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Short Communication

**THE SYNTHESIS, PHYSICO-CHEMICAL PROPERTIES AND
IMMUNOLOGICAL ACTIVITY OF 5-AMINO-3-
METHYLISOXAZOLO[5,4-D]4-PYRIMIDINONE DERIVATIVES**

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Abstract: A series of 5-amino-3-methylisoxazole[5,4-d]4-pyrimidinone derivatives were obtained by reacting substituted 5-amino-3-methylisoxazol-4-carboxylic acid hydrazide with ethyl ortho-formate. The compounds were tested using the models of *in vivo* cellular and humoral immune response in mice and pokeweed mitogen-induced (PWM-induced) polyclonal antibody production in a culture of human peripheral blood mononuclear cells (PBMC). The compounds exhibited differential inhibitory activities in the described models, depending on the character and location of the substituted groups. We suggest that the compounds affect the early stages of the immune response.

Key Words: 5-amino-3-methylisoxazolo[5,4-d]4-pyrimidinone, PBMC, PWM, Immunological Activity

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Abbreviations used: PWM – the pokeweed mitogen; PBMC – peripheral blood mononuclear cells; TLC – thin layer chromatography; DMSO-d₆ – dimethylsulfoxid-d₆; TMS – tetramethylsilan; SRBC – sheep red blood cells; CsA – cyclosporine A; PFC – plaque-forming cells; DTH – delayed type hypersensitivity; i.p. – intraperitoneally; p – Student test.

INTRODUCTION

A number of new, low-molecular weight immune modifiers have become highly valued therapeutic agents. The heterocyclic isoxazole structure has recently been used as the basis for the synthesis of many potential drugs with various biological activities, including AMPA-receptor agonist and antagonist [1-3], antidepressant [2], dopamine 4 activity [3], anticonvulsant [4], antiviral [5], antifungal [6] and immunoregulatory [7-10]. The search for active compounds in the isoxazole family led to the synthesis and investigation of compounds within groups of bicyclic isoxazole[5,4-d]1,2,3-triazine-4-one derivatives (Fig. 1).

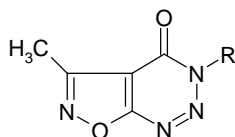


Fig. 1. 5-Substituted 3-methylisoxazole[5,4-d]1,2,3-triazine-4-one derivatives.

Among these compounds, derivatives demonstrating antitumor and immunomodulatory properties were found. Most interestingly, antitumor activities were exhibited by 5-phenyl-substituted compounds [11], one of which is currently in the second phase of preclinical studies (NCI, Bethesda). Other derivatives, such as substituted 3-methylisoxazole[5,4-d]1,2,3-triazine-4-one (Fig. 2), showed interesting immunomodulatory activities [12].

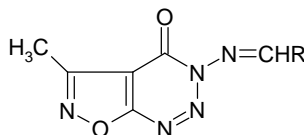


Fig. 2. 5-Substituted 3-methylisoxazole[5,4-d]1,2,3-triazine-4-one derivatives.

Structure-activity studies of these compounds were also reported on [13]. These investigations were followed by biological and structural studies in the family of related bicyclic isoxazole derivatives. Such derivatives have been mainly described in the patent literature as compounds acting on the circulatory system and possessing analgesic and anti-inflammatory properties.

The aim of this investigation was to synthesize and evaluate the immunological activities of a new series of 5-amino-3-methylisoxazole[5,4-d]4-pyrimidinone derivatives (Fig. 3).

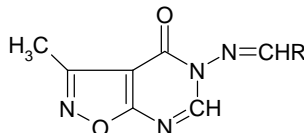


Fig. 3. 5-amino-3-methylisoxazole[5,4-d]4-pyrimidinone derivatives.

MATERIALS AND METHODS**Chemical analytical methods**

Melting points were determined on a Büchi apparatus (Laboratoriums-Technik AG, Flawil, Switzerland) and were uncorrected. TLC was carried out on Kieselgel G-Merck glass silica gel plates (E. Merck, Darmstadt, Germany), using the developing system $\text{CHCl}_3\text{-CH}_3\text{OH} = 9:1$, and detected with UV lamps. IR spectra were recorded with a Specord M-80 spectrophotometer (Carl Zeiss, Jena, Germany) in Nujol mull supported on a KBr disk, and ^1H NMR spectra were obtained in DMSO-d_6 using a Tesla 80 MHz spectrometer (using TMS as the internal standard). The physical data is summarized and presented in Tab. 1. The structural and spectroscopic data is presented in Tab. 2. Elemental analyses were performed within $\pm 0.3\%$ of the theoretical values (Carlo Erba NA, 1500-equipment).

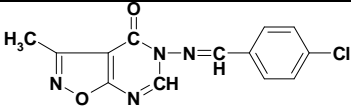
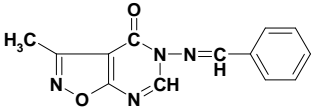
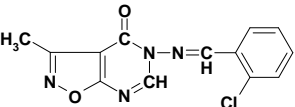
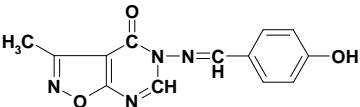
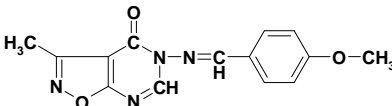
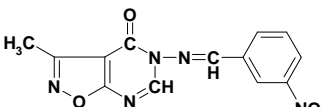
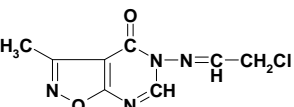
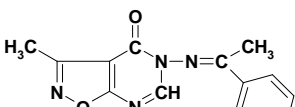
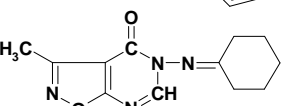
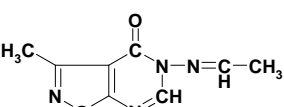
Tab. 1. Physical data for the obtained compounds.

Compound	Melting point (°C)	Yield (%)	Formula Molecular weight
1017	191-192	23.5	$\text{C}_{13}\text{H}_9\text{ClN}_4\text{O}_2$ 288.70
1018	196-197	41.8	$\text{C}_{13}\text{H}_{10}\text{N}_4\text{O}_2$ 254.25
1019	210-211	21.0	$\text{C}_{13}\text{H}_9\text{ClN}_4\text{O}_2$ 288.70
1020	247-248	46.5	$\text{C}_{13}\text{H}_{10}\text{N}_4\text{O}_3$ 270.25
1021	158-159	28.8	$\text{C}_{14}\text{H}_{12}\text{N}_4\text{O}_3$ 284.28
1022	207-208	37.2	$\text{C}_{13}\text{H}_9\text{N}_5\text{O}_4$ 299.25
1023	204-205	25.4	$\text{C}_8\text{H}_7\text{ClN}_4\text{O}_2$ 226.62
1024	229-231	29.0	$\text{C}_{14}\text{H}_{12}\text{N}_4\text{O}_2$ 268.28
1025	227-229	34.6	$\text{C}_{12}\text{H}_{14}\text{N}_4\text{O}_2$ 246.27
1026	214-216	19.8	$\text{C}_8\text{H}_8\text{N}_4\text{O}_2$ 192.18

Synthesis of substituted 5-amino-3-methylisoxazole-4-carboxylic acid hydrazides

10 millilitres of isopropanol and 4.8 mmol of aldehyde or ketone were added to 3.2 mmol of 5-amino-3-methylisoxazole-4-carboxylic acid hydrazide obtained according to a method described in [14]. The solution was stirred and heated for 2 hours. At the end of the reaction (controlled in a TLC), the solution was evaporated to a 5 ml volume under diminished pressure, and cooled. The solid, which separated out, was filtered and washed with ethyl acetate. The crude product was recrystallized from ethanol [15].

Tab. 2. The structural and spectroscopic data for the obtained compounds.

Compound	The structure of compound	Spectroscopic data
1017		IR (nujol) cm^{-1} : C=O 1634, ^1H NMR (DMSO- d_6) ppm: 2.51 (s, 3H, CH_3), 7.70 (q, 4H, aromatic), 8.00 (s, 1H, CH), 9.10 (s, 1H, CH)
1018		IR (nujol) cm^{-1} : C=O 1680, ^1H NMR (DMSO- d_6) ppm: 2.33 (s, 3H, CH_3), 7.60 (m, 5H, phenyl), 7.90 (s, 1H, CH), 9.10 (s, 1H, CH)
1019		IR (nujol) cm^{-1} : C=O 1652, ^1H NMR (DMSO- d_6) ppm: 2.35 (s, 3H, CH_3), 7.60 (q, 4H, aromatic), 7.64 (s, 1H, CH), 9.15 (s, 1H, CH)
1020		IR (nujol) cm^{-1} : C=O 1668, ^1H NMR (DMSO- d_6) ppm: 2.39 (s, 3H, CH_3), 7.55 (s, 4H, aromatic), 7.95 (s, 1H, CH), 8.95 (s, 1H, CH), 10.45 (s, 1H, OH)
1021		IR (nujol) cm^{-1} : C=O 1670, ^1H NMR (DMSO- d_6) ppm: 2.20 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 7.45 (m, 4H, aromatic), 8.30 (s, 1H, CH), 8.90 (s, 1H, CH)
1022		IR (nujol) cm^{-1} : C=O 1668, ^1H NMR (DMSO- d_6) ppm: 2.33 (s, 3H, CH_3), 7.7 (q, 4H, aromatic), 7.95 (s, 1H, CH), 9.30 (s, 1H, CH)
1023		IR (nujol) cm^{-1} : C=O 1678, ^1H NMR (DMSO- d_6) ppm: 2.31 (s, 3H, CH_3), 4.25 (m, 2H, CH_2), 8.21 (s, 1H, CH), 9.10 (s, 1H, CH)
1024		IR (nujol) cm^{-1} : C=O 1635, ^1H NMR (DMSO- d_6) ppm: 2.30 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 7.7 (m, 4H, aromatic), 7.90 (s, 1H, CH), 9.10 (s, 1H, CH)
1025		IR (nujol) cm^{-1} : C=O 1635, ^1H NMR (DMSO- d_6) ppm: 1.65 (m, 8H, cyclohexyl), 2.25 (s, 1H, CH_3), 3.30 (s, 2H, cyclohexyl), 9.15 (s, 1H, CH)
1026		IR (nujol) cm^{-1} : C=O 1640, ^1H NMR (DMSO- d_6) ppm: 2.23 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 7.90 (s, 1H, CH), 9.10 (s, 1H, CH)

General procedure for the synthesis of 5-amino-3-methylisoxazol[5,4-d]4-pyrimidinone derivatives

A mixture of 1.8 mmol of the obtained product, 10 ml of isopropanol and 7.2 mmol of ethyl ortho-formate was stirred and heated to boiling temperature, and 10 drops of conc. HCl were added to the reaction mixture. The solution was stirred and heated for 30 minutes. When the reaction had passed (controlled in a TLC), the mixture was cooled. The crude product was collected on a filter. The unrefined compound was purified by recrystallization in methanol, yielding a pure product. The synthesis of the obtained compounds is schematically presented in Fig. 4.

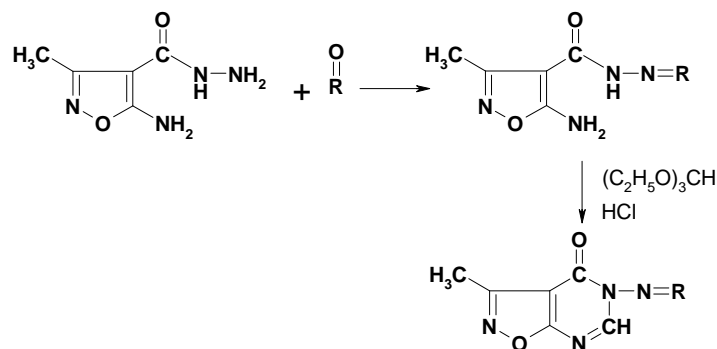


Fig. 4. Preparation of the obtained compounds.

Animals and reagents

12-week old CBA mice of both sexes were used for the experiments. The mice were fed a standard pelleted food and water *ad libitum*.

Sheep red blood cells (SRBC) were delivered by Wrocław Agriculture Academy from a single sheep and were kept in Alsever's solution until use. Pokeweed mitogen (PWM) was purchased from Sigma, and CsA from Sandoz (Switzerland). The fetal calf serum and RPMI 1640 culture medium was from Gibco.

Isolation of peripheral blood mononuclear cells

The venous peripheral blood was taken from a healthy donor into a heparinized tube. The blood, diluted 1:1 with phosphate-buffered saline, was centrifuged on a discontinuous gradient of Ficoll-uropoline (density 1.077 g/ml). The cells from the interphase were washed twice with Hanks' medium and resuspended in a culture medium for the induction of polyclonal antibody formation [16].

Determination of the humoral and the cellular immune response

For the determination of the number of antibody-forming cells, the mice were immunized i.p. with a single dose (0.2 ml) of 5% SRBC suspension. After four days, the spleens were isolated and the number of plaque-forming cells (PFC) in the spleens was determined according to the method of Mishell and Dutton [17].

The data is presented as the mean PFC number per 10^6 viable cells \pm the standard error (SE) from 5 mice per group.

For the determination of the delayed type hypersensitivity (DTH) to SRBS, mice were sensitized intravenously with a dose of 10^5 and four days later, the reaction was elicited by a subcutaneous injection of 10^8 SRBC into the hind foot pad. The antigen-specific foot pad swelling was measured after a further 24 h using a caliper [18]. Control, background responses from non-sensitized mice were subtracted from the reaction results. The results are presented as the mean values from 10 determinations (5 mice per group, two hind feet), expressed as DTH units, \pm SE.

Statistics

For the statistical evaluation of the data, the Student's t test was applied. The results were regarded as significant when p was equal to or less than 0.05.

RESULTS

The effects of the compounds on the humoral immune response to SRBC

Mice were treated with the preparations i.p. at doses of 10 and 100 μ g, 2 hours before immunization with SRBC. 4 days later, the number of PFC in the spleens was determined according to Mishell and Dutton [16]. The results are shown in

Tab. 3. The number of plaque-forming cells (PFC) in the spleens of CBA/liw mice immunized with SRBC and treated i.p. with a given preparation 4 h before antigen administration.

Preparation	Dose μ g/mouse	PFC/ 10^6 cells	\pm SE	p (Student's t-test)
Control		2571	64.53	
1017	10	1673	222.51	< 0.01
	100	815	74.50	< 0.001
1018	10	1703	183.15	< 0.01
	100	1183	97.25	< 0.001
1019	10	1805	188.38	< 0.01
	100	1203	94.96	< 0.001
1020	10	897	87.81	< 0.001
	100	800	86.85	< 0.001
1021	10	647	35.79	< 0.001
	100	856	55.33	< 0.001
1022	10	1515	178.39	< 0.01
	100	1295	51.09	< 0.001
1023	10	974	53.15	< 0.001
	100	718	92.68	< 0.001
1024	10	938	143.42	< 0.001
	100	1265	142.48	< 0.01
1025	10	1336	133.95	< 0.001
	100	703	37.87	< 0.001
1026	10	1183	88.21	< 0.001
	100	662	97.62	< 0.001
CsA	10	1674	96.16	< 0.001
	100	1050	41.31	< 0.001

Tab. 3. The compounds were inhibitory to various degrees, with some being exceptionally active, for example: 1020, 1021, 1023, 1025 and 1026. The suppressory effects of these compounds were stronger than that of CsA.

The effects of the compounds on the delayed type hypersensitivity to SRBC

Mice were treated i.p. with the preparations at doses of 10 and 100 μg , 2 hours before sensitization with SRBC. Four days later, the animals were given the eliciting dose of the antigen, and 24 h later, the foot oedema was measured using the method of Lagrange *et al.* [17]. The results are presented in Tab. 4. All the compounds were inhibitory in this model, except for an insignificant inhibition exhibited by 1022 and 1023. The strongest inhibitory activity was demonstrated by 1024 and 1019, and these effects were more profound than that of CsA, the control drug.

Tab. 4. The DTH reaction (foot pad test) in CBA mice sensitized with SRBC and treated i.p. with the preparation 4 h before the administration of the inductive dose of the antigen.

Preparation	Dose $\mu\text{g}/\text{mouse}$	DTH Units \pm	\pm SE	p (Student's t-test)
Control		12.37	0.24	
1017	10	10.62	0.97	NS
	100	9.50	0.22	NS
1018	10	9.00	0.38	NS
	100	4.87	0.39	< 0.001
1019	10	6.25	0.42	< 0.001
	100	2.12	0.43	< 0.001
1020	10	6.12	0.69	< 0.001
	100	5.50	0.22	< 0.001
1021	10	6.87	1.15	< 0.01
	100	6.37	0.31	< 0.01
1022	10	8.25	1.15	< 0.05
	100	8.75	1.22	< 0.05
1023	10	8.87	0.45	< 0.05
	100	7.25	0.52	< 0.01
1024	10	3.75	0.50	< 0.001
	100	3.75	0.45	< 0.001
1025	10	5.62	0.36	< 0.001
	100	6.12	0.30	< 0.001
1026	10	7.25	0.78	< 0.01
	100	6.37	0.50	< 0.001
CsA	0	7.62	0.71	< 0.01
	100	3.50	0.60	< 0.001

The effects of the compounds on polyclonal antibody production by human peripheral blood mononuclear cells (PBMC) activated by PWM

The compounds were added at doses of 1 and 5 $\mu\text{g/ml}$ to PBMC cultures stimulated by 1 $\mu\text{g/ml}$ PWM for antibody production. After seven days, the number of cells producing polyclonal antibodies (PFC) was determined as described by Fauci and Pratt [18]. The results, presented in Tab. 5, showed that the compounds were inhibitory in a dose-dependent manner. The strongest inhibitory activities at a dose of 5 $\mu\text{g/ml}$ were displayed by 1019, 1021 and 1023. CsA practically blocked the antibody production.

Tab. 5. The effects of the compounds on polyclonal antibody production by human peripheral mononuclear blood cells.

Preparation	Dose $\mu\text{g}/\text{mouse}$	PFC/ 10^6 cells	\pm SE	p (Student's t- test)
Background		96	23.50	
Control PWM only		13333	533.00	
Control of the solvent	1	12600	200.00	
	5	8799	266.50	
1017	1	10933	267.00	NS
	5	3676	56.50	<0.01
1018	1	10766	166.50	< 0.02
	5	3648	84.50	< 0.01
1019	1	9066	533.50	< 0.05
	5	6133	267.00	< 0.02
1020	1	9866	266.50	< 0.02
	5	3466	266.50	< 0.01
1021	1	10133	533.00	< 0.05
	5	4910	85.44	< 0.01
1022	1	10266	133.50	< 0.02
	5	2733	67.00	< 0.01
1023	1	12399	133.50	NS
	5	7199	266.50	NS
1024	1	13466	133.50	NS
	5	2466	200.00	< 0.001
1025	1	11999	266.50	NS
	5	2293	107.00	< 0.01
1026	1	10666	0.00	< 0.02
	5	1000	200.00	< 0.001
CsA	1	83	3.00	< 0.001
	5	26	0.00	< 0.001

DISCUSSION

The compounds studied in this report displayed different effects in the models of cellular and humoral immune response. The largest group of compounds could be characterized as universal suppressors: 1020, 1021, 1025 and 1026. 1025 and 1026 inhibited polyclonal antibody production to a higher degree than 1020 and 1021. Two compounds appeared to be particularly interesting: 1019 was a relatively selective DTH inhibitor and 1023 predominantly inhibited the humoral immune response in mice. On the other hand, 1024 preferentially inhibited the DTH response. In fact, the suppression of DTH by that compound was stronger (at both doses) than that exhibited by 1019. Other compounds (1017, 1018 and 1022) exhibited weak inhibitory properties.

The suppressory activities of the compounds depended on the character and location of the substituents in the basic isoxazole structure. Interestingly, the effects of such substitution on the inhibition of the cellular and humoral immune responses were different. For example, substitution with the 2-chlorobenzylidene group resulted in a very significant inhibition of the DTH response; however, such a change had a relatively small effect on the humoral immune response (compound 1019). Inclusion of the 1-chloro-2-ethenyl group had a minor effect on the DTH response but led to a significant reduction of the anti-SRBC response (1023). Notably, even a minor change in a substituent structure could lead to a significant change in activity. For example, a shift of a Cl atom from position 2 of the benzene ring to position 4 transformed the completely inactive 1017 into the extremely inhibitory 1019 (at a dose of 100 $\mu\text{g/ml}$) in the DTH model. By contrast, the same change did not alter the activity of 1017 in the model of the anti-SRBC humoral immune response in mice or in polyclonal antibody production (an even lower inhibition observed).

All the compounds were, in general, inhibitory with respect to both types of immune response. That suggests that they affected very early signaling events leading to the generation of the immune response, probably by interfering with costimulatory signals delivered by antigen-presenting cells and not by controlling further stages of the immune response, like cell division. Such an assumption is supported by the different effects of CsA and the compounds in the model of polyclonal antibody production, where PWM-cell proliferation is strongly correlated with antibody secretion. For example, in the case of 1026 (at 5 $\mu\text{g/ml}$), the inhibitory action of CsA was 40-fold higher, almost blocking antibody production.

In conclusion, the investigation demonstrated that the isoxazole derivatives were inhibitory in the experimental models used in this study. The inhibitory effects varied and depended on the character and location of the substituted groups, which influenced their preferential inhibition of the humoral or cellular immune response. It is suggested that the compounds affect early stages of the immune response, e.g. costimulatory signals delivered by antigen-presenting cells. The

results allow the selection of the most interesting compounds for further investigation with the ultimate goal of performing preclinical studies.

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