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Original research paper

Synthesis, antibacterial and cytotoxic activity evaluation of hydroxyurea derivatives

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Synthesis and biological evaluation of a series (N = 16) of cyclic and acyclic hydroxyurea derivatives, including benzotriazole-, isocyanuric acid- and biuret-containing compounds, are disclosed. 1-*N*-(benzyloxycarbamoyl)benzotriazole was used as a benzyloxyisocyanate donor, a useful intermediate in the preparation of substituted hydroxyurea. Antibacterial activities of synthesized hydroxyurea derivatives were tested on three *E. coli* strains, *i.e.*, a strain susceptible to antibiotics, a strain resistant to macrolide antibiotics and a strain resistant to aminoglycoside antibiotics. Six compounds (three acyclic and three cyclic hydroxyureas) showed growth inhibition of the tested *E. coli* strains, with different specificity toward each strain. Results of the cytotoxic activity evaluation revealed that twelve out of sixteen test compounds were cytotoxic to human acute monocytic leukemia THP-1 and/or human acute T cell leukemia Jurkat cell line. 1-(*N*-hydroxycarbamoyl)benzotriazole (**5**) increased the metabolic activity of both cell lines. Two compounds, 1-(*N*-hydroxycarbamoyl)benzotriazole (**5**) and *N*,*N*′,*N*″-trihydroxybiuret (**15**), were identified as potential NO donors.

Keywords: hydroxyurea derivatives, synthesis, antibacterial activity, cytotoxicity

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Hydroxyurea and its derivatives exhibit versatile biological activities. Hydroxyurea is currently used in the treatment of various neoplastic and non-neoplastic diseases such as cancer, sickle cell anemia and HIV (1). Derivatives of hydroxyurea were found to inhibit matrix zinc metaloproteinases (MMP), urease, carboanhydrase, carboxypeptidase, cyclooxygenase and 5-lipooxygenase. Early experiments on antibacterial properties and effects on tumor cell lines of hydroxyurea and low molecular mass derivatives were investigated in the 1960s (2–3). Recently, hydroxyurea was recognized as a leading compound in nitric oxide donor synthesis (4).

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† Dedicated to the memory of the late Professors Ivan Butula and Ivan Kos

Continuing our studies on physical, chemical and biological properties of hydroxurea derivatives (5–8), we report here the synthesis of a series of hydroxyurea derivatives along with their effects on metabolic activity on two human cell lines (human acute monocytic leukemia suspension cell line THP-1, and human acute T cell leukemia suspension cell line Jurkat) and their antibacterial activity against three *E. coli* strains.

EXPERIMENTAL

Materials and methods

Melting points were measured on a Stuart SMP3 Melting Point Apparatus (Barloworld Scientific Ltd., UK). NMR spectra were recorded on a Varian Gemini 300 (Varian, USA) spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C nucleus. Samples were measured in DMSO-d_6 solution in 5-mm NMR tubes. Chemical shifts, δ, are given in ppm and referenced to tetramethylsilane (TMS). IR-spectra were recorded on a FT-IR Perkin Elmer Paragon 500 spectrometer (PerkinElmer, USA) for samples dispersed in KBr pellets. Elemental analysis was performed on a CHNS LECO-932 Analyzer (LECO Corporation, USA); the obtained results are within ± 0.4 % of theoretical values. UV-VIS spectra were recorded on a Varian Cary 50 spectrophotometer (Varian, USA).

Benzotriazole, tetrahydrofurane, *O*-ethylhydroxylamine hydrochloride, urea, L-sodium ascorbate, and triethylamine (TEA) were purchased from Fluka (Germany). Triphosgene, *O*-methylhydroxylamine hydrochloride, *O*-benzylhydroxylamine hydrochloride, 4-aminobenzoic acid, imidazole, and hydrochloric acid were purchased from Sigma-Aldrich (USA), while acetone, toluene, methanol, and diethyl ether were from Kemika (Croatia). Dioxane was from Riedel-de Haën (Germany). Dimethyl sulfoxide (DMSO) was purchased from Merck (Germany). Mn(III)-5,10,15,20-tetrakis(*N*-ethylpyridinium-2-yl) porphyrin (MnTE-2-PyP^{5+}) was prepared by a previously published procedure (9). Phosphate-buffered saline (PBS) was from Gibco (USA). LB medium (Luria-Bertani medium) was from Becton Dickinson (UK). RPMI 1640 medium was from Invitrogen (USA). Doubly distilled argon purged CO_2-free water was used. All stock solutions of tested compounds in *E. coli* and cell viability experiments were freshly prepared before use by dissolving an appropriate amount in DMSO to a final concentration of 0.5 mol L^{-1}.

Cell lines. – THP-1 cell line (TIB-202™) and Jurkat cell line (TIB-152™) were obtained from the American Type Culture Collection (USA). All experiments were performed on cells between passages 5 and 10. After 15 passages, cells were discarded and replaced by frozen stocks.

Bacterial cultures. – *Escherichia coli* DH5α, BL21(DE3) and the expression vector pET-25b(+) were obtained from Novagen (USA), pUC18 vector was from GE Healthcare Life Sciences (USA). *E. coli* DH5α (*sup*E44 Δ*lac*U169(Θ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) – a strain with natural sensitivity to antibiotics, DH5α/pUC18-*ermC′* – a strain DH5α carrying a gene for methyltransferase ErmC′, which renders *E. coli* resistant to erythromycin and other macrolide antibiotics (10), and BL21(DE3)/pET25b(+)-*sgm* (*F*$^{-}$ *ompT hsdS*$_B$($r_B^- m_B^-$)*gal dcm* (DE3) – an expression strain of *E. coli* containing a gene for methyltransferase Sgm, which makes *E. coli* resistant to kanamycin and other 4,6-deoxystreptamine aminoglycosides were used (11).

Measurement of bacterial growth in microtiter plates – microdilution assay. – Serial dilutions of test compounds were prepared by dilution of stock solutions, either with LB medium (for *E. coli* strain DH5α) or with LB medium supplemented with 100 mg L^{-1} of ampicillin (for *E. coli* strains DH5α/pUC18-*ermC'* and BL21(DE3)/pET25b(+)-*sgm*). The total volume in each well was 200 μL and final concentrations of test compounds in the wells, after the addition of all components, were 0.001, 0.01, 0.1 and 1 mol L^{-1}. The overnight bacterial culture was diluted in fresh LB medium (1:50) (*E. coli* DH5α) or in LB medium supplemented with 100 mg L^{-1} of ampicillin (*E. coli* DH5α/pUC18-*ermC'* and *E. coli* BL21(DE3)/pET25b(+)-*sgm*) and grown until OD_{600} was 0.8–1. Cultures were diluted to approximately 5×10^5 cells mL^{-1} and an aliquot of 100 μL of the suspension was added to the wells containing serial dilutions of test compounds. Additional wells which contained only the corresponding dilutions of test compounds in growth medium, were used as controls for compounds that were either colored or showed some absorbance in the absence of bacteria. Each microtiter plate contained a control of sterility (200 μL of LB medium), bacterial growth control (100 μL of LB medium, for *E. coli* strain DH5α, or LB medium with ampicillin, for *E. coli* strains DH5α/pUC18-*ermC'* and BL21(DE3)/pET25b(+)-*sgm*) and 100 μL of bacterial inoculum), and a control of compound sterility (150 μL of LB medium and 50 μL of highest concentration of test compound). Microtiter plates were incubated for 18 h at 37 °C and bacterial growth was measured at 570 nm using a microplate reader Biohit BP 800 (Biohit, Finland).

Metabolic MTS assay. – Metabolic activity and the potential cytotoxic effect of test compounds were investigated using the CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA). Fifty μL of the cell culture (2×10^5 cells mL^{-1}, *i.e.*, 10^4 cells per well) or fifty μL of RPMI 1640 medium were transferred into the wells of a 96-microwell plate. Test compounds were initially dissolved in DMSO, serially diluted in culture medium and 50 μL of 2-fold dilution was added into each well to obtain the final concentration of 0.001, 0.01, 0.1 and 1 mmol L^{-1}. Controls contained the test model cells and culture medium (containing the same final concentration of DMSO, < 0.2 %), but no test compounds. A negative control (medium without cells but with test compounds) was used as a blank. Cells were incubated for 24, 48 and 72 hours before addition of 20 μL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) and were additionally incubated in the dark at 37 °C for the following 2 hours. The absorbance was measured at a wavelength of 490 nm using a microplate reader Victor^2-1420 Multilabel Counter (PerkinElmer). Two series of experiments were performed in triplicates. Data were analyzed by the one-way analysis of variance (ANOVA). Because of accuracy of the method any difference was considered significant if larger than ± 20 % and at $p < 0.05$.

Manganese porphyrin test. – Spectrometric assay for nitric oxide was performed by modification of a previously published procedure (12). A 1 μmol L^{-1} manganese(III)-5,10,15,20-tetrakis(*N*-ethylpyridinium-2-yl) porphyrin (MnTE-2-PyP^{5+}) with 1 mmol L^{-1} sodium L-ascorbate in PBS, pH 7.4 was prepared. Stock solutions of tested compounds were prepared by weighing an appropriate amount of compound and dissolving it in distilled water to a final concentration of 1 mmol L^{-1}. PBS solution (1 mL) was mixed with 1 mL of a freshly prepared solution of the tested compound. Spectra were measured in an airtight 1-cm quartz cuvette cell in a range of 350 to 600 nm.

Syntheses

*1,1-Carbonylbisbenzotriazole (1,1-carbonylbis(1*H*-1,2,3-benzotriazole)* (**1**). – It was synthesized by modification of a previously published procedure of Katritzky and coworkers (13), where a phosgene solution (20 % in toluene) was replaced with a solid triphosgene. Compound **1** was prepared by mixing 2.30 g (0.02 mol) of 1-*H*-benzotriazole with 1.19 g (0.004 mol) of triphosgene in dry tetrahydrofurane for 72 h. THF was evaporated and the solid product was suspended in water. The suspension was acidified with HCl (1:1) up to pH 3 and mixed for 3 hours. The solid product was filtered off and dried in vacuo over P_2O_5.

Benzotriazole N-*carbamoyl derivatives* (**2**, **3** and **4**). – *N*-carbamoyl derivatives of benzotriazole were synthesized in the reaction of **1** with the corresponding hydroxylamine derivative in dioxane solution at room temperature (Scheme 1). Compound **1** (2.68 g, 0.01 mol), 0.01 mol of the corresponding hydroxylamine (1.60, 0.83 and 0.97 g of benzyloxyamine hydochloride, methoxyamine hydrochloride, and ethoxyamine hydoxychloride, respectively) and 1.1 g (0.01 mol) TEA were dissolved in 50 mL of dry dioxane. The solution was vigorously mixed for 48 h at room temperature. TEA×HCl was filtered off and washed with 10 mL of dioxane. The filtrate was evaporated to the consistency of oil and crystallized from water. The obtained product was filtered off, washed with HCl (pH 3), water and dried for 24 h in air and in vacuo over P_2O_5 at room temperature.

N-*benzyloxybiuret* (**6**). – Compound **2** (2.68 g, 0.01 mol), 0.60 g (0.01 mol) of urea and 0.34 g (0.005 mol) of imidazole were heated at 130 °C for 10 minutes. After cooling to room temeperature, the solid mixture was suspended in water and acidified with HCl to pH 3. The product was filtered off, washed with water and dried over P_2O_5.

4-(Benzyloxycabamoyl)aminobenzoic acid (**7**). – Compound **2** (2.68 g, 0.01 mol), 1.37 g (0.01 mol) of 4-aminobenzoic acid and 0.68 g (0.01 mol) of imidazole were mixed at 130 °C for 15 minutes. The cooled mixture was suspended in water and acidified with HCl to pH 3. The white product was filtered off, washed with water, recrystallized from ethanol and dried in vacuo over P_2O_5.

N,N′-*bisbenzyloxyurea* (**9**). – Compound **2** (2.68 g, 0.01 mol), 1.60 g (0.01 mol) benzyloxyamine hydrochloride and 1.01 g (0.01 mol) TEA were suspended in 50 mL of dry dioxane. The reaction mixture was mixed for 48 hours at room temperature. TEA×HCl was filtered off and washed with 10 mL of dry dioxane. The solvent was removed and the obtained oil was crystallized with the addition of water. The white solid product was washed with HCl (pH 3) and water, recrystallized from ethyl acetate and hexane and dried over P_2O_5 in vacuo.

*1-(*N*-hydroxycarbamoyl)benzotriazole* (**5**), N-*hydroxybiuret* (**12**), *4-(hydroxyaminocarbonyl)aminobenzoic acid* (**13**), N-*hydroxyurea* (**14**), N,N′,N″-*trihydroxyisocyanuric acid* (**16**) *and* N,N′,N″-*trihydroxibiuret* (**15**)

A mixture of 1-(*N*-benzyloxycarbamoyl)benzotriazole (**2**), *N*-benzyloxybiuret (**6**), 4-(benzyloxycabamoyl)aminobenzoic acid (**7**), *N*-benzyloxyurea (**8**), *N*,*N*′,*N*″-tribenzyloxyisocyanuric acid (**10**) or *N*,*N*′,*N*″-tribenzyloxybiuret (**11**) and 0.05 g palladium on carbon (Pd/C, 10%) in methanol (100 mL) was kept under hydrogen (r. t., normal pressure) for 1–10 hours. Pd/C was filtered off and methanol was evaporated. The obtained products **5**, **12**, **13**, **14**, **16** and **15**, respectively, were dried over P_2O_5 in vacuum and were obtained in practically theoretical yields.

N-*benzyloxyurea* (**8**), *N,N′,N″-trihydroxyisocyanuric acid* (**16**) and its triacetyl ester (**17**) were prepared according to previously published procedures (6, 14, 15).

RESULTS AND DISCUSSION

Chemistry

A reported synthesis of 1-(*N*-benzyloxycarbamoyl)benzotriazole (**2**) included the reaction of 1-(*N*-carbamoylchloride)benzotriazole with benzyloxyamine hydrochloride (15). In our research, 1,1′-carbonylbisbenzotriazole (**1**) was used in reactions with the corresponding oxyamine hydrochlorides (benzyloxyhydroxylamine hydrochloride, methoxyamine hydrochloride and ethoxyamine hydrochloride) and triethylamine in dioxane suspension. Compound **1** was prepared in a suspension of 1-*H*-benzotriazole and a solid triphosgene instead of phosgene in toluene (20 % solution), which is less aggressive, more stable and ecologically more friendly. The use of **1** in reactions with methoxy-, ethoxy- and benzyloxy-amines afforded **2**, 1-(*N*-metoxycarbamoyl)benzotriazole (**3**) and 1-(*N*-ethoxycarbamoyl)benzotriazole (**4**) with yields of 87, 76 and 82 %, respectively (Scheme 1). Among them, only **2** reacted with hydrogen in the presence of palladium/carbon (Pd/C, 10 %), which resulted in 1-(*N*-hydroxycarbamoyl)benzotriazole (**5**) in quantitative yield. Uesato *et al*. (16) reported the use of benzyloxyisocyanate as a useful building block in the synthesis of hydroxyurea derivatives, while Butula and Jadrijević-Mladar Takač (14) reported that **2,** when heated over 100 °C liberates benzyloxyisocyanate which is further trimerized to *N,N′,N″*-tribenzyloxy-triazinone (**10**), and yields a minor product *N,N′,N″*--tribenzyloxybiuret (**11**). In this work, we utilized **2** as a donor of benzyloxyisocyanate, which is very useful for the synthesis of hydroxyurea derivatives. Due to its low solubility in water, **2** and products of its reactions can be easily separated from all water soluble substances and reaction products. When heated up to 130 °C in reactions with urea or 4-aminobenzoic acid, in the presence of imidazole as a catalyst, it afforded *N*-benzyloxybiuret (**6**) and 4-{[(benzyloxy)carbamoyl]amino}benzoic acid (**7**) with yields of 68 and 81 %, respectively. Under the same experimental conditions, **1** in the reaction with *N*-benzyloxyurea (**8**) and *N,N′*-bisbenzyloxyurea (**9**) (17) did not yield the expected di- and trisubstitued biurets (18). Instead, trimerization occurred and only compound **10** was isolated from the reaction mixture.

Compounds **2**, **6**, **7**, **8, 10** and **11** were hydrogenated with hydrogen under atmospheric pressure at room temperature, in the presence of a catalytic amount of palladium on carbon (Pd/C, 10 %) (14). The corresponding hydroxyl derivatives, *i.e.*, 1-(*N*-hydroxycarbamoyl)benzotriazole (**5**), *N*-hydroxybiuret (**12**) (20), 4-{[(hidroxy)carba - moyl]amino}benzoic acid (**13**), *N*-hydroxurea (**14**), *N,N′,N″*-trihydroxybiuret (**15**) (20) and *N,N′,N″*-trihydroxyisocyanuric acid (**16**) were obtained in quantitative yields. The synthetic routes and structures of isolated compounds are presented in Scheme 1, whereas their physicochemical data are summarized in Tables I and II.

The synthesized set of compounds (Table I) represents different derivatives of hydroxyurea, which can be categorized into several subgroups, *i.e.*, benzotriazole *N*-carbamoyl derivatives (**2**, **3**, **4** and **5**), cyclic- (**10**, **16** and **17**) and acyclic hydroxyurea derivatives (**7**, **8**, **9**, **13** and **14**), and biuret derivatives (**6**, **11**, **12** and **15**).

Table I. Physical data of synthesized compounds

Compound	Formula (M_r)	Yield (%)	M.p. (°C)		Elemental analysis Calcd/found (%)		
			Found	Literature value (Reference)	C	H	N
2	$C_{14}H_{12}N_4O_2$ (268.27)	87	113–114	113–114 (15)	62.68 / 63.01	4.51 / 4.48	20.89 / 20.78
3	$C_8H_8N_4O_2$ (192.0)	82	143–145		50.00 / 51.04	4.20 / 4.19	9.15/ 9.13
4	$C_9H_{10}N_4O_2$ (206.2)	76	121–122		52.42 / 52.37	4.89 / 4.90	27.17 / 27.22
5	$C_7H_6N_4O_2$ (178.15)	100	132–134	132–134 (15)	47.19 / 47.52	3.39 / 3.75	31.45 / 31.12
6	$C_9H_{11}N_3O_3$ (209.2)	68	175–177		51.67 / 51.65	5.30 / 5.32	20.09 / 20.07
7	$C_{15}H_{14}N_2O_4$ (286.2)	81	> 350		62.93 / 62.75	4.93 / 4.94	9.79 / 9.81
8	$C_8H_{10}N_2O_2$ (166.18)	85	138–140		57.82 / 67.83	6.07 / 6.05	16.86 / 16.99
9	$C_{15}H_{16}N_2O_3$ (272.30)	89	88–89	86 (18)	66.16 / 66.20	5.92 / 5.90	10.29 / 10.31
10	$C_{24}H_{21}N_3O_6$ (447.43)	83	249–250	249–250 (15)	64.62 / 64.15	4.73 / 4.83	9.39 / 9.74×
11	$C_{23}H_{23}N_3O_5$ (421.45)	91	78–80		65.55 / 65.41	5.50 / 5.45	9.97 / 10.02
12	$C_2H_5N_3O_3$ (119.08)	100	170–171	171 (20)	20.17 / 20.01	4.23 / 4.18	35.29 / 35.18
13	$C_8H_8N_2O_4$ (196.16)	100	>350		48.98 / 48.79	4.11 / 4.15	14.28 / 14.31
14	$CH_4N_2O_2$ (76.06)	100	137–140	133–136 (21)	15.79 / 15.75	5.30 / 5.29	36.83 / 36.79
15	$C_2H_5N_2O_5$ (151.08)	100	65–66		15.90 / 15.73	3.34 / 3.46	27.81 / 27.74
16	$C_3H_3N_3O_6$ ×$2H_2O$ (213.09)	100	255–256	255–256 (15)	16.91 / 17.24	3.31 / 3.30	19.72 / 20.12
17	$C_9H_9N_3O_9$ (303.17)	75	203–204	203–204 (7)	35.65 / 35.70	2.99 / 3.08	13.86 / 13.75

Table II. Spectral data of synthesized compounds

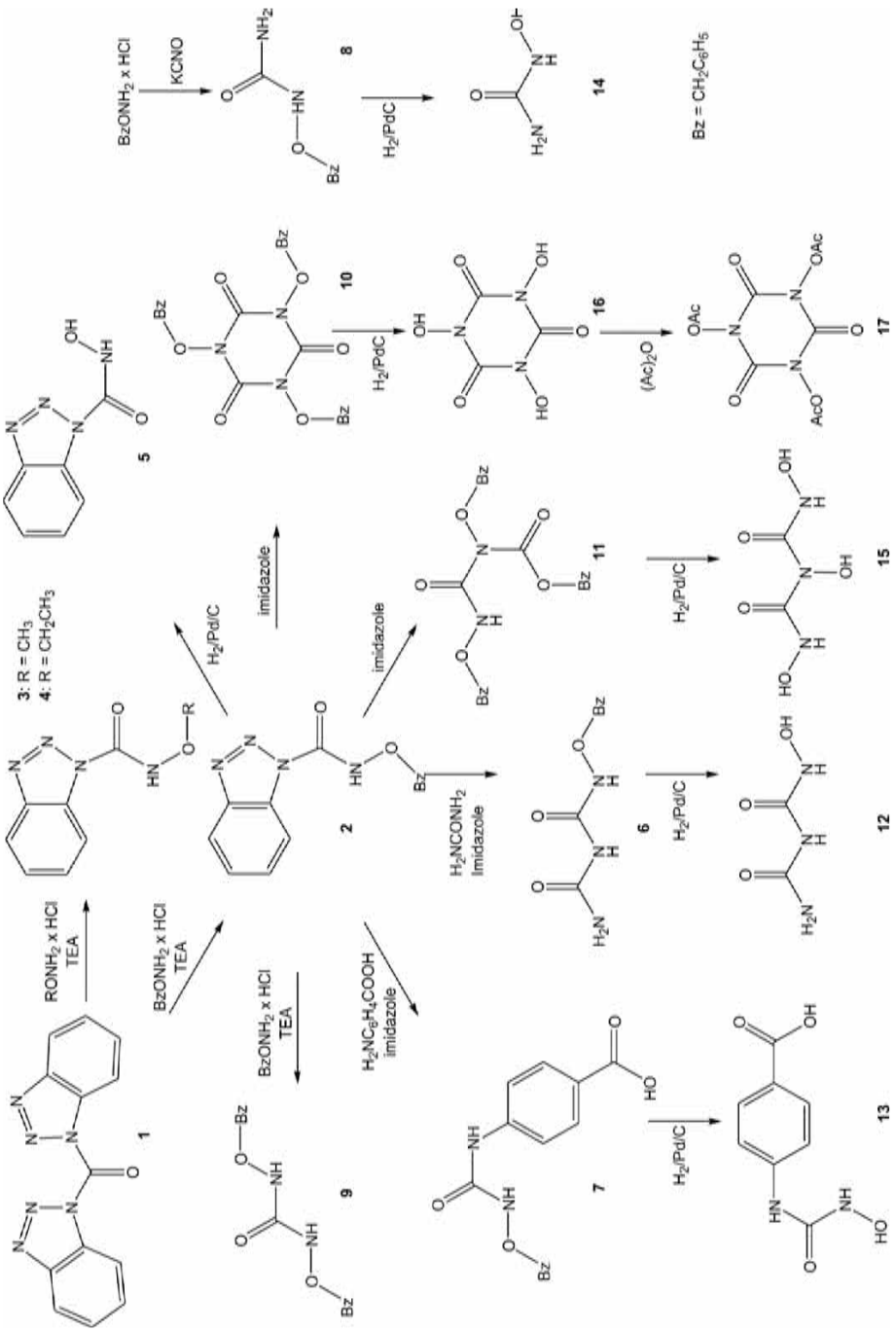
Compound (reference)	FTIR (KBr), ν (cm^{-1})	^{1}H NMR (DMSO-d_6) δ (ppm)	^{13}C NMR (DMSO-d_6) δ (ppm)
2 (15)	3246, 3113, 3030, 2947, 2884, 1728, 1603, 1484, 1367, 1289, 1231, 1132, 1093, 1003, 924, 869, 751, 699, 563	9.95 (s, 1H, NH), 8.20 (d, $^3J_{H,H}$ = 8.2 Hz, 1H), 8.09 (d, $^3J_{H,H}$ = 8.5 Hz, 1H), 7.67 (t, $^3J_{H,H}$ = 7.7 Hz, 2H), 7.48 (t, $^3J_{H,H}$ = 7.7 Hz, 3H), 7.34 (d, $^3J_{H,H}$ = 4.4 Hz, 2H), 5.14 (s, 2H, CH_2)	148.68, 145.72, 134.46, 131.54, 130.23, 129.28, 128.97, 128.63, 125.66, 120.07, 113.49, 79.24
3	3310, 1730, 1492, 1474, 1448, 1320, 1378, 1292, 1234, 1162, 1134, 1092, 1018, 934, 924, 856, 748, 772, 756, 548	9.96 (s, 1H, NH), 8.25 (d, $^3J_{H,H}$ = 8.3 Hz, 1H), 8.1 (d, $^3J_{H,H}$ = 8.1 Hz, 1H) 7.5 (t, $^3J_{H,H}$ = 7.6 Hz, 1H), 7.48 (t, $^3J_{H,H}$ = 7.5 Hz, 1H), 4.01 (s, 3H, CH_3)	148.76, 145.81, 131.60, 130.32, 125.76, 120.16, 65.35
4	3280, 2982, 1756, 1592, 1494, 1450, 1382, 1286, 1298, 1230, 1138, 1022, 960, 926, 876, 854, 816, 780, 774, 758, 538, 512, 490	9.85 (s, 1H, NH), 8.20 (d, $^3J_{H,H}$ = 8.1 Hz, 1H), 8.15 (d, $^3J_{H,H}$ = 8.3 Hz, 1H) 7.2 (t, $^3J_{H,H}$ = 7.7 Hz, 1H), 7.55 (t, $^3J_{H,H}$ = 7.7 Hz, 1H), 4.04 (q, $^3J_{H,H}$ = 7.0 Hz, 2H, CH_2), 1.26 (t, $^3J_{H,H}$ = 7.0 Hz, 3H, CH_3)	148.87, 145.25, 131.62, 130.26, 125.70, 120.13, 113.58, 73.34, 13.43
5 (15)	3297, 3132, 2939, 2876, 1750, 1711, 1610, 1536, 1489, 1451, 1368, 1290, 1240, 1135, 1104, 1017, 936, 870, 785, 744, 644	8.86 (s, 1H, OH), 8.37 (s, 1H, NH), 7.92 (s, 2H, H4 and H7), 7.46 (d, $^2J_{H,H}$ = 3.43 Hz, 2H)	162.68, 138.68, 130.22, 125.65, 120.04, 113.48
6	3360, 3236, 1690, 1682, 1582, 1606, 1496, 1456, 1390, 1366, 1286, 1244, 1132, 1092, 1042, 970, 928, 908, 788, 744, 698, 648, 626, 594, 518, 480, 462	10.09 (s, 1H, NH-O), 8.66 (s, 2H, CONHCO), 7.40 (m, 5H, ar. H), 7.02 (s, 2H, NH_2), 4.80 (s, 2H, CH_2)	155.47, 154.10, 138.09, 135.73, 128.79, 128.23, 77.57
7	3284, 3030, 2676, 2560, 1700, 1676, 1658, 1606, 1552, 1498, 1430, 1324, 1294, 1236, 1178, 1108, 1042, 1002, 926, 916, 876, 860, 788, 764, 748, 728, 696, 668, 668, 638, 610, 552, 504, 427	12.64 (s, 1H, COOH), 9.69 (s, 1H, NH-O-), 9.08 (s, 1H, NH), 7.60 (m, 9H, ar. H), 4.84 (s, 2H, CH_2)	166.92, 156.51, 143.30, 136.16, 130.05, 128.79, 128.12, 128.02, 124.09, 118.17, 77.56.
8	3398, 3224, 1680, 1630, 1456, 1418, 1358, 1210, 1106, 1058, 998, 940, 906, 798, 748, 696, 644, 622, 588, 560, 494	9.05 (s, 1 H, NH), 7.39 (m, 5H, ar.H), 6.38 (s, 2H, $N'H_2$), 4.72 (s, 2H, CH_2)	160.71, 136.46, 128.58, 128.09, 127.86, 77.17
9	3230, 3064, 3030, 2924, 1668, 1466, 1454, 1356, 1306, 1208, 1116, 1072, 1016, 986, 934, 908, 840, 810, 738, 696, 668, 626, 580, 544, 490	9.86 (s, 2H, NH), 7.37 (m, 10H, ar.H), 4.73 (s, 4H, CH_2)	159.39, 136.10, 128.60, 128.03, 127.89, 77.19

10 (15)	3034, 2956, 2924, 1740, 1457, 1402, 1197, 1001, 905, 750, 696, 476	7.56 (d, 2H, $^2J_{H,H}$ = 4.7 Hz, H3′ and H7′), 7.46 (t, 3H, $^3J_{H,H}$ = 4.9 Hz, H4′, H5′ and H6′), 5.13 (s, 2H, CH_2)	145.37, 133.94, 130.01, 129.51, 128.82
11	3287, 3064, 3032, 2943, 2888, 1716, 1566, 1364, 1298, 1215, 1189, 1028, 965, 904, 839, 738, 750, 700, 610, 543, 501	10.92 (s, 2H, NH), 7.39 (m, 15H, ar.H), 4.8 (s, 6H, CH_2)	153.02, 135.56, 134.01, 130.12, 128.97, 128.88, 128.42, 128.38, 128.20, 77.80, 77.49
12	3440, 3394, 3328, 3220, 3126, 2876, 1716, 1676, 1578, 1522, 1412, 1328, 118,1052, 890, 794, 762, 720, 690, 668, 618, 538, 468	9.49 (s, 1H, OH), 9.08 (s, 1H, CONH-O), 8.31 (s, 1H, CONHCO), 6.98 (s, 2 H, NH_2)	156.73, 154.04
13	3352, 3232, 2896, 2660, 2548, 1686, 1652, 1542, 1520, 1420, 1294, 1320, 1234, 1184, 1126, 1092, 998, 940, 882, 862, 840, 772, 720, 696, 658, 638, 596, 556, 510	12.59 (s, 1H, COOH), 9.20 (s, 1H, NOH), 9.05 (s, 2H, NH), 7.79 (m, 4H, ar. H)	166.97, 157.99, 143.70, 130.02, 123.76, 118.02
14	3318, 3208, 2818, 1652, 1594, 1490, 1408, 1112, 824, 764, 626, 560	8.66 (s, 1H, OH), 8.34 (s, 1H, NH), 6.25 (s, 1H, N'H)	162.52
15	3362, 3282, 3204, 1718, 1664, 1552, 1504, 1316, 1180, 1056, 892, 802, 688, 560, 468	10.21 (s, 2H, 2OH), 10.13 (s, 1H, OH), 8.93 (s, 2H, NH)	155.41
16 (15)	3535, 3427, 2916, 2849, 2706, 1720, 1568,1434,1212, 1164, 1010, 702, 478	11.11 (s, 3H)	147.19
17 (7)	1829, 1749, 1403, 1369, 1213, 1146, 1015, 824, 804, 788, 730, 585	1.91 (s, 9H)	174.81, 143.54, 24.21

IR spectra of benzotriazole *N*-carbamoyl derivatives **2** to **5** are characterized with C=O stretching vibrations in the range $\nu_{C=O}$ from 1728 to 1756 cm^{-1}. These vibrations are shifted towards higher frequency values compared to urea stretching vibrations due to dipole characteristics of carbamoyl moiety. The NH stretching vibrations of **2** to **5** were observed in the range ν_{NH} 3248 to 3310 cm^{-1}, while in the spectrum of **5** the NHOH stretching vibrations were revealed from ν 3554 to 2500 cm^{-1} as strong broadbands.

^{1}H NMR spectra of **2** to **5** showed chemical shifts for benzotriazole ring protons in the range from δ 7.00 to 8.50 ppm with two characteristic signals, one doublet and one triplet, in addition to benzyl protons in the spectrum of **2** and methyl and ethyl protons in the spectra of **3** and **4**, respectively. Chemical shifts, δ OH and δ NH in **5** were observed as two separated signals, at 8.86 and 8.37 ppm.

^{13}C NMR spectra of **2** to **5** showed chemical shifts for the C=O group at δ 148.68, 148.76, 148.87 and 162.68 ppm, respectively.


BzONH2 x HCl
KCNO
8
H2/PdC
14
Bz = CH2C6H5
RONH2 x HCl
TEA
BzONH2 x HCl
TEA
BzONH2 x HCl
TEA
H2NC6H4COOH
imidazole
H2NCONH2
Imidazole
H2/Pd/C
imidazole
imidazole
H2/Pd/C
H2/Pd/C
H2/Pd/C
H2/PdC
(Ac)2O
3: R = CH3
4: R = CH2CH3
1
2
5
9
10
11
6
7
12
13
15
16
17

Scheme 1.

IR spectra of cyclic hydroxyurea derivatives (**10**, **16** and **17**) revealed three very strong, almost overlapped, stretching vibrations for cyclic C=O in the range from 1674–1745 cm^{-1} (ν_{max} 1740 cm^{-1}) for **10**, 1648–1720 cm^{-1} for **16** (ν_{max} 1718 cm^{-1}) and 1750–1714 cm^{-1} for **17** (ν_{max} 1749 cm^{-1}). In addition, in the spectrum of **17** the $\nu_{C=O}$ of three ester moieties were found as a single stretching vibration at 1829 cm^{-1}.

Due to high symmetry and equivalence of functional moieties in the molecules of cyclic hydroxyurea derivatives (**10**, **16** and **17**), the ^1H and ^{13}C NMR spectra of these compounds are very simple. In ^1H NMR spectra, the following chemical shifts were revealed at δ 7.56–7.46 ($H_{ar.}$) and 5.13 ppm (benzyl -CH_2-) in **10**, only one signal for proton in OH in **16** at δ 11.11 ppm, and only one signal for CH_3 protons in **17** at δ 1.91. In ^{13}C NMR spectra of **10**, **16** and **17**, chemical shifts of C-atoms in the corresponding carbonyl groups involved in triazintrione ring systems were found at 145.37, 147.19 and 143.54 ppm, respectively. The downfield of chemical shifts for C-atom in C=O of the triazintrione ring was observed in the order **16** > **10** > **17** due to the chemical type of compound and involved substituents (OH, benzyl and acetyl). The chemical shift of ester carbonyl group in the spectrum of **17** was observed more downfield at δ 174.81 ppm.

Compared to stretching vibrations of C=O groups immersed in the triazintrione ring system of cyclic urea derivatives, analogous $\nu_{C=O}$, in hydroxyurea and its acyclic derivatives (**7**, **8**, **9**, **13** and **11**) were observed as a very strong and single stretching vibration at lower frequencies, *i.e.*, at ν 1658, 1680, 1668, 1686 and 1652 cm^{-1}, respectively. The ^1H NMR spectra of compounds **7**, **8**, **9**, **11** and **13**, showed typical chemical shifts that correspond to protons in aromatic moieties (**7**, **8**, **9** and **13**), the carboxylic group (**7** and **13**), and to NH and OH protons in hydroxyurea moieties in **13** and **14**. Chemical shifts of hydroxyurea NH protons were observed in the range 6.24 to 9.86 ppm while chemical shifts of hydroxyl protons (NH-OH) in the spectra of **13** and **14** were found at δ 9.20 and 8.66 ppm, respectively. Comparing these chemical shifts with analogous proton chemical shifts in the spectrum of **16**, $\delta_{N\text{-}OH}$ in ^1H NMR spectra of **13** and **14** were observed in a higher field. The corresponding chemical shifts, $\delta_{C=O}$, of hydroxyurea moieties of these compounds were found at δ 156.91 (**7**), 160.91 (**8**), 159.39 (**9**), 167.997 (**13**) and 162.51 (**14**) ppm, and lower in the field compared to $\delta_{C=O}$ of triazintrione moieties in **10**, **16** and **17** by more than 10 ppm (δ range 13 to 15 ppm) These differences in chemical shifts can be attributed to the strained triazintrione ring with cyclic poly-hydroxyurea moieties.

The basic structure of all synthesized biuret derivatives (**6**, **11**, **12** and **15**) is $R^1(NH)CON(R^2)CON(R^3)$. The IR spectra of these compounds in the part of functional groups are characterized by one strong stretching vibration for both C=O groups in each spectrum of **6** and **11** (ν 1682 and 1716 cm^{-1}, respectively), hydroxyl biuret group is masked by benzyl moiety, while in **12** and **15** the OH group is free, and two very strong vibrations for C=O were observed at ν 1716 and 1676 cm^{-1} in the spectrum of **12,** and at 1718 and 1664 cm^{-1} in the spectrum of **15**. Additionally, in the spectra of **12** and **15**, in part of OH, NH_2 and NH stretching vibrations, several broadband vibrations were overlapped in the range from 3340 to 3000 cm^{-1}, while in the spectra of **6** and **11**, only two sharp vibrations in each spectrum were observed, *i.e.*, at ν 3360 and 3236 cm^{-1} in **6** and at 3350 and 3288 cm^{-1} in **11**.

The ^1H NMR spectra of biuret derivatives **6** and **11** were represented by NH_2 and NH in the range from 8.66 to 10.98 ppm, δ of H_{ar} in the range from 7.05 to 7.40 ppm, while δ of CH_2 benzyl protons were observed in the range from 4.80 to 4.88 ppm. Proton

NMR spectra of **12** and **15** consisted only of chemical shifts for OH, NH_2 and NH protons, which were observed in the range from 8.66 to 10.09 ppm. In the molecule **11** only one signal was observed for both NH protons, while in **15** three signals were observed at δ 10.21 ppm for two –NH-OH, 10.13 ppm for N-OH and 8.927 ppm for both NH protons. In ^{13}C NMR spectra of compounds **11** and **15**, only one signal for both C=O groups was revealed at δ 153.02 and 155.41 ppm, in each spectrum, while in asymmetrically substituted biuret compounds **6** and **12**, two chemical shifts were observed for each C=O in each spectrum, *i.e.*, 154.10 and 155.47 (**6**), and δ 154.04 ppm and 156.73 ppm (**12**).

Metabolic activity

To date, there is no data on the cytotoxic effects of synthesized compounds **2**–**17** on human acute monocytic leukemia THP-1 and human acute T cell leukemia Jurkat cell line, so we investigated the effects of compounds in the 0.001, 0.01, 0.1 and 1 mmol L^{-1} concentrations of the compounds on the metabolic status of the cells using the MTS assay.

The results of this study showed that compounds **6**, **8** and **10** were not cytotoxic up to 1 mmol L^{-1} to either leukemia cell line tested, nor changed the metabolic status of the cells. Only compounds **7**, **14** and **15** influenced the metabolic status of the cells at concentrations lower than 1 mmol L^{-1} in a manner described below: the toxic effect (~50 %) of 0.1 mmol L^{-1} of the compound **13** was observed only on THP-1 cells, while compound **15** showed a stimulative effect at 0.1 mmol L^{-1} concentration on both cell lines, and increased metabolic activity of THP-1 cell line by 45 % and Jurkat cell line by 39 % (data not shown). Compound **7** decreased the metabolic activity of Jurkat cells by ~34, 31 and 66 % in respect to control cells, when applied at 0.01, 0.1 (data not shown) and 1 mmol L^{-1} concentrations (Fig. 1b), respectively. Such effect was not observed on THP-1 cells; just a slight decrease of metabolic activity was detected at 1 mmol L^{-1} (Fig. 1a).

Among sixteen investigated compounds, eleven compounds showed a significant effect on the metabolic status of the cells at a concentration of 1 mmol L^{-1} (Figure 1). Ten compounds were toxic either to both or at least to one type of cells, while compound **5** showed a stimulative effect (of ~55 % on THP-1 cells and ~25 % on the Jurkat cell line). An extensive (more than 70 %) toxic effect on both cell lines was detected at 1 mmol L^{-1} for compounds **9**, **11**, **12**, **14** and **15**. Compound **16** decreased the metabolic activity by ~54 % of the Jurkat cell line, while its acetyl derivative, **17**, reduced the metabolic activity of THP-1 by 34 % and of Jurkat cell lines by 43 %. Compounds **2**, **3** and **4** showed a decrease in metabolic activity only for THP-1 cells, by 43, 35 and 39 %, respectively.

The cytotoxic effect of *N*-hydroxyurea **14** is well known and is based on its ability to inhibit the M2 subunit of ribonucleotide reductase by quenching the tyrosine radical (21). Gale *et al.* (22) reported a cytotoxic effect of *N*-hydroxybiuret, **12**. In this study, both compounds, **12** and **14**, showed a strong cytotoxic effect on THP-1 and Jurkat cells, while their corresponding benzyloxy derivatives (**6** and **8**) showed no activity, presumably because of the blocked OH group and consequently inability to quench the tyrosine radical. On the other hand, structurally similar derivatives, *N*,*N*′-bisbenzyloxyurea (**9**), and *N*,*N*′,*N*″-tribenzyloxybiuret (**11**), were highly toxic, compared to their hydroxy derivatives **6** and **7**, which could imply another mode of action for these compounds. *N*,*N*′,*N*″-trihydroxybiuret (**15**) showed a dose-dependent, opposing effect, being cytotoxic at 1 mmol

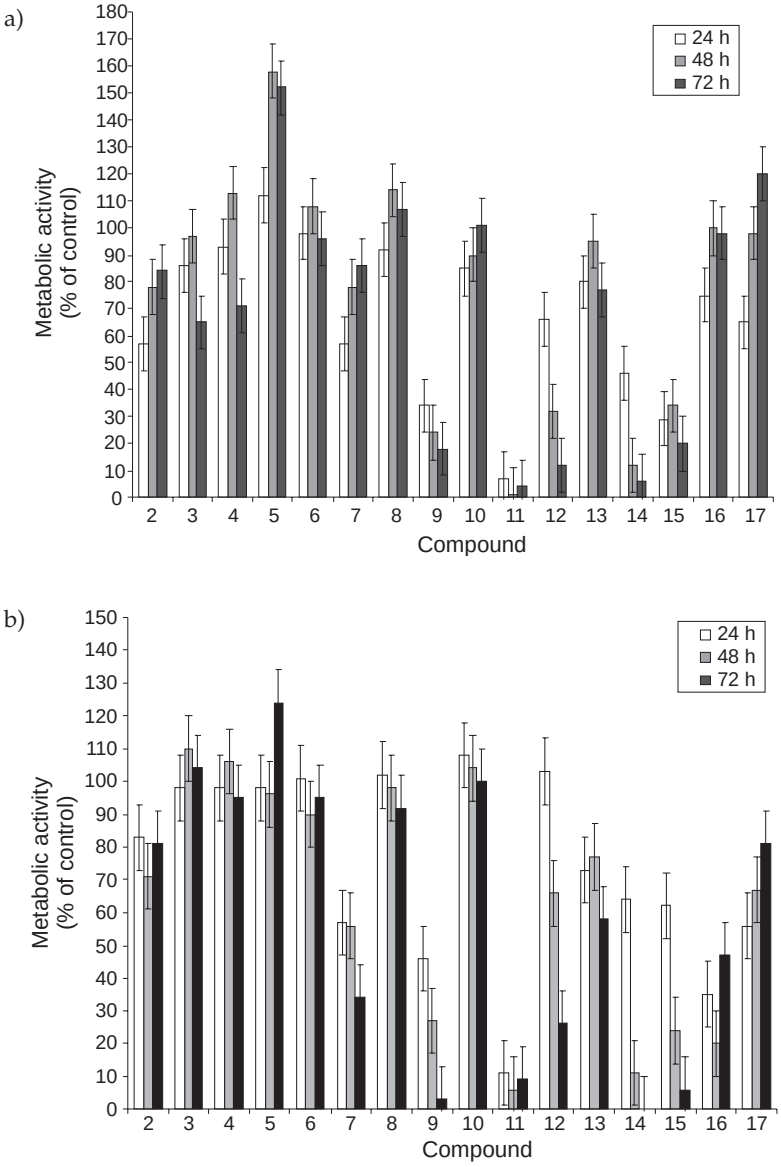


Fig. 1. Effect of compounds **2-17** on the metabolic activity of THP-1 and Jurkat cells. a) THP-1 and b) Jurkat cells were cultivated in the presence of 1 mmol L^{-1} compounds **2–17.** Experiments performed in triplicate are presented as percentage of metabolic activity of treated *vs.* untreated cells (corrected for the corresponding media) ± SEM, $n = 3$. Changes in the metabolic activity of at least 20 % and $p < 0.05$ are marked with an asterisk.

L^{-1} but stimulative at 0.1 mmol L^{-1} on both cell lines. The *N*′-benzyloxyurea derivative of 4-amino benzoic acid, **7**, decreased the metabolic activity of the Jurkat cell line at 1 mmol L^{-1} by 66 %, while its corresponding *N*′-hydroxyurea derivative, **13**, decreased the metabolic activity of both THP-1 and Jurkat cell lines at 1 mmol L^{-1} by 23 and 42 %, respectively. In addition, compound **7** was toxic at 0.01 and 0.1 mmol L^{-1} on Jurkat cells. These results suggest that compounds **7** and **13** decrease cell metabolic activity regardless of the hydroxylamine OH moiety being unprotected or derivatized with a benzyloxy moiety. Compounds **2**–**5**, which belong to a subgroup of benzotriazole analogues, can be considered as reactive ureas. Unlike the insignificant toxic effect of compounds **2**, **3** and **4** on Jurkat cell lines at 1 mmol L^{-1} (19, 4 and 5 %, respectively) and the slight toxic activity on monocytic THP-1 cells at 1 mmol L^{-1} (34, 35 and 39 %, respectively), compound **5**, which is characterized by unprotected OH moiety, showed a stimulative metabolic effect on both cell lines (61 % for THP-1 and 24 % for Jurkat). Different effects were also reveled among isocyanuric acid derivatives **10**, **16** and **17**. The *N*,*N*′,*N*″-tribenzylisocyanuric acid (**10**) did not show any effect on either cell line, *N*,*N*″,*N*″-trihydroxyisocyanuric acid (**16**) was toxic only to the Jurkat cell line (1 mmol L^{-1}, 54 %), while its more lipophylic triacetic acid ester derivative, **17**, was toxic to both cell lines at 1 mmol L^{-1} concentration with a decrease of metabolic activity by 34 % for THP and 43 % for the Jurkat cell line.

Antibacterial activity

The activity of compounds **2**–**17** was tested on three different *E. coli* strains at concentrations of 0.001, 0.01, 0.1 and 1 mmol L^{-1}. Several compounds showed a significant activity (defined as at least 20 % of decrease in bacterial growth) at concentrations of 1 mmol L^{-1} and the results are shown in Figure 2. No activity was observed at lower concentrations. The most efficient compounds were **5**, **10** and **15**. They decreased the bacterial growth of *E. coli* DH5a from 63 to 96 %, respectively, of *E. coli* strain DH5a/pUC18-*ermC*′ from 60 to 86 %, and of strain BL21(DE3)/pET25b(+)-*sgm* from 50 to 93 %, respectively.

Among the investigated compounds, **10**, **16** and **17**, showed antibacterial activity. They probably have similar mechanisms of action, though yet unknown, since they are structural analogues. The activity of the two structurally different compounds (**5** with a benzotriazole ring and **15** as a biuret derivative) could not be assigned to a similar mechanism like in the case of *N*,*N*′,*N*″-trihydroxyisocyanuric acid analogues, hence some other modes of action had to be considered. It is interesting, however, that compounds **5**, **7** and **15** showed less evident antibacterial activity, either on the strain resistant to macrolide antibiotics or on the sensitive DH5a strain, while **10**, **16** and **17** showed stronger antibacterial activity on the strain resistant to aminoglycoside antibiotics. Aminoglycoside resistant strain expressed 16S rRNA methyltransferase Sgm, a member of the Arm family of enzymes that methylate the N^7 position of residue G1405 within the decoding- or A-site in the small ribosomal subunit (23). This modification prevents 4,6-disubstituted 2-deoxystreptamine aminoglycosides from binding to the A-site, thus rendering them bacteria resistant (12).

The additional methyl group changes the form and depth of the aminoglycoside binding pocket, but this rearrangement could simultaneously enable binding of different

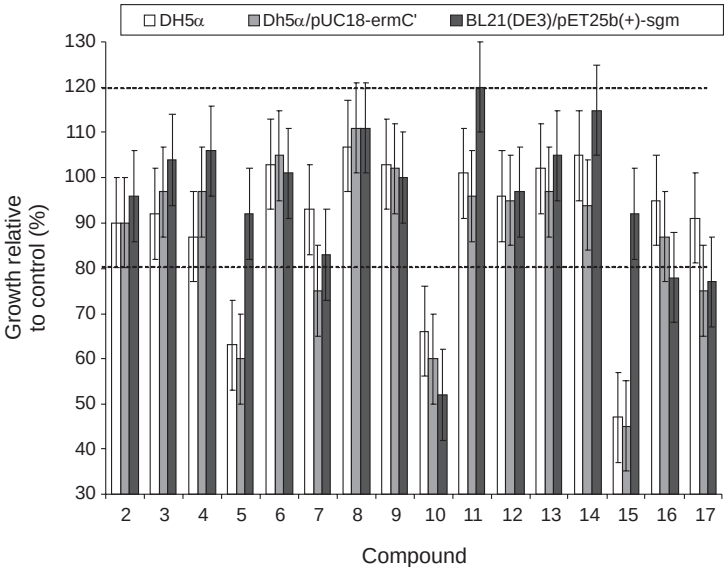


Fig. 2. The relative bacterial growth of *E. coli* strains DH5a, DH5a/pUC18-*ermC′* and BL21(DE3)/pET25b(+)-*sgm* in the presence of compounds **2–17** at concentration of 1 mmol L^{-1} represented as the percentage of growth obtained for a control sample (bacterial strains grown in the absence of tested compounds) ± SEM, $n = 3$.

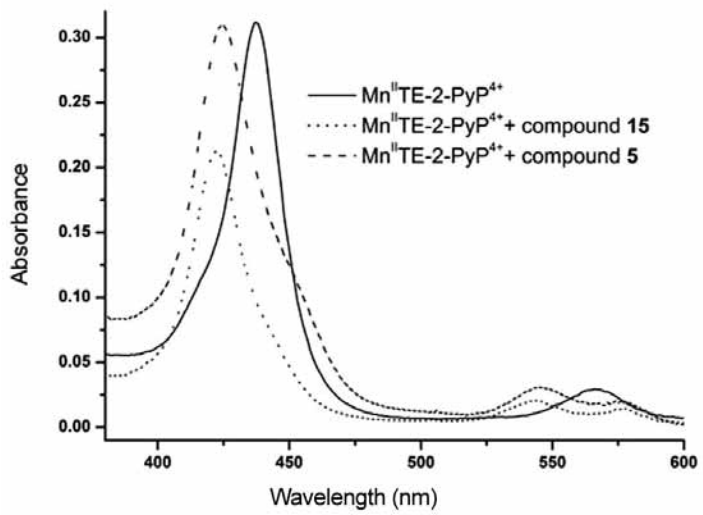


Fig. 3. Spectra Mn(II)-5,10,15,20-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP^{4+}) with compounds **5** and **15**. Hypsochromic shifts from 438 nm to 422 nm indicate coordinations of NO. c(MnTE-2-PyP^{4+}) = 0.5 μmol L^{-1}, c(**5** or **15)** = 0.5 mmol L^{-1}.

chemical entities with similar properties. Compounds **10**, **16** and **17** are all molecules that could mimic parts of aminoglycoside molecule and could still interact with nucleotides in the modified A-site *via* nitrogen atoms in a manner similar to kanamycin or related 4,6-disubstituted 2-deoxystreptamine antibiotics. This could result in interference with the protein synthesis and in turn affect the bacterial growth. In this respect, **10**, **16** and **17** could even be considered as lead molecules for drug development in the treatment of resistant bacteria that carry Arm methyltransferases as aminoglycoside resistance determinants. Compounds **5**, **7** and **15,** on the other hand, could accommodate better into unmodified A-site, thus having a more prominent effect on any bacterial strain that is either susceptible to antibiotics, or carries any other resistance determinants, except the Arm enzyme.

Manganese-porphyrin test

Dimethylsulfoxide solutions of compounds **5** and **15** showed instability and release of gas bubbles a few hours after solubilization. Due to a high content of nitrogen and oxygen in both compounds and findings of hydroxyurea as a nitric oxide donor, the corresponding compounds were tested for NO formation using the manganese-porphyrin test (5, 13, 24). Both **5** and **15** gave positive results (Figure 3), while all the other tested compounds were negative in the manganese-porphyrin test. Effects on the metabolic status of eukaryotic cells (25) and bacteriostatic activity (26) of NO have been demonstrated earlier and these results are in accord with our findings. Mechanisms and kinetics of NO production from **5** and **15** are out of scope of this paper and will be published elsewhere.

CONCLUSIONS

In this paper, the synthesis of new hydroxyurea cyclic and acyclic derivatives [1-(*N*-metoxycarbamoyl)benzotriazole (**3**), 1-(*N*-ethoxycarbamoyl)benzotriazole (**4**), *N*-benzyloxybiuret (**6**), 4-{[(benzyloxy)carbamoyl]amino}benzoic acid (**7**), 4-{[(hydroxy)carbamoyl]amino}benzoic acid (**13**) and *N*,*N*′,*N*″-trihydroxybiuret (**15**)] and new synthetic routes for these groups of compounds [1,1′-carbonylbisbenzotriazole (**1**), *N*-benzyloxycarbamoyl)benzotriazole (**2**), *N*,*N*′-bisbenzyloxyurea (**9**), *N*-hydroxybiuret (**12**) and *N*-hydroxurea (**14**)] are disclosed. Metabolic activity test showed the cytotoxic potential of twelve compounds (**2–4**, **7**, **9** and **11–17**). In addition, 1-(*N*-hydroxycarbamoyl)benzotriazole (**5**) stimulated metabolic activity, while compounds 1-(*N*-hydroxycarbamoyl)benzotriazole (**5**) and *N*,*N*′,*N*″-trihydroxybiuret (**15**) were identified as potential NO donors. Six compounds showed antibacterial activity (**5**, **7**, **10** and **15–17**). Findings described in this work could be used as guidelines for the preparation of new and modification of existing compounds, offering a model for tested biological effects in the drug discovery process.

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