), 130.17 (5'), 129.19 (7, 11), 128.36 (8, 10), 126.55 (4'), 125.76 (9), 119.91 (3'), 113.47 (2'), 54.98 (2), 35.78 (5)

Table II, continuations

3a	H	10.90 (s, 1H, OH), 9.33 (t, 1H, 3, J = 5.8 Hz), 8.22 (t, 2H, 2', 5', J = 9.3 Hz), 7.77-7.69 (m, 2H, 2'', 6''), 7.58 (t, 2H, 3', 4', J = 7.7 Hz), 7.41 (t, 2H, 3'', 5'', J = 8.0 Hz), 7.18 (t, 1H, 4'', J = 7.4), 4.47 (d, 2H, 2, J = 5.7 Hz)	167.29 (1), 149.47 (4), 145.42 (1'), 141.23 (1''), 131.16 (6'), 130.09 (5'), 128.51 (3'', 5''), 125.60 (4'), 124.73 (4''), 119.82 (3') 119.18 (2'', 6''), 113.40 (2'), 42.51 (2)
3b	$^5CH_3$	11.00 (s, 1H, OH), 8.80 (s, 1H, 3), 7.92-7.91 (m, 2H, 2', 5', J = 9.3 Hz), 7.56-7.33 (m, 7H, 3', 4', 2''-6''), 4.34 (q, 1H, 2), 1.37 (d, 3H, 5)	167.80 (1), 153.91 (4), 139.91 (1'), 138.19 (1''), 130.21 (5'), 129.86 (6'), 128.91 (3'', 5''), 126.37 (4'), 126.23 (4''), 123.84 (3'), 121.34 (2'', 6'') 115.93 (2'), 50.50 (2), 16.49 (5)
3c		11.00 (s, 1H, OH), 8.84 (s, 1H, 3) 8.23-7.20 (m, 14H, arom.), 4.50-4.45 (m, 1H, 2), 2.60-2.50 (m, 2H, 5)	165.25 (1), 152.47 (4), 148.44 (1'), 140.00 (6), 135.78 (1''), 130.34 (6'), 129.84 (5'), 129.16 (3'', 5''), 127.91 (7, 8, 10, 11), 125.62 (9, 4'), 124.38 (3', 2'', 4'', 6''), 115.63 (2'), 55.95 (2), 38.77 (5)
4a	H	8.72 (s, 1H, 3), 7.56-7.34 (m, 5H, arom.), 4.10 (s, 2H, 2)	164.56 (1), 152.83 (4), 137.39 (1''), 129.14 (3'', 5''), 127.56 (4''), 122.41 (2'', 6''), 44.20 (2)
4c		8.84 (s, 1H, 3), 7.51-7.27 (m, 10H, arom.), 4.49 (t, 1H, 2) 3.19-3.05 (m, 2H, 5)	164.85 (1), 151.69 (4), 137.09 (1''), 135.59 (6), 129.78 (8, 10), 129.13 (3'', 5''), 128.48 (7, 11), 127.95 (4''), 127.10 (9), 123.16 (2'', 6''), 55.48 (2), 37.34 (5)

[a] NMR data for compounds **1a-c** are included as well since they have not been reported until now.
[b] DMSO-d_6 solution, δ (ppm)

while cell proliferation was observed at concentrations c_2 and c_3. Statistically significant growth inhibition of HeLa cells was observed also for **4c**, ranging from 50% to 75% (Fig. 2). The higher growth inhibition of the tested compounds on HeLa cells, compared to GMK cells, was probably due to the programmed cell death phenomena permanently present in HeLa cells (data not shown) (16). Contrary to the tested compounds, acyclovir stimulated both GMK and HeLa cell growth at all concentrations (Figs. 1 and 2).

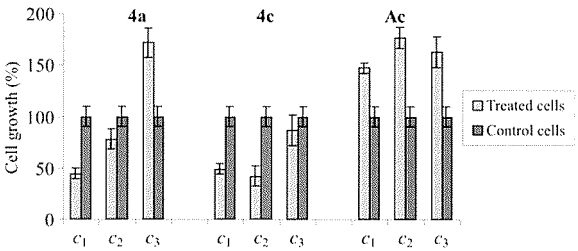


Fig. 1. Percentage of growth of GMK cells determined by MTT-test in the absence (control) or in the presence of **4a**, **4c** and acyclovir (Ac) ($c_1 = 10^{-4}$ mol L^{-1}, $c_2 = 10^{-5}$ mol L^{-1}, $c_3 = 10^{-6}$ mol L^{-1}) (mean ± SD, $n = 12$).

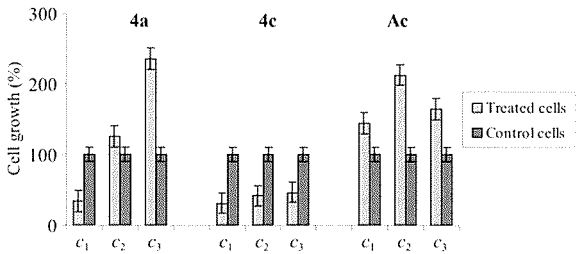


Fig. 2. Percentage of growth of HeLa cells determined by MTT-test in the absence (control) or in the presence of **4a**, **4c** and acyclovir (Ac) ($c_1 = 10^{-4}$ mol L^{-1}, $c_2 = 10^{-5}$ mol L^{-1}, $c_3 = 10^{-6}$ mol L^{-1}) (mean ± SD, $n = 12$).

As the cytostatic effect of **4a** and **4c** was observed, IC_{50} values (17) were calculated and are presented in Table III. A compound **4a** showed higher cytostatic effect on GMK than on HeLa cells, while **4c** was more cytotoxic on HeLa than on GMK cells.

Results of the antiviral assay (percentage of CPE inhibition) are shown in Table IV. The inhibition of CPE (approximately 5–20%) for **4a** was observed at concentration c_1 for all tested viruses, while the inhibition of coxsackievirus CPE (approximately 5–15%) was observed only at concentrations c_2 and c_3. Compound **4c** inhibited CPE of adenovirus at concentration c_1 (approximately 20%) as well as CPE of herpesvirus and enteroviruses at

*Table III. IC_{50} for compounds **4a** and **4c***

Cell line	IC_{50} (mol L^{-1})	
	4a	**4c**
GMK	1.125×10^{-4}	5.800×10^{-5}
HeLa	0.897×10^{-5}	0.824×10^{-4}

concentrations c_1, c_2 and c_3 (approximately 5–20%). A satisfactory inhibitory effect ranging from 20 to 40% of acyclovir, as expected (18), was observed on herpesvirus at all tested concentrations and virus titres.

Poor CPE inhibition of **4a** and **4c** observed for all tested viruses (maximum 20%) and the lack of CPE inhibition at minor virus titres and at lower concentrations of the tested compounds was probably not an antiviral effect but more likely their inhibitory effect upon cell growth (shown previously by the MTT-test). Thus, the lack of CPE inhibition at low virus titres, where it is usually possible to observe the inhibition of CPE for antiviral compounds (*e.g.* acyclovir), may confirm the previous statement.

Table IV. Cytopathic effect (CPE) on cell culture of different viruses[a] upon **4a**, **4c** *and acyclovir (Ac)[b] compared to control (solvent DMEM)*

Virus/titre	CPE inhibition (%)								
	4a			**4c**			Ac		
	c_3	c_2	c_1	c_3	c_2	c_1	c_3	c_2	c_1
Adenovirus 7									
V-1	0	0	20	0	0	20	–	–	–
V-2	0	0	20	0	0	20	–	–	–
V-3	10	0	0	0	0	0	–	–	–
V-4	0	0	0	0	0	0	–	–	–
V-5	0	0	0	0	0	0	–	–	–
Herpesvirus 1									
V-1	0	0	10	0	0	10	20	20	20
V-2	0	0	10	10	10	10	40	40	50
V-3	0	0	10	0	0	0	20	30	30
V-4	0	0	5	0	0	0	25	25	25
V-5	0	0	0	0	0	0	20	20	25
Echovirus 7									
V-1	0	0	10	5	10	10	–	–	–
V-2	0	0	10	0	10	10	–	–	–
V-3	0	0	10	0	10	10	–	–	–
V-4	0	0	10	0	0	10	–	–	–
V-5	0	0	0	0	0	0	–	–	–
Coxsackievirus B5									
V-1	0	0	0	0	0	0	–	–	–
V-2	15	15	5	0	20	10	–	–	–
V-3	15	15	5	20	20	20	–	–	–
V-4	5	5	0	15	5	5	–	–	–

[a] Viruses titres V-1 to V-5: serial dilution 1:2 for adenovirus 7 and herpesvirus 1 and 1:10 for echovirus 7 and coxsackievirus B5.

[b] $c_1 = 10^{-4}$ mol L^{-1}, $c_2 = 10^{-5}$ mol L^{-1}, $c_3 = 10^{-6}$ mol L^{-1}.

CONCLUSIONS

1,3,5-Oxadiazine-3,6-diones can be successfully prepared by cyclisation of the appropriate hydroxamic acids. Since various methods for the preparation of a wide array of *N*-monosubstituted hydroxylamines are available, this procedure constitutes a new general method for construction of the 1,3,5-oxadiazine ring system. Selected biological studies indicated that oxadiazines **4a** and **4c** had no cytotoxic effect on HeLa and GMK cells at tested concentrations, but they had a pronounced cytostatic effect, *i.e.*, they inhibited cell growth in the 25–75% range, depending on the compound, cell line and concentration. A minor inhibition of CPE on adenovirus, herpesvirus and enteroviruses, for compounds **4a** and **4c**, was observed. These preliminary findings indicate that potential antiviral drugs could be found within oxadiazine derivatives. Thus, further investigation should be undertaken.

Acknowledgements. – This study was supported by grants (No: 0006543 and 0098104) from the Ministry of Science and Technology of the Republic of Croatia. The authors are grateful to Josip Begovac for providing the acyclovir used herein.

REFERENCES

1. P. T. Berkowitz, R. A. Long, P. Dea, R. K. Robins and T. R. Matthews, Synthesis and antimicrobial activity of certain 6*H*-1,2,4-oxadiazin-3(2*H*)-ones, *J. Med. Chem.* **20** (1977) 134–138.
2. K. M. Khan, S. Rahat, M. I. Choudhary, A. Rahman, U. Ghani, S. Perveen, S. Khatoon, A. Dar and A. Malik, Synthesis and biological screening of 2-substituted 5,6-dihydro-5-oxo-4*H*-1,3,4-oxadiazine-4-propanenitriles and of their intermediates, *Helv. Chim. Acta* **85** (2002) 559–570.
3. M. J. Kornet, Synthesis and anticonvulsant activity of 2-aryl-3,4-dialkytetrahydro-1,3,4-oxadiazines and 2-aryl-3,4-dialkyltetrahydro-1,3,4-oxadiazin-5-ones, *J. Heterocyclic Chem.* **33** (1996) 2047–2049.
4. R. P. Robinson and K. M. Donahue, Synthesis of *N*-alkoxycarbonyl and *N*-carboxamide derivatives of anti-inflammatory oxindoles, *J. Heterocyclic Chem.* **31** (1994) 1541–1544.
5. M. B. Gravestock, *Preparation of 8-Oxo-3-oxa-1,4-diazabicyclo[4,2,0]oct-4-enes as Penem and Carbapenem Intermediates*, Eur. Pat. Appl. EP 265,169, 27 Apr 1987; ref. *Chem. Abstr.* **109** (1988) 110–160d.
6. R. K. Smalley, *1,2,5-Oxadiazines and 1,2,5-Thiadiazines*, in *Comprehensive Heterocycle Chemistry* II (Ed. A. J. Boulton), Vol. 6, Elsevier, Oxford 1996, pp. 681–694.
7. R. J. Bergeron, Synthesis and solution structure of microbial siderophores, *Chem. Rev.* **84** (1984) 587–602.
8. S. Hanessian and S. Johnstone, Synthesis of hydroxamic esters via alkoxyaminocarbonylation of β-dicarbonyl compounds, *J. Org. Chem.* **64** (1999) 5896–5903.
9. M. Whittaker, C. D. Floyd, P. Brown and A. J. H. Gearing, Design and therapeutic application of matrix metalloproteinase inhibitors, *Chem. Rev.* **99** (1999) 2735–2776.
10. *Fundamental virology*, 2nd ed. (Eds. B. N. Fields and D. M. Knipe), Raven Press, New York 1990.
11. S. Uršić, V. Vrček, D. Ljubas and I. Vinković, Interaction of L-ascorbate with substituted nitrosobenzenes. Role of the ascorbate 2-OH group in antioxidant reactions, *New J. Chem.* **1998**, 221–223.
12. I. Butula, B. Zorc and V. Vela, Reaktionen mit 1-Benzotriazol carbonsaurechlorid. VII. Die Umsetzung mit Aminosauren, *Croat. Chem. Acta* **54** (1981) 435–440.

13. B. Zorc and I. Butula, Reaktionen mit *N*-(1-Benzotriazolylcarbonyl)-aminosauren. I. Synthese von Hydantoinen und Hydantoinsauren-amiden, *Croat. Chem. Acta* **54** (1981) 441–449.
14. T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65** (1983) 55–63.
15. D. F. Smee, A. C. Morrison, L. Dale, B. W. Sidwell and R. W. Sidwell. Comparison of colorimetric, fluorimetric, and visual methods for determinig anti-influenza (H1N1 and H3N2) virus activities and toxicities of compounds, *J. Virol. Methods* **106** (2002) 71–79.
16. A. F. McGettrick, R. C. Barnes and D. M. Worrall, SCCA2 inhibits TNF-mediated apoptosis in transfected HeLa cells, *Eur. J. Biochem.* **268** (2001) 5868–5875.
17. J. S. Soothill, R. Ward and A. J. Girling, The IC50: an exactly defined measure of antibiotic sensitivity, *J. Antimicrob. Chemother.* **29** (1992) 137–139.
18. *Virological Methods Manual* (Eds. B. W. J. Mahy and H. O. Kango), Academic Press, London 1996.

SAŽETAK

Novi derivati 1,2,5-oksadiazina – Sinteza i biološko djelovanje u *in vitro* uvjetima

MONIKA BARBARIĆ, SANDRA KRALJEVIĆ, MAGDALENA GRCE i BRANKA ZORC

U radu je opisan novi sintetski put za pripravu 1,2,5-oksadiazinskog heterocikličkog sustava. 2-Supstituirani i 2,4-disupstituirani 2*H*-1,2,5-oksadiazin-3,6(4*H*,5*H*)-dioni **4** sintetizirani su intramolekulskom ciklizacijom odgovarajućih hidroksamskih kiselina **3**, derivata *N*-(1-benzotriazolilkarbonil)-aminokiselina **1**. Strukture sintetiziranih spojeva u potpunosti su karakterizirane IR, ^{1}H i ^{13}C NMR spektroskopijom i elementarnom analizom. Ispitano je biološko djelovanje spojeva **4a** i **4c** u *in vitro* uvjetima. Citotoksično i citostatsko djelovanje testirano je na dvije stanične linije (HeLa i GMK) i evaluirano MTT-testom. Antiviralni testovi provedeni su na dva humana DNA virusa (adenovirus 7 i herpesvirus 1) i dva humana RNA virusa (coxsackievirus B5 i echovirus 7). Ustanovljeno je da 2-fenil-2*H*-1,2,5-oksadiazin-3,6(4*H*,5*H*)-dion (**4a**) i 4-benzil-2-fenil-2*H*-1,2,5-oksadiazin-3,6(4*H*,5*H*)-dion (**4c**) statistički značajno inhibiraju rast stanica GMK i stanica HeLa, te da imaju slabi protuvirusni učinak na ispitane viruse.

Ključne riječi: 1,2,5-oksadiazin derivati, sinteza, citotoksični učinak, citostatski učinak, protuvirusno djelovanje, citopatski učinak

Farmaceutsko-biokemijski fakultet Sveučilišta u Zagrebu, Zagreb

Institut Rudjer Bošković, Zagreb