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Synthesis, immunomodulating effects and structure–activity relationships of new N-phenyl-5-amino-3-methylisoxazole-4-carboxamides †

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isoxazoles / N-phenyl-5-amino-3-methylisoxazole-4-carboxamides / immunological activity / structure–activity relationships

1. Introduction

Immunostimulatory drugs are considered as a useful supplement in the chemotherapy of cancer and diseases related to immunodeficiencies. In 1971 Renoux discovered that anthelmintic drug levamisole was able to increase the immune response [1]. Many synthetic substances, structurally relevant, or not, to levamisole, have been prepared and their immunological profiles investigated [2–9]. A new immunomodulating compound used for treatment of autoimmune disorders, named leflunomide **I** (HWA-486), was synthesized [10–13] which structurally resembles the compounds studied in this paper. In the course of our studies on the reactivity and the biological properties of the isoxazole derivatives we have found significant antiinflammatory, antibacterial and antitumor activities of some amido derivatives of 5-amino-3-methylisoxazole-4-carboxylic acid **II** [14] (see *figure 1*).

In the present paper we describe the synthesis of a new series of N-phenyl-5-amino-3-methylisoxazole-4-carboxamides containing different substituents in the phenyl ring. These compounds were tested in several immunological tests, such as: humoral immune response to sheep red blood cells in vivo and in vitro and delayed type hypersensitivity reaction to SRBC. The structure–reactivity relationships were also described.

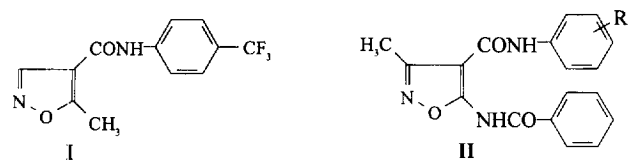

Figure 1.

2. Chemistry

The 5-amino-3-methylisoxazole-4-carboxamide **4** and their phenyl substituted derivatives **5–9** were prepared in different ways, by the sequence of reactions shown in *figure 2*.

The starting material, 5-amino-3-methylisoxazole-4-carboxylic acid **1** was prepared according to the method described by Shaw and Sugowdz [15]. Aminoacid **1** was converted to phenylamides by condensation with phenylamines in the presence of dicyclohexylcarbodiimide. Reaction of compound **1** with the thionyl chloride formed

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† Dedicated to the memory of Professor J.L. Mokrosz
Abbreviations: PFC, plaque forming cells; DTH, delayed type hypersensitivity; SRBC, sheep red blood cells; DCC, dicyclohexylcarbodiimide; SGM, standard growth medium; PBS, phosphate buffered saline; Cremophor®, a derivative of castor oil and ethylene oxide (registered trademark of BASF)

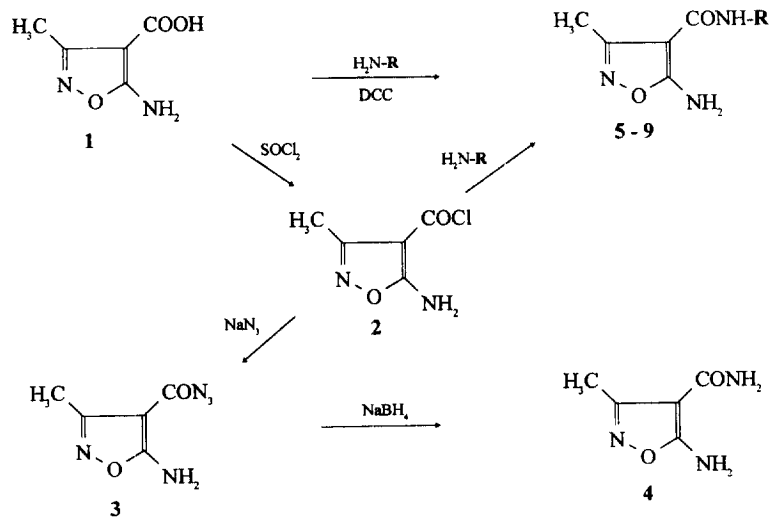
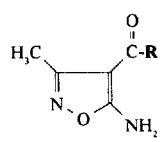
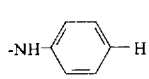
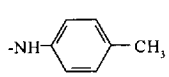
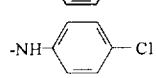
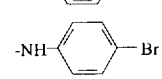
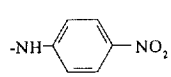


Figure 2.

Table I. Preparation of compounds **4–9**.



Compound	R	Yield (%)	M.p. (°C)	Formula and molecular weight
4	$-NH_2$	16	204–205	$C_5H_7N_3O_2$ 141.05
5		82	177–178	$C_{11}H_{11}N_3O_2$ 217.10
6		86	187–188	$C_{12}H_{13}N_3O_2$ 231.10
7		87	196–197	$C_{11}H_{10}N_3O_2Cl$ 251.04
8		84	207–208	$C_{11}H_{10}N_3O_2Br$ 294.99
9		92	221–222	$C_{11}H_{10}N_4O_4$ 262.07

intermediate chloride **2** which was subsequently condensed into the target phenylamides **5–9**. This process can also be performed in a one-pot procedure for synthesis of the amide **4**, but in this case the workup and isolation of the final compound was more difficult and the overall yield was lower. A more productive method of synthesis the 5-amino-3-methylisoxazole-4-carboxamide **4** appeared to be the reduction of 5-amino-3-methylisoxazole-4-carboxylic acid azide **3** obtained according to Ryng and Machoń [16, 17] with sodium borohydride in dry tetrahydrofuran in the presence of glacial acetic acid.

Table II. Spectral data of compounds **4–9**.

Compound	IR $\nu_{C=O}$ (cm^{-1})	MS (70 eV) *m/z* (%)	^{1}H-NMR (DMSO-d_6), TMS δ, *J* (Hz)
4	1665	209	2.4 (s, 3H, CH_3); 3.9 (s, 2H, NH_2); 6.2 (s, 2H, NH_2); 7.4 (s, 1H, NH)
5	1680	211	1.8 (s, 3H, CH_3); 2.3 (s, 3H, CH_3 isox.); 6.0 (s, 2H, NH_2); 7.6 (m, 5H, aromat); 8.1 (s, 1H, NH)
6	1664	156	2.4 (s, 3H, CH_3); 6.0 (s, 2H, NH_2); 7.7 (dd, 4H, aromat, 6.25); 7.9 (s, 1H, NH)
7	1658	231	2.35 (s, 3H, CH_3); 6.1 (s, 2H, NH_2); 7.4 (dd, 4H, aromat, 6.5); 7.9 (s, 1H, NH)
8	1652	254	2.45 (s, 3H, CH_3); 6.1 (s, 2H, NH_2); 7.5 (dd, 4H, aromat, 6.5); 8.0 (s, 1H, NH)
9	1650	170	2.3 (s, 3H, CH_3); 5.9 (s, 2H, NH_2); 7.6 (s, 4H, aromat); 8.3 (s, 1H, NH)

Physical constants and NMR spectral data for compounds **4–9** are summarized in *tables I* and *II*. Infra-red spectra for these compounds show a broad absorption near 1650 cm^{-1}, attributable to carbonyl group.

3. Biological results and discussion

3.1. Effects of the compounds on the magnitude of the humoral immune response to SRBC in vitro and in vivo

The compounds were added to the spleen cell cultures at the beginning of a 4-day incubation period, together with antigen (SRBC). The results presented in *table III* show that preparates **7** and **8**, and to a lesser degree **9**, exhibited stimulatory activities, comparable or even higher to that of the control drug, levamisole. Other compounds (**5** and **6**) were also stimulatory (not shown).

The studies on the stimulatory action of the compounds were subsequently extended to the in vivo model of generating anti-SRBC response since anticipated in vivo effects of the drugs are more relevant to a potential application in clinic. This is particularly important in the case of oral treatment (*table V*). The compounds, given intraperitoneally (*table IV*) or per os (*table V*), significantly stimulated the humoral immune response to SRBC. These effects were most pronounced in cases of compounds **7** and **8**.

Significant immunostimulatory activities of the compounds in the generation of the humoral immune response in vitro and in vivo suggest that the compounds affect the afferent phase of the immune response by enhancing processes of antigen processing, recognition and differentiation/proliferation of antigen-specific cells.

3.2. Effect of the preparates, given intraperitoneally, on the DTH reaction to SRBC

These effects are shown in *table VI* and were stimulatory, as in the case of humoral immune response. Best enhancing effects on the DTH reaction were observed with **7** and **8**, somewhat lower in the case of compound **5**.

Table III. The effects of the compounds added to the in vitro system of generating anti-SRBC response.

Compound	Dose (μg/mL)	PFC/10^6	± SE	*P* (Student's *t*-test)
Control of 0.9% NaCl		1481	136	
Solvent only [a]		2186	216	
Levamisol	0.1	2949	782	< 0.05
	1.0	2970	141	< 0.01
7	0.1	5025	307	< 0.01
	1.0	5482	572	< 0.001
8	0.1	4234	1188	< 0.01
	1.0	5901	440	< 0.001
9	0.1	2423	199	NS
	1.0	4392	532	< 0.001

The results are expressed as a mean ± SE of 6 wells. [a] The compounds were dissolved in a mixture of ethanol/cremophor (0.64:0.36). Concentration of the mixture in the control as in the probe containing 1 μg/mL of a compound.

Table IV. The number of plaque-forming cells in the spleen of CBA/liw mice immunized with SRBC and treated intraperitoneally with the compounds 3 h before and 24 h after antigen administration.

Compound	Dose (μg/mouse)	PFC/10^6	± SE	*P* (Student's *t*-test)
Control of 0.9% NaCl	1315	114		
Solvent only [a]		1247	90	
7	1	3808	217	< 0.001
	10	5864	292	< 0.001
8	1	3324	543	< 0.05
	10	4442	693	< 0.001
9	1	1581	187	NS
	10	2882	463	< 0.01

The results are expressed as mean ± SE of 7 mice. [a] The compounds were dissolved in a mixture of ethanol/cremophor (0.64:0.36). Concentration of the mixture in the control as in the probe containing 10 μg/mL of a compound.

Table V. The number of plaque-forming cells in the spleen of CBA/liw mice immunized with SRBC and treated per os 3 h before and 24 h after antigen administration with the compound dissolved in ethanol/cremophor mixture.

Compound	Dose (μg/mouse)	PFC/10^6	± SE	*P* (Student's *t*-test)
Control of 0.9% NaCl		546	58	
Solvent only [a]		497	46	
Levamisol	10	740	100	NS
	100	1746	159	< 0.01
7	10	767	64	< 0.05
	100	1542	134	< 0.01
8	10	1035	90	< 0.02
	100	1815	193	< 0.001
9	10	1230	192	< 0.01
	100	1070	203	< 0.02

The results are expressed as mean ± SE of 7 mice. [a] The compounds were dissolved in a mixture of ethanol/cremophor (0.64:0.36). Concentration of the mixture in the control as in the probe containing 100 μg/mL of a compound.

Table VI. DTH reaction (foot pad test) in 129/liw mice sensitized with SRBC and treated intraperitoneally with the compound 3 h before and 48 h after antigen administration.

Compound	Dose (μg/mouse)	Units	± SE	*P* (Student's *t*-test)
Control of 0.9% NaCl		11.02	1.23	
Solvent only [a]		10.80	1.21	
Levamisol	100	15.08	1.03	< 0.02
4	100	11	1.47	NS
		10		
5	100	15.00	1.01	< 0.02
6	100	11.20	0.98	NS
7	100	16.00	1.26	< 0.01
8	100	17.00	1.97	< 0.001
9 [b]	100	10.80	1.09	NS

The results are expressed as mean ± SE of 10 mice. [a] The compounds were dissolved in a mixture of ethanol/cremophor (0.64:0.36). Concentration of the mixture in the control as in the probe containing 100 μg of a compound. One unit = 10^{-2} cm. [b] Toxic.

The above presented data on the activities of compounds **7** and **8** reveal their universal stimulatory properties in both humoral and cellular immune responses. It indicates that they activate generation of both TH2 and TH1 cell subsets [18], leading to overall stimulation of the immune system. On the other hand, the stimulatory activity of compound **9**, was restricted only to the humoral immune response.

The accessibility of the studied compounds by per os route makes them attractive potential drugs, for example in immunodeficiences, perioperative treatment and in immunosuppressed patients.

4. Structure–activity relationship

The discussion on the relationship between the structure and activity has to be limited to compounds **7**, **8** and **9** since the determination of activities of derivatives **4**, **5** and **6** was fragmentary. These compounds derive from a nonactive precursor ($-NH_2$) by addition of phenyl ring **5**, and substituted with $-CH_3$, $-Cl$, $-Br$ or NO_2, respectively. The results show that substitution of aromatic rings with $-Cl$ or $-Br$ (preparates **7** and **8**) results in similar activities, i.e. the compounds are active in all tests. Presence of the nitro group renders compound **9** not active in DTH assay.

It was a particularly interesting finding that the substitution of the CH_3 in the basic structure of leflunomide, a known immunosuppressive compound, resulted in an opposite (immunostimulatory) property. It suggests that the nature and location of a substituent rather than the basic structure in a given class of compounds predicts a given immunological property. In conclusion, some of the compounds appeared to exhibit higher immunostimulatory activity than levamisole, therefore they may be of potential therapeutical use.

5. Experimental protocols

5.1. 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 fogg.

IR spectra were measured in nujol mulls with a Specord M-80 spectrometer. ^1H NMR spectra were recorded with a Tesla 80 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 analytical results were within ±0.4% of the theoretical values. Commercially available reagents and solvents were used without further purification. Starting 5-amino-3-methylisoxazole-4-carboxylic acid **1** and 5-amino-3-methylisoxazole-4-carboxylic acid azide **3** was prepared according to previously reported methods [16].

*5.1.1. General procedure for the synthesis N-phenyl-5-amino-3-methylisoxazole-4-carboxamides **5–9***

The sample of 5-amino-3-methylisoxazole-4-carboxylic acid **1** (0.43 g, 0.3 mmol), 30 mL DMSO, appropriate phenylamine (0.3 mmol) and (0.6 g, 0.3 mmol) DCC were placed in a 100 mL round-bottom flask. The reaction mixture was smoothly stirred for 10 h at the room temperature using an magnetic stirrer. After the course of the reaction a solid (dicyclohexylurea) was separated. The clear solution was poured into 100 mL of water and the product was filtered off after half an hour. The unrefined compound was purified by recrystallization in methanol. As a result, a white crystalline product was obtained.

*5.1.2. 5-Amino-3-methylisoxazole-4-carboxamide **4***

To a stirred solution of 5-amino-3-methylisoxazole-4-carboxylic acid azide (0.5 g, 0.3 mmol) in dry THF (50 mL) and (0.18 g, 0.3 mmol) of glacial acetic acid was slowly added (0.12 g, 0.3 mmol) of sodium borohydride over within 20 min. The mixture was stirred and heated under reflux for 0.5 h, then cooled and carefully treated with 0.5 g anhydrous potassium carbonate. After filtration, the filtrate was evaporated and the residue was extracted with ether. The organic layer was washed with water, dried and evaporated. The crude product was crystallized from i-PrOH. As a result 0.23 mmol of **4** were obtained.

5.2. Immunology

5.2.1. Animals

CBA/liw mice were used for PFC and 129/liw mice were used for DTH experiments.

5.2.2. Humoral immune response

The influence of the compounds on the humoral immune response to SRBC was tested by the Jerne test in a modification of Mishell and Dutton [19] performed both in vitro and in vivo. The number of plaque-forming cells was determined and compared to the control.

5.2.3. Cellular immune response

The influence of compounds **4–9** on the cellular immune response to SRBC was examined in vivo by delayed type hypersensitivity test, using the methodological approach of Lagrange et al. [20]. The results are expressed in units (1 unit = 10^{-2} cm of the increase of foot pad thickness). Levamisole was used as a reference substance in both PFC test and DTH tests. Only the inductive phase of DTH was examined in this test. Statistical analysis of these data was performed using the Student's *t*-test.

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