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SYNTHESIS AND IMMUNOMODULATORY ACTIVITES OF NEW 5-HYDRAZINO-3-METHYL-4-ISOTHIAZOLECARBOXYLIC ACID ETHYL ESTERS

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Abstract: Several new derivatives of 5-hydrazino-3-methyl-4-isothiazolecarboxylic ethyl esters were synthesized. Using 4-aminoacetophenone, the hydrazine group was transformed in position 5 in the hydrazone which reacted with the isocyanates, aldehydes and sugars. Thirteen newly synthesized compounds were tested for their ability to affects the immunological response *in vitro* in several rodent models. The immunoregulatory properties of the compounds were differential and dose-dependent. The strongest activity was exhibited by 5-{N'–[1-4{-4-[3-(-methoxyphenyl)-ureido]–phenylethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (compound **3a**). The compound strongly inhibited the secondary, humoral immune response to sheep erythrocytes and the proliferative response of mouse splenocytes to concanavalin A and pokeweed mitogen. The immunotropic activities of the new isothiazole derivatives and potential application of the compounds in therapy are discussed.

Keywords: ethyl 5-hydrazino-3-methyl-4-isothiazolecarboxylate hydrazone derivatives, mice, humoral immune response, mitogens, TNF-α, IL-6

Isothiazole derivatives showed differential pharmacological activities such as anti-inflammatory (1, 2), antiviral (3, 4), analgesic and antibacterial (5) and immunomodulatory ones (6- 8).

Our earlier investigations revealed that the presence of –CH=N- (Schiff's base) moiety was crucial for the pharmacological activity of isothiazole derivatives (9-11). Considering this fact we decided to use ethyl ester of 5-hydrazino-3-methyl-4-isothiazolecarboxylic acid and ethyl ester of [N'-1-(4-aminophenyl)-ethylidenohydrazino]-3-methyl-4-isothiazolecarboxylic acid (9, 12), synthesized by us previously, for the synthesis of Schiff's bases and hydrazones with sugars as carbonyl component. Substituted urea derivatives, as a consequence of reaction with appropriate isocyanates, were also obtained.

The isothiazole derivatives were subjected to evaluation of their immunological activities. More specifically, the compounds were tested for their ability to affect the humoral immune response to sheep red blood cells *in vitro*, the proliferative response of mouse splenocytes to T- and B-cell mitogens and lipopolysaccharide-induced production of tumor necrosis factor alpha and interleukin-6 by rat peritoneal cell cultures.

EXPERIMENTAL

Immunology
Material and Methods

Animals

CBA mice, 12-weeks old, derived from the breeding centre in Ilkowice/Kraków. 2-month old Wistar rats were from the Animal Facility of The Medical University in Wrocław. The animals were fed a commercial pelleted food and water *ad libitum*. The local Ethics Committee approved the study.

Reagents and reference compounds

Sheep red blood cells (SRBC) were delivered by Wrocław University of Life and Environmental Sciences, Concanavalin A (ConA), pokeweed mito-

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gen (PWM), lipopolysaccharide (LPS) *E. coli* serotype O111:B4, DMSO, levamisole and leflunomide were from Sigma. Thiazolyl blue (MTT) was from Serva, RMPI 1640 and fetal calf serum (FCS) were from Gibco. Cyclosporine A (CsA) was from Sandinum (Switzerland).

Preparation of the compounds for the *in vitro* experiments

The preparations were initially dissolved in DMSO and subsequently in the culture medium. For a control, appropriate dilutions of DMSO were applied, corresponding to respective preparation concentrations.

The secondary humoral immune response to SRBC

Mice were primed with 0.2 mL of 1% SRBC suspension, intraperitoneally. After 4 days, the splenocytes were isolated and a single cell suspension was prepared in a medium (referred later as the culture medium) consisting of RPMI 1640, supplemented with 10% fetal calf serum, glutamine, sodium pyruvate and antibiotics. The cells were incubated in 24-well culture plates (5×10^{-6}/mL/well) with addition of 50 µL of 0.005% SRBC. After 4 days, the number of antibody forming cell number (AFC) in the cultures was determined (13). The results are shown as the mean values of AFC number from 4 wells ± standard error, calculated per 10^6 viable cells.

The proliferative response of splenocytes to mitogens

Spleens were pressed through a plastic screen into a pre-cooled Hanks' medium. Erythrocytes were lyzed using 0.84% NH_4Cl, the lymphocytes were washed 3 times with Hanks' medium and re-suspended in the culture medium. The cells were then distributed into 100 µL aliquots (2×10^5 cells) in 96-flat-bottom well plates. ConA and PWM were used at a concentration of 2.5 µg/mL. Control cultures contained solvent (DMSO) at appropriate dilutions. After 3-day culture, cell proliferation was determined using MTT colorimetric method (14).

Induction of cytokines in the culture of rat peritoneal cells

Peritoneal cavities of rats were lavaged with 10 mL of Hanks' medium, the cells were washed twice with Hanks' medium, counted and re-suspended in the culture medium at a density of 5×10^6/mL. The cells were distributed to 24-well culture plates in 1 mL aliquots. The compounds were added at a concentration of 10 µg/mL and LPS at a dose of 5 µg/mL. After 24 h culture in a cell culture incubator the supernatants were harvested for cytokine determination.

Determination of tumor necrosis factor alpha and interleukin-6 activities

The activities of cytokines were measured by bioassays. TNF-α concentration was determined using a highly sensitive WEHI 164.13 clone (ATCC CRL 1751) (15). IL-6 production was determined using 7TD1 indicator cells (16).

Statistics

The results are presented as the mean values ± standard error (SE). The Levene's and Brown-Forsyth's tests were used for determination of the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (One Way ANOVA) was applied, followed by post hoc comparison with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data were evaluated by the Kruskal-Wallis' analysis of variance, as indicated in the text. Significance was determined at $p = 0.05$. The statistical analysis was performed using STATISTICA 6.1 for Windows.

Chemistry

The melting points of all new compounds were measured in a Boetius hot stage (VEB Kombinat Negema Wägetechnik Rapido, Germany) and were uncorrected. Elemental analysis, carried out at the Microlaboratory of the Pharmacy Department, Wrocław Medical University, showed that all analytical results were in accord with the calculated ones within ± 0.5%.

The following equipment was employed: IR spectra – Specord M 80 apparatus, in KBr discs. ^{1}H NMR spectra – 300 MHz Bruker (Germany). The chemical shifts are expressed in δ (ppm) using TMS (tetramethylsilane) as an internal standard, coupling constants are given in Hz. TLC was performed with Polygram SIL G/UV_{254} plates (Macherey-Nagel, Düren, Germany, using toluene : methanol (9:1, v/v) as a mobile phase. Detection of the compounds on the chromatograms was performed with iodine vapor.

General method of synthesis of Schiff's bases (2a-c)

0.01 mole of compound **1** in 70 mL of anhydrous ethanol and 0.01 mole of respective aldehyde was warmed up for 4 h. The course of the reaction was controlled by TLC. Then, the reaction mixture was left for 12 h at room temperature.

General methods of synthesis of 3(a-d) derivatives

0.005 mole of compound **1** in 35 mL of anhydrous mixture $CH_3COOC_2H_5$: DMF (1:1, v/v) and 0.0055 mole of respective isocyanate (RNCO) were heated under reflux for about 4 h. The mixture was cooled, the precipitate was filtered, washed with ethanol (2 mL) and crystallized from ethanol or EtOH/DMF.

General method of synthesis of hydrazones (5a-e)

Compound **4** (0.01 mole) was dissolved in 80 mL of ethanol, treated with a solution of sugar (0.01 mole in water) and few drops of glacial acetic acid. The reaction mixture was refluxed for 4 h. An excess of the solvent was removed under reduced pressure. The residue was triturated with 20 mL of ether, the solid product was filtered, dried and crystallized from ethanol.

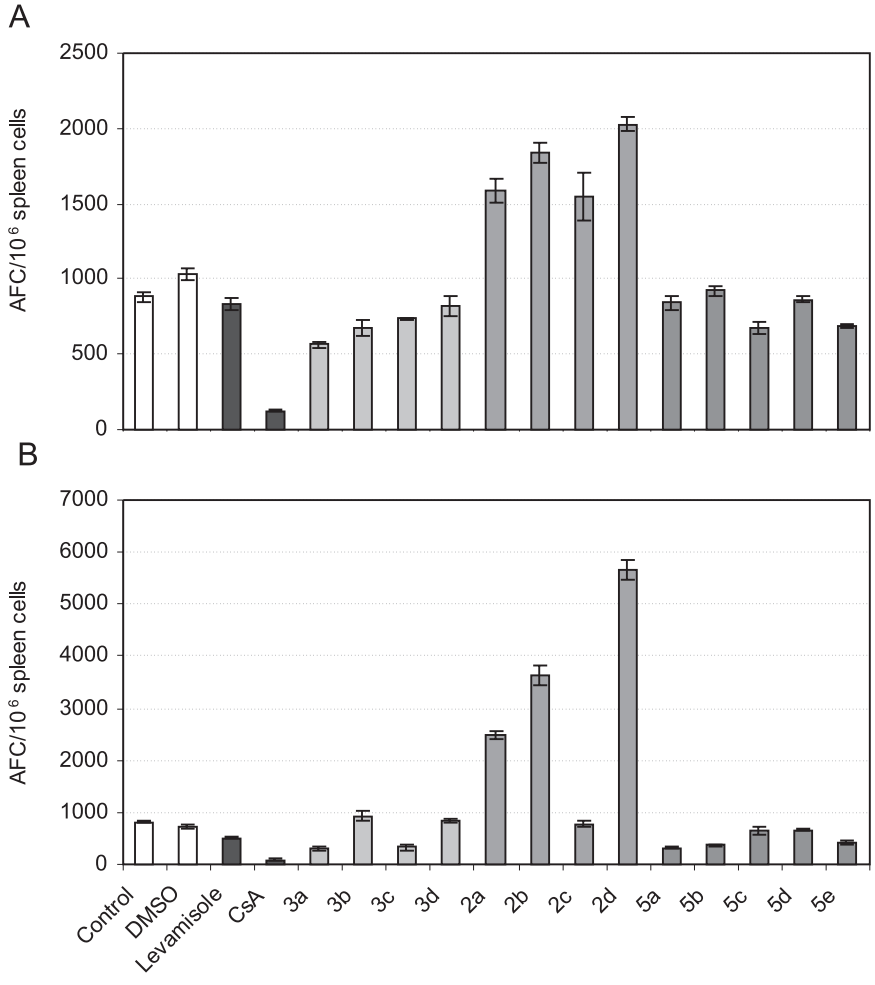


Figure 1. Immunoregulatory effects of the compounds on the secondary humoral response of mouse splenocytes to SRBC. The compounds were added to the splenocyte cultures at 1 (**A**) and 10 (**B**) μg/mL. After four days the number of antibody-forming cells was determined. Statistics: **A:** Control *vs*. DMSO NS; DMSO *vs*. levamisole NS; DMSO *vs*. CsA p = 0.0001; DMSO *vs*. **3a** p = 0.0002; DMSO *vs*. **3b** p = 0.0036; DMSO *vs*. **3c** p = 0.0415; DMSO *vs*. **3d** NS; DMSO *vs*. **2a** p = 0.0001; DMSO *vs*. **2b** p = 0.0001; DMSO *vs*. **2c** p = 0.0001; DMSO *vs*. **2d** p = 0.0001; DMSO *vs*. **5a** NS; DMSO *vs*. **5b** NS; DMSO *vs*. **5c** p = 0.0036; DMSO *vs*. **5d** NS; DMSO *vs*. **5e** p = 0.0065 (ANOVA); **B:** Control *vs*. DMSO NS; DMSO *vs*. levamisole NS; DMSO *vs*. CsA p = 0.0002; DMSO *vs*. **3a** p = 0.0466; DMSO *vs*. **3b** NS; DMSO *vs*. **3c** NS; DMSO *vs*. **3d** NS; DMSO *vs*. **2a** p = 0.0001; DMSO *vs*. **2b** p = 0.0001; DMSO *vs*. **2c** NS; DMSO *vs*. **2d** p = 0.0001; DMSO *vs*. **5a** NS; DMSO *vs*. **5b** NS; DMSO *vs*. **5c** NS; DMSO *vs*. **5d** NS; DMSO *vs*. **5e** NS (ANOVA). NS – not significant.

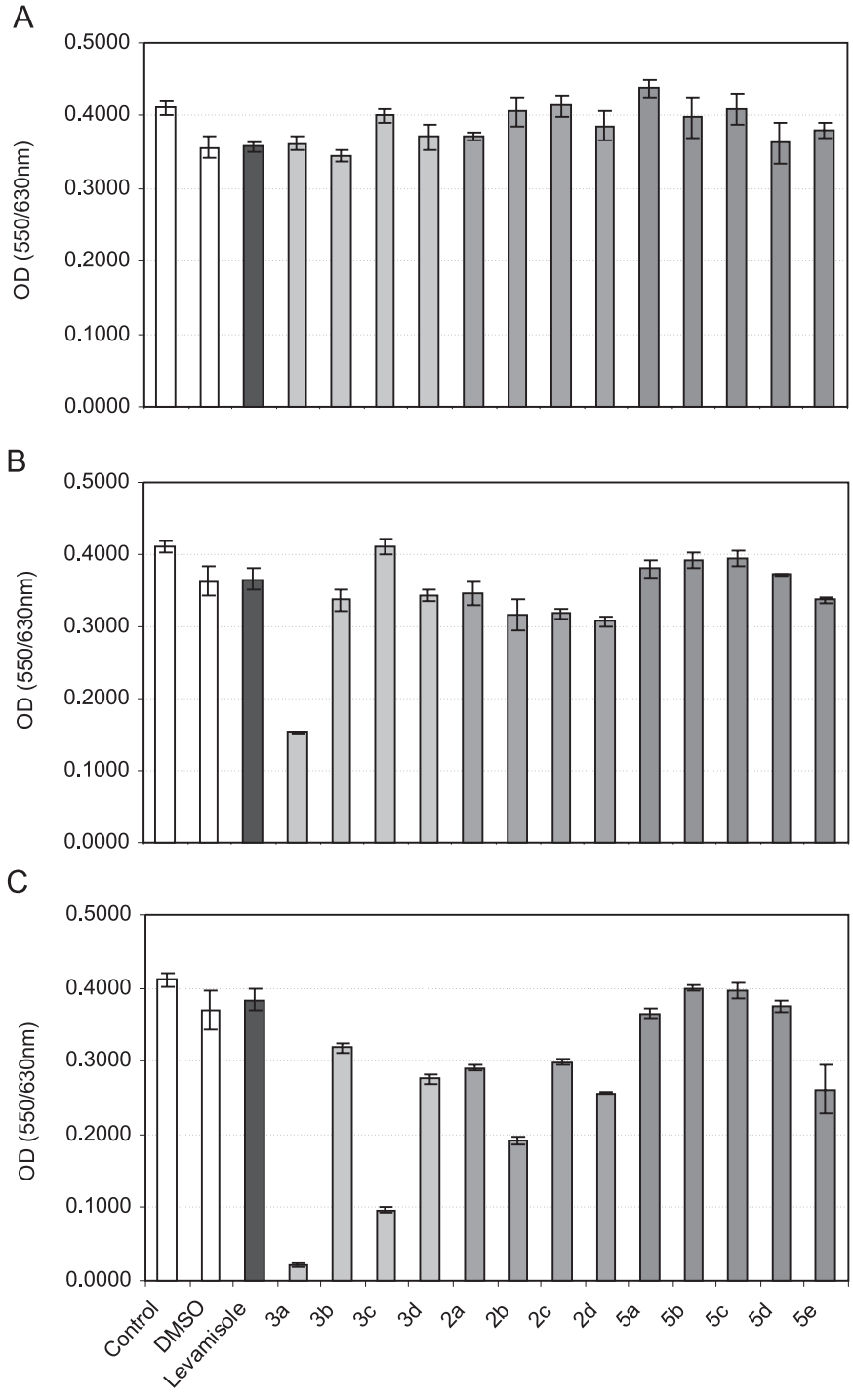


Figure 2. Effects of the compounds on the proliferative response of mouse splenocytes to concanavalin A. The compounds were added to the cultures at concentrations of 0.1 (**A**), 1 (**B**) and 10 (**C**) µg/mL. Statistics: **A:** all comparisons NS (ANOVA); **B:** DMSO *vs*. **3a** p = 0.0001; other comparisons NS (ANOVA); **C:** Control *vs*. DMSO NS; DMSO *vs*. levamisole NS; DMSO *vs*. **3a** p = 0.0000; DMSO *vs*. **3b** NS; DMSO *vs*. **3c** p = 0.0001; DMSO *vs*. **3d** p = 0.0020; DMSO *vs*. **2a** p = 0.0027; DMSO *vs*. **2b** p = 0.0003; DMSO *vs*. **2c** NS; DMSO *vs*. **2d** p = 0.0010; DMSO *vs*. **5a** NS; DMSO *vs*. **5b** NS; DMSO *vs*. **5c** NS; DMSO *vs*. **5d** NS; DMSO *vs*. **5e** p = 0.0179 (ANOVA). NS – not significant.

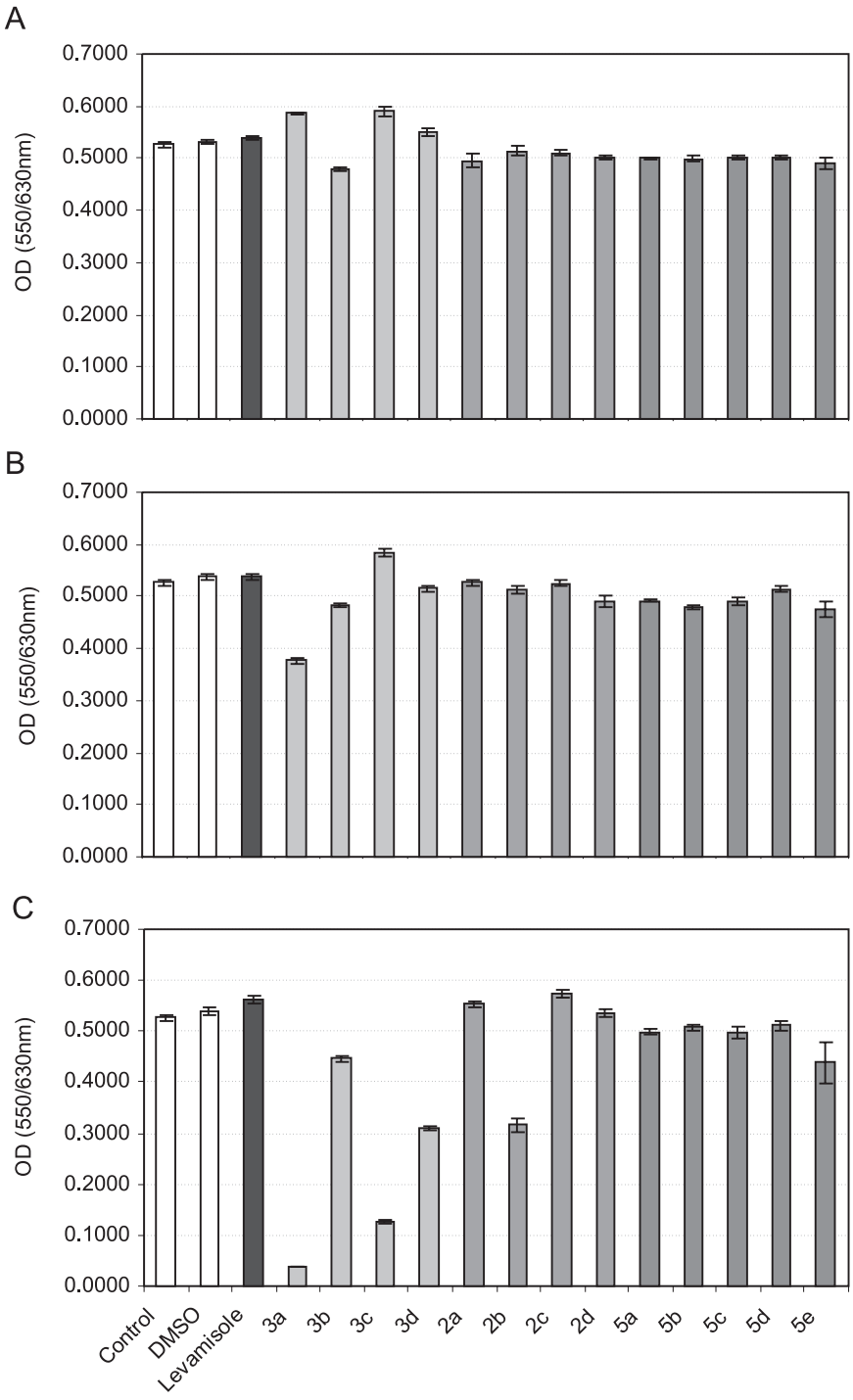


Figure 3. Effects of the compounds on the proliferative response of mouse splenocytes to pokeweed mitogen. The compounds were added to the cultures at concentrations of 0.1 (**A**), 1 (**B**) and 10 (**C**) μg/mL. Statistics: **A:** DMSO *vs*. **3a** p = 0.0156; DMSO *vs*. **3c** p = 0.0207; other comparisons NS (ANOVA); **B:** Control *vs*. DMSO NS; DMSO *vs*. levamisole NS; DMSO *vs*. **3a** p = 0.0016; DMSO *vs*. **3b** p = 0.0180; DMSO *vs*. **3c** p = 0.0198; DMSO *vs*. **3d** NS; DMSO *vs*. **2a** NS; DMSO *vs*. **2b** NS; DMSO *vs*. **2c** NS; DMSO *vs*. **2d** NS; DMSO *vs*. **5a** NS; DMSO *vs*. **5b** NS; DMSO *vs*. **5c** p = 0.0237; DMSO *vs*. **5d** NS; DMSO *vs*. **5e** p = 0.0301 (ANOVA); **C:** Control *vs*. DMSO NS; DMSO *vs*. levamisole NS; DMSO *vs*. **3a** p = 0.0000; DMSO *vs*. **3b** p = 0.0120; DMSO *vs*. **3c** p = 0.0008; DMSO *vs*. **3d** p = 0.0100; DMSO *vs*. **2a** NS; DMSO *vs*. **2b** p = 0.0090; DMSO *vs*. **2c** NS; DMSO *vs*. **2d** NS; DMSO *vs*. **5a** NS; DMSO *vs*. **5b** NS; DMSO *vs*. **5c** NS; DMSO *vs*. **5d** NS; DMSO *vs*. **5e** p = 0.0310 (ANOVA). NS – not significant.

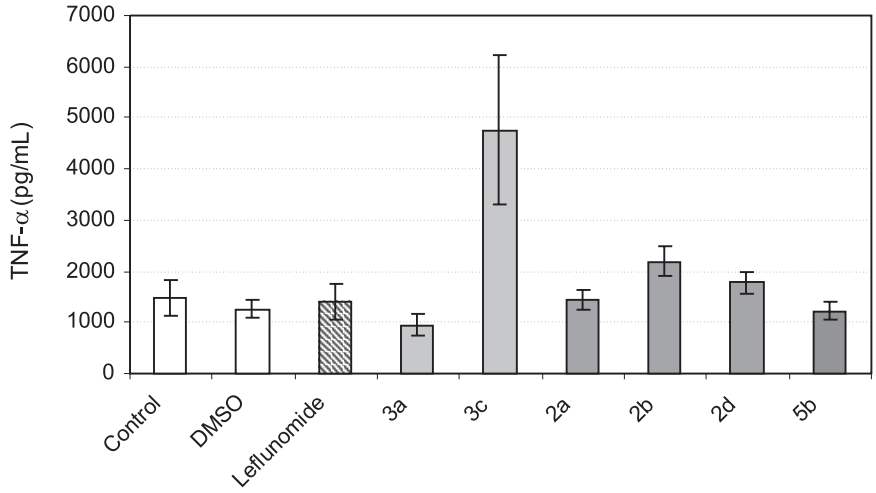


Figure 4. Effects of the compounds on lipopolysaccharide-induced TNF-alpha production by rat peritoneal cells. The compounds were added to the cultures at concentrations of 10 μg/mL. After 24 h incubation the activities of TNF-alpha were measured in the supernatants using bioassays. Statistics: DMSO *vs.* **3c** p = 0.0007; other comparisons NS (ANOVA). NS – not significant.

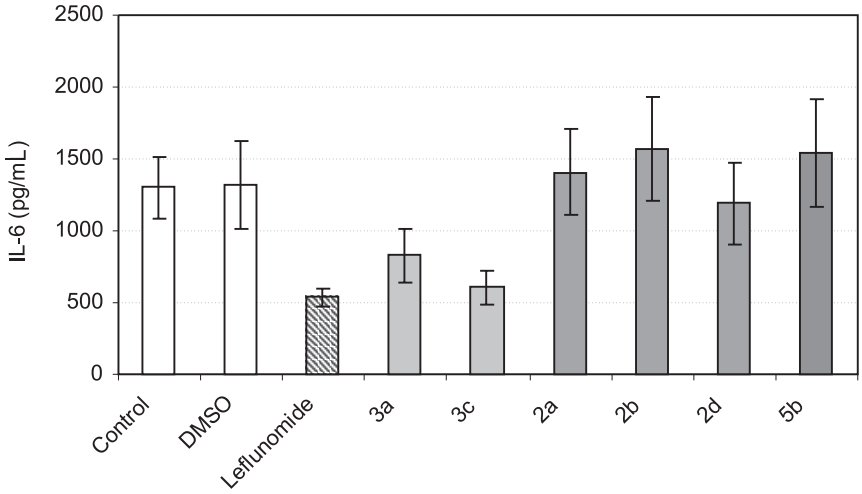


Figure 5. Effects of the compounds on lipopolysaccharide-induced IL-6 production by rat peritoneal cells. The compounds were added to the cultures at concentrations of 10 μg/mL. After 24 h incubation the activities of IL-6 were measured in the supernatants using bioassays. Statistics: all comparisons NS. NS – not significant.

RESULTS AND DISCUSSION

Immunology

Effects of the compounds on the secondary humoral immune response *in vitro* to SRBC

The compounds, at concentrations of 0.1 and 10 μg/mL, were tested for their potential immunoregulatory activity in the *in vitro* model of the secondary humoral immune response to SRBC. The results (Figure 1A) revealed differential effects of the compounds. Compounds **3a-3d** and **5a-e** were moderately inhibitory whereas **2a-2d** were stimulatory at 1 μg/mL. The inhibitory effects of **3a**, **3b**, **3c**, **5c** and **5e** were statistically significant, similarly as the stimulatory effects of compounds **2a**, **2b**, **2c** and **2d** . At a dose of 10 μg/mL (Figure 1B), **3a** was moderately inhibitory, on the other hand, **2a**, **2b** and **2d** exhibited remarkable stimulatory activities (3.5, 5.0 and 7.9-fold stimulation over the control response, respectively). Whereas CsA exhibited expected strong inhibitory action, levamisole did not elevate the immune response.

Table 1. Analytical data for the obtained compounds.

Compound	Formula – molecular weight (g/mol)	Elemental analysis Calculated/Found		
		C%	H%	N%
2a	$C_{21}H_{21}N_5O_2S$ – 407	61.91/62.05	5.15/5.40	17.19/16.82
2b	$C_{22}H_{21}ClN_4O_2S$ – 440.5	59.93/59.21	4.76/5.02	12.71/12.30
2c	$C_{22}H_{22}N_4O_3S$ – 422	62.55/61.98	5.21/5.15	13.27/13.70
2d	$C_{22}H_{22}N_4O_2S$ – 406	65.02/65.50	5.14/4.98	13.79/13.55
3a	$C_{23}H_{25}N_5O_4S$ – 469	59.10/58.67	5.35/5.63	14.99/14.80
3b	$C_{22}H_{23}N_5O_3S$ – 437	60.41/61.07	5.26/5.50	16.02/16.39
3c	$C_{23}H_{22}N_5O_3F_3S$ – 504.97	54.65/54.12	4.36/4.63	13.86/14.01
3d	$C_{18}H_{22}ClN_5O_3S$ – 423.5	51.01/50.95	5.19/5.35	16.53/16.28
5a	$C_{13}H_{21}N_3O_7S$ – 363	42.98/42.65	5.78/5.52	11.57/11.98
5b	$C_{13}H_{21}N_3O_7S$ – 363	42.98/42.59	5.78/5.63	11.57/12.00
5c	$C_{13}H_{21}N_3O_7S$ – 363	42.98/43.02	5.78/5.87	11.57/11.42
5d	$C_{13}H_{21}N_3O_7S$ – 363	42.98/42.49	5.78/5.98	11.57/11.77
5e	$C_{12}H_{19}N_3O_6S$ – 333	43.24/43.56	5.71/5.84	12.61/12.60

Effects of the compounds on the proliferative response of mouse splenocytes to mitogens

In the next step we analyzed the effects of the compounds on the proliferative response of mouse splenocytes to concanavalin A (ConA), a T-cell mitogen, and pokeweed mitogen (PWM), a B-cell mitogen (Figures 2A-C and 3A-C). It appeared that the inhibitory actions of the compounds were strongly dose-dependent. Whereas no effects on the proliferative response to ConA were observed at 0.1 µg/mL concentration, at 1 µg/mL dose only **3a** compound was significantly inhibitory. However, at 10 µg/mL dose, a statistically significant inhibitory activities were registered for compounds **3a**, **3c**, **3d**, **2b**, and **5e**. An exceptionally strong suppression of proliferation was noted in cases of compounds **3a** and **3c** (Figure 2A-C).

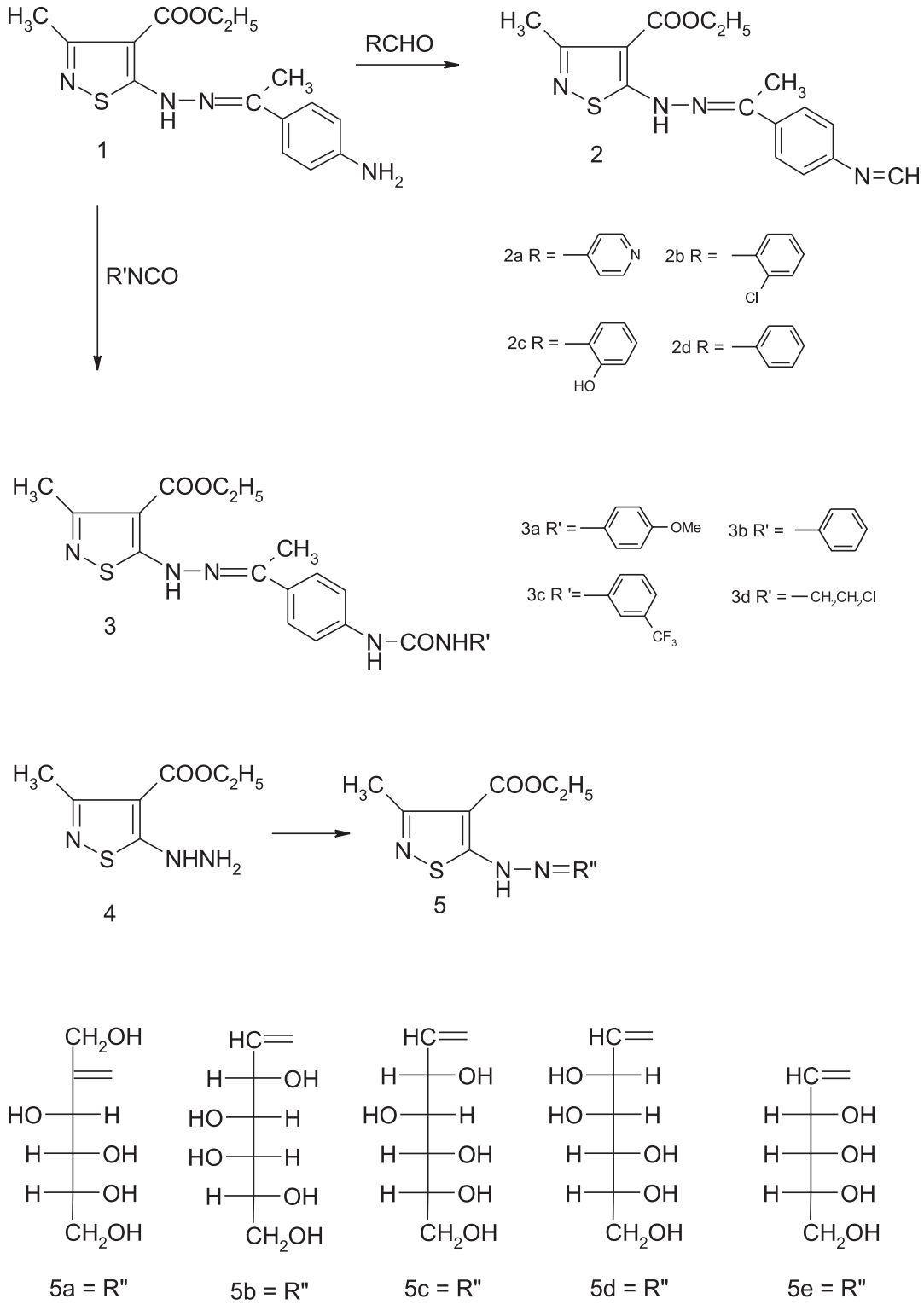
A similar but not identical activity pattern was observed for the effects on the proliferative response of splenocytes to PWM (Figure 3A-C). The inhibition was also distinctly dose-dependent. Interestingly, at 0.1 µg/mL dose, a small, stimulatory effect was seen for compounds **3b** and **3c**. At 1 µg/mL dose, four compounds demonstrated small inhibitory activity (**3a**, **3b**, **5b** and **5d**), whereas at 10 µg/mL a very strong suppression of the proliferative response was found for compounds **3a** and **3c**, a moderate for **2b** and **3d** and a negligible one for **3b** and **5e**. Levamisole was without any effect on the proliferative response of splenocytes induced by ConA or PWM.

Effects of the compounds on lipopolysaccharide-induced production of TNF-alpha and interleukin-6 by rat peritoneal cells

For these assays we selected several active compounds and, for control, compound **5b** which was inactive in the previously described models. As a reference, drug leflunomide was chosen. The compounds were applied at concentrations of 10 µg/mL. The results (Figure 4) showed that among the studied compounds only **3c** expressed significant stimulatory action on TNF-alpha production. Leflunomide, at this dose, was not suppressive.

On the other hand, compounds **3a** and **3c** were to some degree inhibitory with regard to LPS-induced IL-6 production (Figure 5) although these effects were not statistically significant. The suppressive action of leflunomide was marked but not statistically significant.

In summary, several compounds exhibited very interesting, strong, directional actions, which are of potential therapeutic value. Compound **3a** is a hydrazone and represents a substituted derivative of urea. The residue at the urea nitrogen atom is a phenyl, substituted at position 4 with methoxy group, containing two free electron pairs at the oxygen atom. Compound **3a**, at higher doses, demonstrated very strong, antiproliferative actions against T and B cells. That property was correlated with its ability to inhibit the humoral immune response *in vitro* which was not, however, so profound as in the


RCHO
R'NCO
1
2
3
4
5
2a R =
2b R =
2c R =
2d R =
3a R' =
3b R' =
3c R '=
3d R' =
5a = R"
5b = R"
5c = R"
5d = R"
5e = R"

Scheme 1.

case of CsA, suggesting a lower toxicity of **3a** in comparison to CsA. Until now we have not found such a strong, antiproliferative action among other, strongly suppressive synthetic compounds, for example isoxazoles (17). Compound **3c**, similarly as compound **2**, also contains a phenyl group at the urea nitrogen atom, substituted at position 3 with electronegative trifluoromethyl group. This compound, characterized by the ability to strongly stimulate TNF-alpha production, could be of potential value in the search for new anticancer drugs. On the other hand, compound **3d** has a chloroethyl group at the urea nitrogen atom, where the carbon atom is characterized by sp^3 hybridization. That structural configuration would be probably the cause of low activity of compound **3** since, as we demonstrated previously (11), the pharmacological activity can be only ensured by existence of sp^2 carbon hybridization. Lastly, compound **2d**, the exceptionally strong stimulator of the immune response, may be of potential interest in treatment of immunodeficiencies or in immunocompromised patients, as recently suggested for an isoxazole derivative RM-11 (18).

In conclusion, the isothiazole system may serve as a rich source of new compounds with differential properties in the immunological tests and potential therapeutic value.

Chemistry

The starting materials 5-hydrazino-3-methyl-4-isothiazolecarboxylic acid ethyl esters **4** were synthesized by treating 5-chloro-3-methyl-4-isothiazolecarboxylic acid ethyl ester with hydrazine hydrate (12) (Scheme 1). Compound **4** was treated with *p*-aminoacetophenone in the presence of acetic acid (6) and a respective hydrazone **1** was obtained. The latter, in a reaction with aldehydes, gave Schiff's bases **2a-d**. The structures of **2a-d** products were established from their IR spectra and their ^{1}H NMR spectra show a singlet at δ 8,7-8,8 ppm (-CH=N-R) and the absence of the $–NH_2$ signal. The reaction of compound **1** with arylo- and ethyl chloroisocyanate gave corresponding **3a-d** derivatives. Their IR spectra show a characteristic NH-C=O absorption at 1670-1680 cm^{-1} and $–COOC_2H_5$ at 1720-1730 cm^{-1} and their ^{1}H NMR spectra agree with the structures. Condensation of hydrazinoester **4** with monosaccharides: glucose, galactose, mannose, fructose and ribose, gave the corresponding **5a-e** sugar hydrazones. Their IR spectra show the following frequecies: –OH at 3300-3500; **NH**CO at 3200-3250 and >C=O at 1720 cm^{-1}. The ^{1}H NMR spectra are characterized by a doublet at 5,2-5,4 ppm corresponding to the proton at C-1 of the sugar. The protons of the sugar hydroxyl groups appear at 3,7-3,9 ppm. Elemental analyses of the synthesized compounds are in agreement with the assigned structures. The assignments of NH and OH groups in these compounds were determined by D_2O exchange. The analytical data for series **2**, **3**, **5** were presented in Table 1.

The physical and spectroscopic data of the obtained compounds

5-{N'-[1-(4-{[(4-pyridin-4-yl)-methylidene]-amino}phenyl)-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**2a**)

M.p. 206-207°C, yield 78.13%. IR (KBr, cm^{-1}): 1700 (>C=O), 1600 (Ar), 1550 (C=N), 1260 (-C-O-C_2H_5). ^{1}H NMR (DMSO, δ ppm): 1.1-1.3 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH_2-), 2.23-2.40 (s, 6H, C**H$_3$**-C=N), 4.23-4.33 (q, *J*=7.5 Hz, 2H, CH_3-C**H$_2$-**), 7.38-7.42 (d, *J* = 8.3 Hz, 2H, Ar), 7.48-7.51 (d, *J* = 8.5 Hz, 2H, C_5**H$_4$**-N), 7.79-7.81 (d, *J* = 8.3 Hz, 2H, Ar), 8.7-8.76 (d, *J* = 8.5 Hz, 2H, C_5**H$_4$**-N), 8.82 (s, 1H, C**H**=N), 10.05 (s, 1H, N**H**-N=).

5-{N'-[1-(4-{[1-(2-chlorophenyl)-methylidene]-amino}-phenyl)-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**2b**)

M.p. 176-177°C, yield 60.87% (EtOH/DMF). IR (KBr, cm^{-1}): 1695 (>C=O), 1600 (Ar), 1555 (C=N), 1260 (-C-O-C_2H_5). ^{1}H NMR (DMSO, δ ppm): 1.31-1.37 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH_2-), 2.23-2.40 (s, 3H, C**H$_3$**-C=N), 3.28 (s, 3H, C**H$_3$**-C=N), 4.26-4.31 (q, *J* = 7.5 Hz, 2H, CH_3-C**H$_2$-**), 6.58-7.25 (m, 4H, C_6**H$_4$**-2-Cl), 7.48-7.53 (d, *J* = 8.3 Hz, 2H, Ar); 7.8-7.87 (d, *J* = 8.3 Hz, 2H, Ar), 8.88 (s, 1H, C**H**=N), 10.05 (s, 1H, N**H**-N=).

5-{N'-[1-(4-{[1-(2-hydroxyphenyl)-methylidene]-amino}-phenyl)-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**2c**)

M.p. 177-178.5°C, yield 60.87%. IR (KBr, cm^{-1}): 3100-3250 (-OH), 1695 (>C=O), 1610 (Ar), 1550 (C=N), 1250 (-C-O-C_2H_5). ^{1}H NMR (DMSO, δ ppm): 1.29-1.33 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH_2-), 2.36 (s, 3H, C**H$_3$**-C=N), 3.3 (s, 3H, C**H$_3$**-C=N), 4.2-4.33 (q, *J* = 7.5 Hz, 2H, CH_3-C**H$_2$-**), 6.84-6.9 (m, 4H, Ar), 7.2-7.64 (dd, *J* = 8.8 Hz, 4H, C_6**H$_4$**-4-OH), 8.73 (s, 1H, C**H**=N); 10.05 (s, 1H, N**H**-N=), 11.2 (s, 1H, ArO**H**).

5-{N'-[1-(4-(benzylideneaminophenyl)-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**2d**)

M.p. 174-175°C, yield 62.8%. IR (KBr, cm^{-1}): 1690 (>C=O), 1600 (Ar), 1550 (C=N), 1255 (-C-O-C_2H_5).

^{1}H NMR (DMSO, δ ppm) 1.30-1.35 (t, *J* = 7.4 Hz, 3H, C**H$_3$**-CH$_2$-), 2.34 (s, 3H, C**H$_3$**-C=N), 3.2 (s, 3H, C**H$_3$**-C=N), 4.3-4.42 (q, *J*,=,7.4 Hz, 2H, CH$_3$-C**H$_2$**-), 6.9-7.1 (m, 5H, Ar), 7.2-7.8 (dd, *J* = 8.4 Hz, 4H, C$_6$**H$_4$**-); 8.7 (s, 1H, C**H**=N); 10.02 (s, 1H, N**H**-).

5-{N'-[1-{4-[3-(4-methoxyphenyl)-ureido]-phenyl}-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**3a**)

M.p. 205-206°C, yield 89.4% IR (KBr, cm^{-1}): 3280 (CONH), 3250-2950 (CH), 1720 (**CO**OC$_2$H$_5$), 1670 (**CO**NH), 1550 (C=N), 1160 (C-O). ^{1}H NMR (DMSO, δ ppm): 1.32-1.37 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH$_2$-), 2.26-2.48 (s, 6H, C**H$_3$**-C=N), 3.33 (s, 3H, C**H$_3$**O-), 4.25-4.32 (q, *J* = 7.5 Hz, 2H, -C**H$_2$** – CH$_3$), 6.95-6.99 (d, *J* = 8.3 Hz, 2H, Ar), 7.41-7.46 (d, *J* = 8.3 Hz, 2H, Ar), 7.51-7.54 (d, *J* = 8.4 Hz, 2H, Ar); 7.63-7.72 (d, *J* = 8.4 Hz, 2H, Ar); 8.7, 8.9 (s, 1H, -N**H**-CON**H**-), 10.12 (s, 1H, N**H**–N=).

5-{N'-[1-{4-(3-phenylureido)-phenyl}-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**3b**)

M.p. 257-258°C, yield 81.7% (DMF). IR (KBr, cm^{-1}): 3290 (CO**NH**), 1730 (**CO**OC$_2$H$_5$), 1670 (**CO**NH), 1570 and 1600 (Ar), 1520 (C=N). ^{1}H NMR (DMSO, δ ppm): 1.33-1.37 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH$_2$-), 2.31-2.43 (s, 6H, C**H$_3$**-C=N), 4.23-4.28 (q, *J* = 7.5 Hz, 2H, -C**H$_2$**–CH$_3$), 7.49-7.52, 7.7-7.9 (dd, *J* = 8.3 Hz, 4H, Ar), 8.3, 8.5 (s, 2H, -N**H**-CON**H**-); 10.16 (s, 1H, N**H**–N=).

5-{N'-[1-{4-[3-(3-trifluromethylphenyl)ureido]-phenyl}-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**3c**).

M.p. 265-266°C, yield 50.1%. IR (KBr, cm^{-1}): 3300 (CO**NH**), 1720 (**CO**OC$_2$H$_5$), 1670 (**CO**NH), 1580 (Ar), 1550 (C=N). ^{1}H NMR (DMSO, δ ppm): 1.32-1.37 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH$_2$-), 2.32 (s, 6H, C**H$_3$**-C=N), 4.27-4.30 (q, *J* = 7.5 Hz, 2H, -C**H$_2$**–CH$_3$), 6,9-7,1 (m, 3H, Ar), 7.64-7.67 (d, *J* = 8.3 Hz, 2H, Ar), 7.72-7.75 (d, *J* = 8.3 Hz, 2H, Ar); 8.0 (s, 1H, Ar); 9.0-9.1 (s, 2H, N**H**CON**H**), 10.1 (s, 1H, N**H**–N=).

5-{N'-[1-{4-[3-(2-chloroethyl)ureido]-phenyl}-ethylidene]-hydrazino}-3-methyl-4-isothiazolecarboxylic acid ethyl ester (**3d**)

M.p. 218-219°C decomp, yield 38.3%. IR (KBr, cm^{-1}): 3320 (CO**NH**), 3000-2950 (C-H), 1725 (**CO**OC$_2$H$_5$), 1680 (**CO**NH), 1590 (Ar), 1550 (C=N). ^{1}H NMR (DMSO, δ ppm) 1.31-1.35 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH$_2$-), 2.35 (s, 6H, C**H$_3$**-C=N); 4.27-4.31 (q, *J* = 7.5 Hz, 2H, -C**H$_2$**–CH$_3$), 6.3-6.7 (m, 4H, -C**H$_2$**-C**H$_2$**-), 7.58-7.62 (d, *J* = 8.35 Hz, 2H, Ar), 7.78-7.80 (d, *J* = 8.35 Hz, 2H, Ar), 8.5, 8.8 (s, 2H, N**H**CON**H**), 10.1 9.9 (s, 1H, N**H**–N=).

3-Methyl-5-[N'-2-(1,3,4,5,6-pentahydroxyhexylidene)-hydrazino]-4-isothiazolecarboxylic acid ethyl ester (**5a**) with fructose

M.p. 196-198°C decomp., yield 56.3%. IR (KBr, cm^{-1}): 3450-3330 (OH), 3230 (NH), 2950 (CH), 1720 (C=O), 1550 (C=N), 1160 (C-O). ^{1}H NMR (DMSO, δ ppm): 1.31-1.35 (t, 3H, C**H$_3$**-CH$_2$-), 2.35 (s, 3H, C**H$_3$**-C=N), 4.27-4.31 (q, *J* = 7.5 Hz, 2H, -C**H$_2$**-CH$_3$), 3.78 (m, 3H, 5'H, 2 × 6'H), 3.92 (s, 2H, 2 × 1'H), 4.2-4.6 (m, 7H, C**H**OH and -C**H$_2$**-CH$_3$), 8.8 (s, 1H, N**H**).

3-Methyl-5-[N'-2-(1,3,4,5,6-pentahydroxyhexylidene)-hydrazino]-4-isothiazolecarboxylic acid ethyl ester (with galactose **5b**)

M.p. 237-239°C decomp., yield 49.6%. IR (KBr, cm^{-1}): 3450-3320 (OH), 3250 (NH), 2920 (CH), 1725 (C=O), 1560 (C=N), 1120 (C-O). ^{1}H NMR (DMSO, δ ppm): 1.33-1.35 (t, 3H, C**H$_3$**-CH$_2$-), 2.35 (s, 3H, C**H$_3$**-C=N), 3.75 (m, 3H, 5'H and 2 × 6'H), 4.1-4.6 (m, 10H, -C**H**OH and C**H$_2$**-CH$_3$), 5.2 (d, *J* = 6 Hz, 1H, -C**H**-); 8.7 (s, 1H, N**H**).

3-Methyl-5-[N'-2-(1,3,4,5,6-pentahydroxyhexylidene)-hydrazino]-4-isothiazolecarboxylic acid ethyl ester (with glucose **5c**)

M.p. 208-209°C decomp., yield 45.5%. IR (KBr, cm^{-1}): 3550-3360 (OH), 3200-3160 (NH), 2930 (CH), 1725 (C=O), 1210 (C-O). ^{1}H NMR (DMSO, δ ppm): 1.3 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH$_2$-), 2.4 (s, 3H, C**H$_3$**-C=N), 3.73 (m, 3H, 5'H and 2 × 6'H), 3.9-4.6 (m, 10H, -C**H**OH and C**H$_2$**-CH$_3$), 5.4 (d, *J* = 6 Hz, 1H, -C**H**-), 8.5 (s, 1H, N**H**).

3-Methyl-5-[N'-2-(1,3,4,5,6-pentahydroxyhexylidene)-hydrazino]-4-isothiazolecarboxylic acid ethyl ester (with mannose **5d**)

M.p. 210-211°C decomp., yield 42.3%. IR (KBr, cm^{-1}): 3500-3400 (OH), 3180-3200 (NH), 2930 (CH), 1720 (C=O), 1215 (C-O). ^{1}H NMR (DMSO, δ ppm): 1.29 (t, *J* = 7.5 Hz, 3H, C**H$_3$**-CH$_2$-), 2.35 (s, 3H, C**H$_3$**-C=N), 3.7 (m, 3H, 5'H and 2 × 6'H). 4.0-4.55 (m, 10H, -C**H**OH and C**H$_2$**-CH$_3$), 5.4 (d, *J* = 6 Hz, 1H, -C**H**-), 8.6 (s, 1H, N**H**).

3-Methyl-5-[N'-2-(1,3,4,5,6-pentahydroxyhexylidene)-hydrazino]-4-isothiazolecarboxylic acid ethyl ester (with ribose **5e**)

M.p. 168-170°C decomp., yield 60.3%. IR (KBr, cm^{-1}): 3350-3500 (OH), 3200 (NH), 2960

(CH), 1720 (C=O), 1550 (C=N). ^{1}H NMR (DMSO, δ ppm): 1.35 (t, *J* = 7.5 Hz**,** 3H, C**H**$_3$-CH$_2$-), 2.42 (s, 3H, C**H**$_3$-C=N), 3.65 (m, 3H, -C**H**OH), 4.2-4.6 (m, 6H, C**H**$_2$-CH$_3$ and 4-OH); 5.4 (d, *J* = 6 Hz, 1H, -C**H**-); 8.8 (s, 1H, N**H**).

REFERENCES

1. Kuczyński L., Kuriata M., Ciupka B.: Pol. J. Pharmacol. Pharm. 36, 485 (1984).
2. Regiec A., Machoń Z., Międzybrodzki R., Szymaniec S.: Arch. Pharm. Chem. Life Sci. 339, 401 (2006).
3. Garozzo A., Cutri C.C., Pannecouque C., Castro A., Guerrera F., De Clercq E. : Antivir. Chem. Chemother. 18, 277 (2007).
4. Machoń Z.: Drugs Future 13, 426 (1988).
5. Malinka W., Sieklucka-Dziuba M., Rajtar G., Zgodzinski W, Kleinrok Z.: Pharmazie 55, 416 (2000).
6. Lipnicka U., Zimecki M.: Acta Pol. Pharm. Drug Res. 64, 233 (2007).
7. Lipnicka U., Regiec A., Sułkowski E., Zimecki M.: Arch. Pharm. Chem. Life Sci. 338, 322 (2005).
8. Machoń Z., Wieczorek Z., Zimecki M.: Pol. J. Pharmacol. 52, 377 (2001).
9. Lipnicka U., Machoń Z.: Acta Pol. Pharm. Drug Res. 54, 207 (1997).
10. Machoń Z., Giełdanowski J., Wieczorek Z., et al. : Arch. Immunol. Ther. Exp. 31, 769 (1983).
11. Lipnicka U., Regiec A., Machoń Z.: Pharmazie 49, 652 (1994).
12. Machoń Z.: Dissert. Pharm. Pharmacol. 21, 135 (1969).
13. Dutton R. W., Mishell R. I.: J. Exp. Med. 126, 443 (1967).
14. Hansen, Nielsen S. E., Berg K.: J. Immunol. Methods 119, 203 (1989).
15. Espevik T., Nissen-Meyer J.: J. Immunol. Methods 95, 99 (1986).
16. Van Snick J., Cayphas S., Vink A., Uyttenhove C., Coulie P. G., Rubira M. R., Simpson R. J.: Proc. Natl. Acad. Sci. USA 83, 9679 (1986).
17. Ryng S., Zimecki M., Mączyński M., Chodaczek G., Kocięba M.: Pharmacol. Rep. 57, 195 (2005).
18. Zimecki M, Artym J, Ryng S, Obmińska-Mrukowicz B.: Pharmacol. Rep. 60, 183 (2008).

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