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Synthesis and immunological activity of new 5-amino-3-methyl 4-amido and 4-ureilene isoxazole derivatives

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5-Amino-3-methylisoxazole-4-carboxylic acid amides and ureilenes have been synthesized from 5-amino-3-methylisoxazole-4-carbonyl azide. The compounds were investigated for potential immunotropic activity in several immunological tests. The most interesting suppressory activities in the humoral and cellular immune response were compared to activities of analogous compounds previously described as immunostimulatory.

1. Introduction

In our earlier reports we described immunostimulatory and suppressive activities of new isoxazole derivatives. We have found that amido derivatives of a basic structure **I** exhibit immunostimulatory action [1], and compounds substituted at position 5 of a basic structure **II**, possess immunosuppressive activity [2]. The new immunomodulating compound lefunomide (HWA-486) **III** obtained in the Hoechst research laboratories possesses a structure similar to **I** [3, 4].

High immunostimulatory activity of the compounds **I** prompted us to synthesize 4-substituted isoxazol derivatives with increased hydrophilicity and to investigate their immunological properties. By an earlier elaborated method, new amides were obtained (2-hydroxyethylaminoethylamine and diethylaminopropylamine). In addition, other compounds of the basic structure **IV** were obtained, where the carbonyl group in position 4 is not linked to the isoxazole ring directly, as in compounds of type **I**, but via an amino group. Such a modification of the structure and its effect on the biological activity of the compounds seemed to be of particular interest.

In the earlier paper [5] we described the synthesis and antileucemic activity of the compounds **IV**. In the structures investigated we preserved a free amino group in position 5 and a secondary one at the amido bond. In addition, in these compounds, aromatic hydrophobic residues remained. These factors contributed to the immunostimulatory activity of the previously described amides.

2. Investigations, results and discussion

2.1. Synthesis of the derivatives

Previous syntheses of amides of 5-amino-3-methylisoxazolecarboxylic acid were based mainly on the chlorination of 5-amino-3-methyl-4-isoxyzolecarboxylic acid and ensuing reaction with amines and other standard procedures [1–2]. In this paper we report new amides obtained according to a recently described procedure [6]. The binary reactivity of the 5-amino-3-methyl-5-isoxazolecarboxylic acid azide was easy to obtain in the compounds tested (Scheme).

The starting material, 5-amino-3-methyl-4-isoxazolecarboxylic acid azide **1** was prepared according to a method described earlier [7]. Compound **1** was converted to ureides **2** by reaction with amines in a Curtius rearrangement. This process can also be performed in a one-pot procedure for synthesis of the amide **3**. Reaction of compound **1** with amines possessing a basicity higher than pH 10.2 afforded amides **3** running without a Curtius rearrangement [6].

2.2. Immunological tests

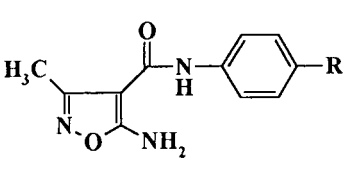
2.2.1. Effects of intraperitoneal treatment of CBA/liw mice with the compounds on the humoral immune response to sheep red blood cells

The effects of an i.p. treatment of mice on the development of the humoral immune response is presented in Ta-

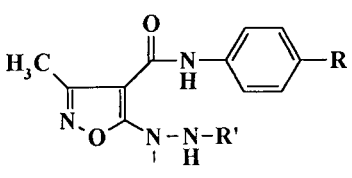
Table 1: Number of the plaque forming cells (PFC) in the spleen cells of CBA/liw mice immunized with SRBC and treated intraperitoneally (i.p.) with the compounds 3 h before and 24 h after antigen administration

Compd.	μg/mouse	PFC/10^6	±SE	P. Student test
Control of the solvent		4461	608	
CSA	10	2976	164	<0.05
	100	2208	214	<0.01
4	10	2460	55	<0.02
	100	1767	299	<0.01
5	10	2511	523	<0.05
	100	3682	314	NS
6	10	3183	211	NS
	100	3200	332	<0.01
7	10	2656	170	<0.05
	100	2014	99	<0.01
8	10	1624	348	<0.01
	100	2832	227	<0.05

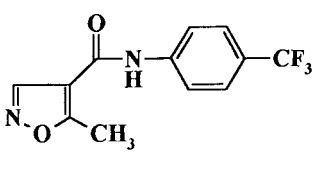
The results are expressed as a mean ±SE of 5 mice
The compounds were dissolved in the mixture EtOH/cremophor (0.64:0.36 respectively)
Concentration of the mixture in the control as in the samples containing 10 μg or 100 μ/ml of the compounds, respectively



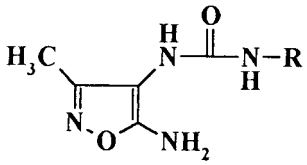
I



II



III



IV

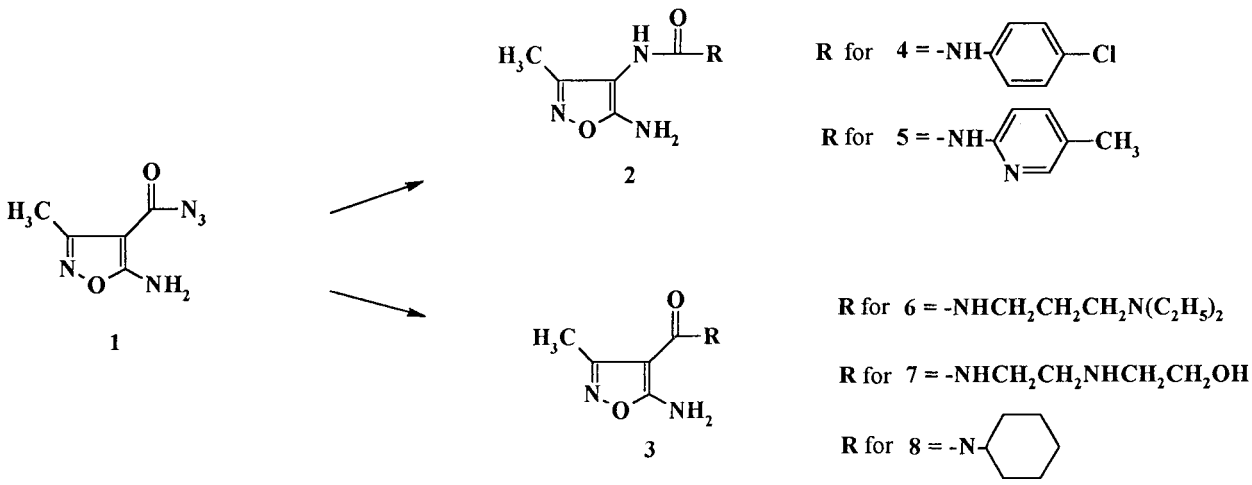
ble 1. As expected, cyclosporine A, used as a reference drug exerted a significant inhibitory action, particularly at the dose of 100 μg/mouse. Among the test compounds, two preparates require special attention. Compound **4** exerted a significant inhibitory action already at a dose of 10 μg/mouse and was even more effective at a dose of 100 μg/mouse. Compound **8** was more active at the low dose (10 μg/ml). On the other hand, the high activity of compound **6** and **7** at the dose of 100 μg/mouse might be due to the toxicity of these compounds as indicated by a weight loss and a worsening condition of the mice.

Table 2: DTH reaction (foot pad test) in 129/liw mice sensitized with SRBC and treated with the compounds intraperitoneally 3 h before and 24 h after antigen administration

Compd.	μg/mouse	Units	±SE	P. Student test
Control of the solvent		12.10	1.17	
CSA	100	4.80	1.14	<0.01
4	100	5.70	1.21	<0.01
5	100	6.30	1.49	<0.05
6	100	4.70	0.81	<0.01
7	100	8.54	0.53	NS
8	100	8.80	1.53	NS

The results are expressed as a mean ±SE of 9 mice
The compounds were dissolved in the mixture (EtOH/cremophor 0.64:0.36 respectively)
Concentration of the mixture in the control as in the samples containing 100 μg of the compounds

Scheme



2.2.2. Effects of intraperitoneal treatment of 129/liw mice with the compounds on the generation of delayed type hypersensitivity to sheep red blood cells

The effects of the compounds on the delayed type hypersensitivity (DTH) in mice are presented in Table 2. Similarly to the case of humoral immune response, compound **4** exhibited a strong, statistically significant, suppressor activity. High inhibitory action of compound **6** could again be caused by a high toxicity, therefore this compound cannot be taken into consideration for further investigations. Of particular interest is the lack of activity of compound **8**, which was strongly inhibitory in the humoral immune response. Thus, we can conclude that compound **4** demonstrates universal inhibitory activity (for both humoral and cellular immune response) and compound **8** affects only the humoral immune response. The obtained results indicate that compounds **4** and **8** may be interesting in terms of immunotropic activities and should be further investigated as potential drugs for general inhibition of the immune response or for selective inhibition of the cellular immune response.

Interestingly, the 4-chlorophenylamide of the 5-amino-3-methylisoxazole-4-carboxylic acid exhibited an immunostimulatory activity higher than levamisole, being less toxic at the same dose [1]. In these studies we demonstrated that in this group of compounds the presence of a primary amino group at position 5 and a −CONH-group at position 4 of the isoxazole structure is essential for immunostimulatory activity.

Introduction of a −NH-group between the isoxazole ring and the carbonyl group causes fundamental change in the immunological reactivity, i.e. from strongly stimulatory to deeply inhibitory. Ureilene derivatives possess in position 4 the −NHCONH-group and exhibit a significant immunosuppressive activity. For example, 5-amino-3-methylisoxazole-4-carboxylic acid 4-chlorophenylamide is a strong immunostimulator (stronger than the reference drug levamisole) [1] and its ureilene analogue showed very high immunosuppressive activity − higher with that of cyclosporine A.

3. Experimental

3.1. Chemistry

M.p.'s were determined on Boetius apparatus and were uncorrected. TLC was carried out on glass silica gel plates Kieselgel G-Merck, using the developing system: $CHCl_3/CH_3OH$ (9:1), detected with J_2 fog.

IR spectra were measured in nujol mulls with a Specord M-80 spectrometer.

^1H NMR spectra were recorded with a Tesla 60 MHz instrument: chemical shifts are reported in ppm from an internal tetramethylsilane standard.

MS were measured with an LKB 9000 spectrometer. All compounds were analyzed for C, H, N and the results were within ±0.4% of the theoretical values. Commercially available reagents and solvents were used without further purification. The starting compound 5-amino-3-methyl-4-isoxazolecarboxylic acid azide (**1**) was prepared according to a previously reported method [7].

3.1.1. 5-Amino-3-methyl-4-ureileneisoxazoles (general method)

To 0.05 mol of **1** in 200 ml of xylene 0.05 mol of the appropriate amine were added in a 500 ml round-bottomed flask fitted with a condenser. The resulting mixture was heated gradually to boiling temperature (during 1 h) at continuous stirring and refluxed for the reaction time of 1 h. During the reaction time the precipitated product was formed and was filtered off after cooling.

3.1.1.1. 5-Amino-4-(4-chlorophenyl)ureilene-3-methylisoxazole (**4**)

Yield: 98%, colorless prisms (EtOH); m.p. 216–217 °C; IR (nujol, cm^{-1}); 1634-CON-, 1543 −C=N- (isoxazole), ^1H NMR ($DMSO_{d\text{-}6}$): 2.32 (s, 3 H, $-CH_3$); 6.7 (s, 1 H, −NH); 7.6 (q, 4 H, aromat); 8.3 (s, 2 H, $-NH_2$); 8.9 (s, 1 H, −NH−).
$C_{11}H_{11}ClN_4O_2$ (267.0)

3.1.1.2. 5-Amino-4-(4-methyl–2-pyridyl))ureilene-3-methylisoxazole (**5**)

Yield: 92%, colorless plates (EtOH); m.p. 214–215 °C; IR (nujol, cm^{-1}); 1656-CON-, 1552 −C=N- (isoxazole), ^1H NMR ($DMSO_{d\text{-}6}$): 1.92 (s, 3 H, piryd. $-CH_3$); 2.43 (s, 3 H, isoxaz. $-CH_3$); 6.5 (s, 1 H, −NH); 7.4 (m, 3 H, aromat); 8.2 (s, 2 H, $-NH_2$); 8.9 (s, 1 H, −NH−).
$C_{11}H_{11}N_5O_2$ (247.3)

3.1.2. 5-Amino-3-methyl-4-isoxazolecarboxylic acid amides (general method)

The sample of 5-amino-3-methyl-4-isoxazolecarbocylic acid azide (**1**, 0.1 mol) was dissolved in 20 ml of EtOH and 0.1 mol of the appropriate amine was placed in a 100 ml round-bottomed flask fitted with a condenser. The reaction mixture was smoothly refluxed with stirring, using a hot plate. During a course of the reaction (controlled in TLC) the precipitated amide was filtered off. Unrefined compound was purified by recrystallization in EtOH. As a result, a colorless crystalline product was obtained.

3.1.2.1. 5-Amino-3-methyl-4-isoxazolecarboxylic acid 3-diethylaminopropylamide (**6**)

Yield: 68%, colorless needles (EtOH); m.p. 158–159 °C; IR (nujol, cm^{-1}); 1666-CON-, 1552 −C=N- (isoxazole), ^1H NMR ($DMSO_{d\text{-}6}$): 1.42 (t, J = 7 Hz, 6 H, 2 CH_3); 2.32 (s, 3 H, isoxaz. $-CH_3$); 3.12–3.86 (m, 6 H, $CH_2CH_2CH_2$); 4.5–4.8 (q, J = 6.5 Hz, 4 H, 2 CH_2); 6.2 (s, 1 H, −NH); 7.12 (s, 2 H, −NH−).
$C_{12}H_{22}N_4O_2$ (254.3)

3.1.2.2. 5-Amino-3-methyl-4-isoxazolecarboxylic acid 3-hydroxyethylaminoethylamide (**7**)

Yield: 67%, colorless prisms (EtOH); m.p. 149–150 °C; IR (nujol, cm^{-1}); 1668-CON-, 1555 −C=N- (isoxazole), ^1H NMR ($DMSO_{d\text{-}6}$): 2.40 (s, 3 H, $-CH_3$); 4.23–4.67 (m, 8 H, 2 CH_2CH_2); 6.1 (s, 1 H, −NH−); 6.84 (s, 2 H, $-NH_2$); 7.23 (s, 1 H, −NH−); 10.12 (s, 1 H, OH).
$C_9H_{16}N_4O_3$ (228.2)

3.1.2.3. 5-Amino-3-methyl-4-isoxazolecarboxylic acid piperidylamide (**8**)

Yield: 90%, colorless plates (EtOH); m.p. 199–200 °C; IR (nujol, cm^{-1}); 1672-CON-, 1548 −C=N- (isoxazole), ^1H NMR ($DMSO_{d\text{-}6}$): 2.38 (s, 3 H, $-CH_3$); 3.23–3.86 (m, 10 H, piperidin); 6.44 (s, 2 H, $-NH_2$).
$C_{10}H_{15}N_3O_2$ (209.2)

3.2. Immunological tests

3.2.2. Animals

CBA/liw mice were used for the determination of plaque forming cells (PFC) in the humoral *in vivo* and *in vitro* immune response, 129/liw mice were used for delayed type hypersensitivity (DTH) experiments, Balb/c/liw mice were used for acute toxicity. Antigen: sheep red blood cells (SRBC).

3.2.3. Humoral immune response

The effect of the compounds on the humoral immune response to SRBC was tested by the PFC test. The details of PFC number determination were described previously [8]. Treatment of mice and cell cultures with the studied compounds is described in Tables 1 and 2.

3.2.4. Cellular immune response

The influence of compounds **4**–**8** on the cellular immune response to SRBC was examined *in vivo* by the DTH test, using the methodological approach of Lagrange et al. [9]. The results are expressed in units of 10^{-2} cm of the increase of foot pad test thickness. The details of this procedure were presented elsewhere [10].
Cyclosporin A (CSA) was used as a reference substance in both PFC and DTH tests. Effects of the compounds were examined only during the inductive phase of DTH. Statistical analysis of these data was performed using t Student's test.

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