Synthesis and Structure Elucidation of 5-Aminomethinimino-3-methyl-4-isoxazolecarboxylic Acid Phenylamides and Their Immunological Activity

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Key Words: *Isoxazoles; 3-methyl-4-isoxazolecarboxylic acid amide 5-derivatives; immunological activity*

Summary

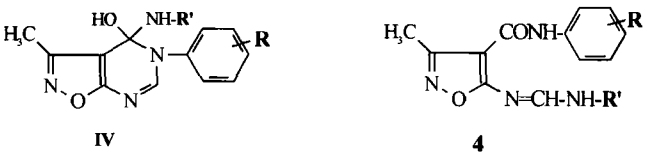
A series of 5-aminomethinimino-3-methyl-4-isoxazolecarboxylic acid phenylamides **4** has been prepared by condensation of 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides **1** with trichloroacetic aldehyde. Alcoholysis of trichloro derivatives **2** gave 5-alkoxymethine derivatives **3** which, on reaction with an appropriate amine, formed the corresponding compounds **4**. The compounds obtained were evaluated for their immunological activity. The properties of three compounds, described in this report, permitted inhibition of the immune response in all possible ways: diminishing both types of immune response (**4d**), humoral immune response (**4a**), or cellular immune response (**4c**). Preparation **4d** is comparable in its effectiveness to CsA, so it may be potentially used as an agent for prolongation of the function of transplanted organs. Two other compounds may potentially be used in cases where only one type the immune response is required for combating pathogen invasion.

Introduction

Demand for therapeutic agents able to restore a normal immune response in immunocompromised patients (primary and acquired immuno-deficiency) has led to the discovery of a number of substances, collectively defined as immunomodulators. In our research program, aimed at the synthesis of immunomodulating agents, we focused our attention on 5-amino-3-methyl-4-isoxazolecarboxylic acid derivatives. The isoxazole moiety is indeed frequently contained in few immunoactive compounds [1–3]. In addition, our study has demonstrated antitumor activity of some isoxazole derivatives[4].

As a continuation of our previous studies on 5-substituted-3-methyl-4-isoxazolecarboxylic acid we present in this article potential immunomodulatory activities of the compounds, in particular those containing an aminomethinimino group in position 5 of the isoxazole cycle.

Some time ago we described the preparation and pharmacological properties of compounds to which the isoxazolo[5,4-*d*]-6,7-dihydropyrimidine structure was assigned[4]. Owing to our continuing interest in this heterocyclic system, we have repeated the original work and found that although the reactions proceed ostensibly as described, the final product **4** was the 5-aminomethine-3-methyl-4-isoxazolecarboxylic acid phenylamide instead of the 4-amino- 3-methyl-isoxazolo[5,4-*d*]-6,7-dihydropyrimidine **IV**.



IV **4**

Figure 1

The revised pathway for the formation of compound **4** from 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamide **1** is shown in Scheme 1. The NMR spectrum of **2a** and **2b** showed the presence of a NH proton and a methine proton. The IR spectrum showed a NH band and an amide carbonyl absorption peak. These data are consistent with the structures of compound **2** and **II** shown in Scheme 1.

We established that solvolysis, together with decarboxylation of the trichloromethyl moiety of the intermediate product **2** (see Scheme 1), took place and cyclization to pyrimidino-isoxazole did not occur. Compound **2**, after heating with methyl alcohol, evolving hydrochloric acid *in situ*, resulted in formation of compound **3**. The 5-methoxymethinimino-3-methyl-4-isoxazole-carboxylic acid phenylamide **3** reacts with amines to give the final 5-aminomethinimino-3-methyl-4-isoxazolecarboxylic acid phenylamides **4** which, on reaction with lithium aluminium hydride under anhydrous conditions, is converted into 5-aminomethyleneamino-3-methyl-4-isoxazolecarboxylic acid phenylamides **6**. Since interpretation of the spectral data on the final products **IV** did not allow a definite assignment of the structure, we performed X-ray crystallographic investigations, which confirmed that the resulted structure is indeed compound **4** (Figure 2).

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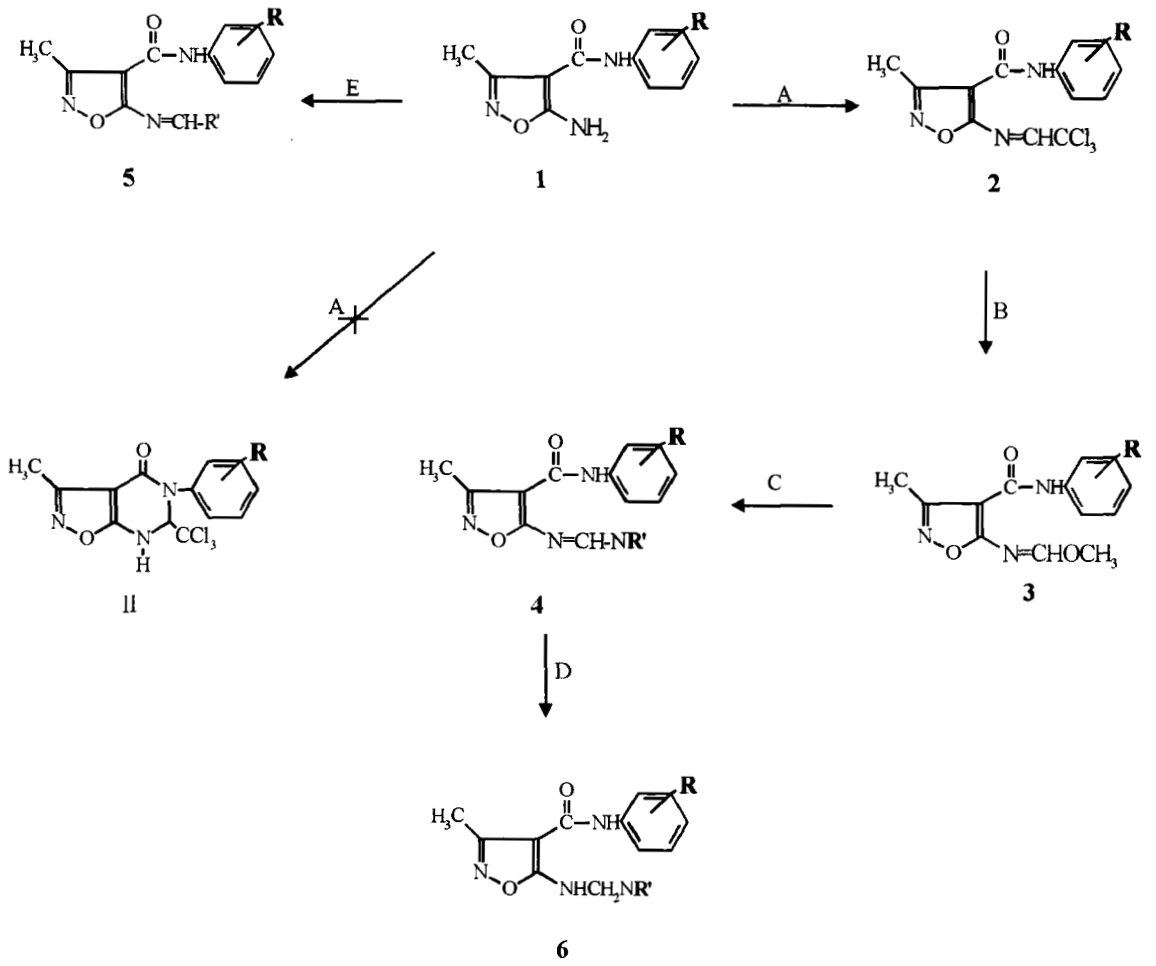


Scheme 1. A: Cl_3CCHO; B: CH_3OH; C: NH-R'(NR_2); D: $LiAlH_4$; E: ArCHO.

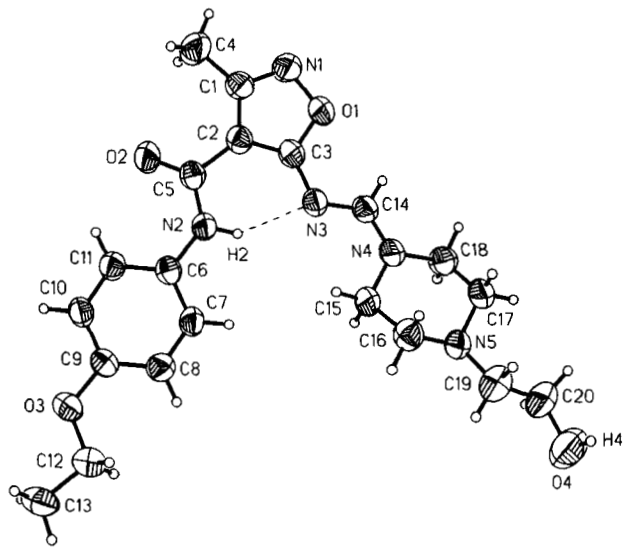


Figure 2. An ORTEP drawing of **IVd** with the atom numbering scheme. Thermal ellipsoids are drawn at the 50% probability level.

Results and Discussion

Chemistry

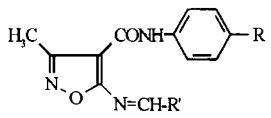
The compounds were synthesized according to Scheme 1.

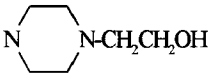
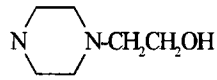
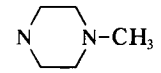
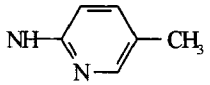
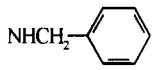
The 5-amino-3-methyl-4-isoxazolecarboxylic acid phenyl amide **1** is treated with anhydrous trichloroacetyl aldehyde in toluene suspension, forming **2** in high yield. Reaction of product **2** with boiling methanol under reflux gives 5-methoxymethinimino-3-methyl-4-isoxazolecarboxylic acid phenylamide **3**. On treatment of this compound with amine in ethanole solution under reflux, 5-aminomethinimino-3-methyl-4-isoxazolecarboxylic acid phenylamide **4** (Table 1) is obtained in good yield. When product **4** was subjected to reaction with lithium aluminium hydride in THF solution, it yielded the dihydro derivative **6** (Table 3). On starting with the initial product **1**, reaction with phenylaldehyde gives compound **5** (Table 2) in a good quantitative yield.

Acknowledgment

This work was supported by the State Committee for Scientific Research (Grant 0270/P2/94/06 and 2 0729 91 01).

Table 1. Physical properties for 5-aminometinimino-3-methyl-4-isoxazolecarboxylic acid phenylamides.



Compound	R	R'	Yield [%]	Mp [°C]	Formula M.w.
2 a	OC_2H_5	CCl_3	87	146–7	$C_{15}H_{14}N_3O_3Cl_3$ 390.66
2 b	Cl	CCl_3	62.9	149–50	$C_{13}H_9N_3O_2Cl_4$ 381.05
3 a	OC_2H_5	OCH_3	65	108–9	$C_{15}H_{19}N_3O_4$ 305.33
3 b	Cl	OCH_3	63	121–2	$C_{13}H_{14}N_3O_3Cl$ 245.73
4 a	Cl		87	235–7	$C_{16}H_{19}N_4O_3Cl$ 350.80
4 b	OC_2H_5		85	203–5	$C_{18}H_{24}N_4O_4$ 360.40
4 c	Cl		64	192–4	$C_{18}H_{24}N_5O_3Cl$ 393.87
4 d	OC_2H_5		67	176–7	$C_{20}H_{27}N_5O_4$ 401.46
4 e	Cl		64	192–4	$C_{17}H_{22}N_5O_2Cl$ 363.85
4 f	Cl		63	203–4	$C_{18}H_{18}N_5O_2Cl$ 371.82
4 g	Cl		75	222–4	$C_{19}H_{19}N_4O_2Cl$ 370.83
4 h	Cl	$NHCH_2CH_2NHCH_2$-CH_2OH	72	196–7	$C_{16}H_{22}N_5O_3Cl$ 367.84
4 i	Cl	$NHCH_2CH_2OH$	75	220–2	$C_{14}H_{17}N_4O_3Cl$ 324.77
4 k	Cl	$NHCH_2CH_2CH_2OH$	82	206–8	$C_{15}H_{19}N_4O_3Cl$
4 l	Cl	$NHCH_2CH{=}CH_2$	74	218–9	$C_{15}H_{17}N_4O_2Cl$ 320.78
4 m	Cl	$NHCH_2CH_2N(CH_2CH_3)_2$	85	257–8	$C_{13}H_{15}N_6O_3Cl$ 338.80

Immunological Part

Inhibition of the humoral immune response to sheep red blood cells (SRBC) in vitro and in vivo

Table 4 shows effects of the compounds, tested in the splenocyte cultures of CBA mice, on the magnitude of the humoral immune response, as measured by the number of plaque-forming cells (PFC) to SRBC. In control cultures a solvent (cremophor – Sigma) or cyclosporin A – Sandoz (CSA) were added instead of the compounds tested.

The results show that the stimulatory action on the number of PFC was exhibited by compound **4d** (1 μg/ml). Most notably, downregulatory effects were exhibited by compounds: **4c** (10 μg), **4b, 4d, 4f, 4m, 6b** at a concentration of 5 μg/ml. The inhibitory action of **4b** could be due to the low cytotoxicity of this compound.

Table 5 demonstrates the effect of the compounds, administered i.p. 3 h before and 24 h after immunization with SRBC, on the number of PFC. Similarly, as in the *in vitro* system, the compounds exhibited inhibitory activities on the magnitude of the immune response. Most significant effects were exerted by preparations: **4d, 4f, 4i** (10 μg) and **4b**. Other products: **5a, 5b,** and **6b** were less inhibitory. One compound – **4c** – showed even slight upregulatory activity.

Inhibition of delayed type hypersensitivity (DTH) to SRBC by the compounds

Table 6 illustrates suppressive effects of the tested compounds on the magnitude of DTH reaction, as measured by the foot pad test. The compounds were dissolved in a mixture of alcohol and cremophor and given intraperitoneally (i.p.) 3 h before and 24 h after the sensitizing dose of antigen (SRBC). The results show that best inhibitory actions were exhibited by the compounds **4c** and **4d.** Other compounds were less inhibitory and **4b, 4h, 4m, 5b**, and **6b** were not active.

*Acute toxicity in BALB/c mice for preparates **4d, 4f** given once intraperitoneally*

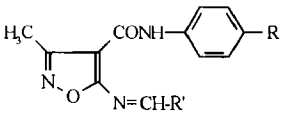
The results are presented in Table 7. The LD100% values for the compounds **4d** and **4f** were 500 mg and 750 mg per kg/body weight, respectively.

The LD50% values were: 412.5 mg and 550 mg per kg/body weight, respectively.

Screening of the studied compounds required performance of several experiments because it was technically impossible to accomplish it in one experiment a day. Therefore, the results are presented as a combination of usually 3 experiments (Tables 3–6).

The compounds, tested in this study for immunotropic activity, showed differential immunosuppressive actions. Among 18 compounds studied, 3 compounds attract special attention - **4a, 4c** and **4d.** Compound **4d** exhibited immunosuppressive activity in both the humoral as well as in the cellular immune response. In this regard the compound resembles CsA. The ac-

Table 2. Physical properties for 5-aminomethyleneamino-3-methyl-4-isoxazolecarboxylic acid 4-phenylamides



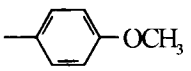
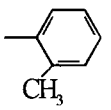
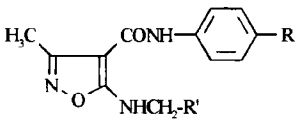
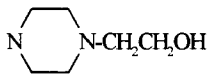
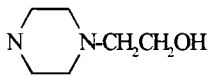
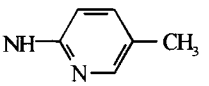
Compound	R	OCH	Yield [%]	Mp [°C]	Formula M.w.
5 a	Cl		88	225–7	$C_{19}H_{16}N_3O_3Cl$ 369.81
5 b	Cl		88	225–7	$C_{19}H_{16}N_3O_2Cl$ 353.81

Table 3. Physical properties for 5-benzylidene-3-methyl-4-isoxazolecarboxylic acid phenylamides



Compound	R	R'	Yield [%]	Mp [°C]	Formula M.w.
6 a	Cl		69	212–4	$C_{18}H_{24}N_5O_3Cl$ 393.87
6 b	OC_2H_5		74	194–6	$C_{20}H_{29}N_5O_4$ 403.48
6 c	Cl		68	223–4	$C_{18}H_{18}N_5O_2Cl$ 371.83

tions of **4a** and **4c** compounds were strictly directional; **4a** selectively inhibited the humoral and **4c** the cellular response. Lack of inhibition of the humoral immune response *in vitro* by several compounds **4h, 4i, 4k** may be due to the fact that for the *in vitro* assay the cells are already sensitized *in vivo with the antigen*, so addition of the compounds to the cell cultures was less effective. The inhibition of PFC number *in vitro* by compound **4m** may be caused by its toxicity.

The observed activities of the compounds are associated with their structure. Interestingly, the complete lack of activity of compounds **5a,b** may be linked to the absence of amino group in this compound, compared with other preparations. The universal suppressive activity of compound **4d**, compared with **4c**, which inhibits only the cellular response, may be associated with a more hydrophilic nature of **4d** (-OC_2H_5 instead of -Cl). On the other hand, product **4a**, exhibiting strong inhibitory action on antibody production, is more lipophilic compared with **4c** (which contains the morpholine group in the place of hydroxyethylpiperazine).

The properties of three compounds, described in this report, allow for inhibition of the immune response in all possible ways: diminishing both types of the immune response (**4d**), the humoral response (**4a**), or the cellular response (**4c**). Compound **4d** is comparable in its effectiveness to CsA, so it may be potentially used as an agent for prolongation of the function of transplanted organs. Two other compounds may potentially be used in cases where only one type of immune response is required for combating pathogen invasion. Studies are underway to further establish the mechanism of the inhibitory action of compounds **4a**, **4c**, and **4d**. It is also important to stress that the toxicity of the studied compounds (LD50 and LD100) is many times lower than that described for CsA.

Crystallographic Part

The molecular structure and atom numbering scheme are shown in Figure 2. The isoxazole ring is planar within the limits of experimental error. The methyl C(4) atom and carbonyl C(5) - O(2) group are situated in plane of the isoxazole ring. The torsion angle C(4)–C(1)–C(2)–C(3) is –179.3(3)°.

In piperazine moiety, C(15), N(4), C(18) atoms are in opposite sides of C(15), C(16), C(17), C(18) plane. This plane makes an angle of 145° with the isoxazole ring. The conformation of the molecule can be characterized by the relative orientation of the isoxazole and phenyl ring. The planes through these two rings make an angle of 6.8° with each other. The packing of the molecules in the crystals is stabilized by a system of hydrogen bonds. There is an intramolecular hydrogen bonding between imino N(2) and azo N(3) atoms [N(2)–H(2)···N(3) = 2.844(4) Å, < 143(2)°], essentially in the plane of the isoxazole ring system.

The hydroxyl group O(4)–H(4) is involved as donor to carbonyl O(2) atom at x, $1+y$, $1+z$ in an intermolecular hydrogen bonding [O(4)–H(4)···O(2) = 2.878(4)Å, < 170(2)°].

Experimental Part

Chemistry

All reagents were of commercial quality from freshly opened containers or distilled before use. TLC plates (silica gel 60 F_{254} were purchased from Merck, Darmstadt. All melting points (Boetius apparatus) are uncorrected. Microanalyses were obtained with a Carlo Erba Instruments. IR spectra were measured for nujol mulls with a Specord M-80. ^{1}H NMR spectra were recorded on a Tesla 80 MHz instrument. Mass spectra were measured with an LKB 9000s spectrometer.

5-Amino-3-methyl-4-isoxazolecarboxylic acid phenylamides 1 was obtained according to ref. [5].

3-Methyl-5-phenylmethinimino-4-isoxazolecarboxylic acid phenylamides 5 was obtained according to ref. [5].

5-Trichloromethylmethinimino-3-methyl-4-isoxazolecarboxylic acid 4-phenylamides 2; General Procedure

To a 12.6g (0.05 mol) 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamide **2** was added 7.7g (0.05 mol) CCl_3CHO in the 30 mL of PhMe and the resulting suspension was refluxed for 2 h. After cooling, the precipitate was filtered off. The yield of the crude product was 83–87% of the theoretical value.

*5-Trichloromethylmethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-ethoxyphenylamide) **2a***

From **1a** and trichloroacetaldehyde; as colorless solid.

*5-Trichloromethylmethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chlorophenylamide) **2b***

From **1b** and trichloroacetaldehyde; as colorless solid.

Table 4. The number of plaque forming cells in the spleen cell cultures of CBA/liw mice treated with the indicated doses of compounds or cyclosporine A.

Compound	µg/well	PFC/10^6	±SE	*P* Student test
		Experiment 1		
PBS only		2241	54	
Control of the solvent	1	2829	76	
	10	2431	175	
CSA	1	1045	44	<0.001
	10	331	22	<0.001
4c	1	2676	89	NS
	10	1757	188	<0.02
5a	1	2250	181	<0.05
	10	2226	204	NS
5b	1	2841	135	NS
	10	2157	48	NS
		Experiment 2		
PBS only		1180	44	
Control of the solvent	1	1431	76	
	10	1500	44	
CSA	1	255	38	<0.001
	10	159	9	<0.001
4d	1	751	129	<0.01
	10	165	33	<0.001
4e	1	2131	251	<0.01
	10	965	63	<0.05
4f	1	562	23	<0.001
	10	252	5	<0.001
4g	1	255	53	<0.001
	10	150	22	<0.001
4h	1	1530	187	NS
	10	1189	235	NS
4i	1	989	77	<0.05
	10	113	6	<0.001
4l	1	360	50	<0.001
	10	265	103	<0.001
4m	1	971	99	<0.05
	10	111	13	<0.001
		Experiment 3		
PBS[1] only		2440	144	
Control of the solvent	1	2622	144	
	5	2051	263	
6a	1	1867	204	<0.05
	5	1280	102	<0.01

The results are expressed as a mean ±SE of 6 wells.
Compound were dissolved in the mixture (ethanol/cremophor 0.64:0.36 respectively).
Concentration of the mixture in the control as in the probes containing 1µg or 10 µg of the compounds, respectively.
[1] PBS - phosphate buffered saline

Table 5. The number of the plaque forming cells in the spleen cells of CBA/liw mice immunized with SRBC and treated intraperitoneally (i.p.) with the compounds before 3 h and 24 h after antigen administration.

Compound	µg/mouse	PFC/10^6	±SE	*P* Student test
		Experiment 1		
Control of the solvent		4461	608	
CSA	10	2976	164	<0.05
	100	2208	214	<0.01
4c	10	5963	520	<0.05
	100	6431	556	<0.05
5a	10	2511	523	<0.05
	100	3682	314	NS
5b	10	3357	713	NS
	100	3968	537	NS
		Experiment 2		
Control of the mixture		2414	280	
CSA	10	1198	105	<0.01
	100	516	37	<0.001
4d	10	901	102	<0.01
	100	760	102	<0.001
4f	10	1156	226	<0.01
	100	764	192	<0.001
4i	10	1276	296	<0.01
	100	2915	238	NS
4e	10	1573	90	<0.02
	100	1929	175	<0.05
4h	10	1275	150	<0.01
	100	2808	390	NS
4m	10	1841	262	NS
	100	2010	223	NS
4l	10	698	193	<0.001
	100	1790	194	<0.05
4g	10	1223	124	<0.01
	100	1770	292	<0.05
		Experiment 3		
Control of the solvent		2537	143	
6a	10	1737	152	<0.01
	100	1918	127	<0.05

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

Table 6. DTH reaction (foot pad test) in 129/liw mice sensitized with SRBC and treated with the compounds intraperitoneally before 3 h and 24 h after antigen administration.

Compound	µg/mouse	Units	±SE	P Student test
		Experiment 1		
Control of the solvent		12.10	1.17	
CSA	100	4.80	1.14	<0.01
4e	100	6.30	1.28	<0.01
4h	100	11.80	1.33	NS
4m	100	10.20	0.99	NS
		Experiment 2		
Control of the solvent		11.40	1.42	
CSA	100	3.97	0.70	<0.01
5a	100	6.30	1.49	<0.05
5b	100	9.44	1.54	NS
4c	100	4.84	0.89	<0.01
		Experiment 3		
Control of the solvent		10.5	1.49	
CSA	100	2.39	0.42	<0.01
4d	100	2.15	0.64	<0.01
4f	100	6.00	0.50	<0.05
4i	100	5.80	1.00	<0.05
4l	100	6.90	0.73	NS
4g	100	4.00	0.59	<0.01
		Experiment 4		
Control of the solvent		6.14	0.72	
4m	10	4.57	0.46	NS
	100	7.47	0.81	NS
		Experiment 5		
Control of the solvent		10.44	0.89	
CSA	10	7.70	0.49	<0.05
	100*	5.47	1.20	<0.01
	1000**	5.87	0.69	<0.01
4d	10	3.24	1.03	<0.001
	100	3.07	0.87	<0.001
	1000	7.23	1.52	<0.05

The results are expressed as a mean ±SE of 9 mice.
The compounds were dissolved in the mixture (ethanol/cremophor 0.64:0.36 respectively).
Concentration of the mixture in the control as in the probes containing 100µg of the compounds.
One unit = 10^{-2} cm
* one mouse died
** three mice died

Table 7. Acute toxicity in BALB/c mice after intraperitoneal administration of one dose of the compounds.

Compound	LD 100%		LD 50%*	
	mg/mouse	mg/kg	mg/mouse	mg/kg
4d	10	500	8.25	412.5
6a	15	700	11.00	550.0

Time of observation – 10 days.

5-Methoxymethylmethinimino-3-methyl-4-isoxazolecarboxylic acid phenylamides **3***; General Procedure*

To a crude 12.0 g 5-trichloromethylmethinimino-3-methyl-4-isoxazolecarboxylic acid phenylamide 30 mL of MeOH was added and refluxed for 2 h. The resulting solution was concentrated at diminished pressure to 10 mL of total amount. After cooling and filtration, the crude product was obtained. The yield was 59–65% of the theoretical value. The crude products were crystallized from MeOH.

5-Methoxymethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-ethoxy-phenylamide) ***3a***

From **2a** and methanol; as colorless plates.

5-Methoxymethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chloro-phenylamide) ***3b***

From **2b** and methanol; as colorless needles.

5-Aminomethinimino-3-methyl-4-isoxazolecarboxylic acid 4 phenylamide 3; General procedure:

To (0.02 mol) 5-methoxymethinimino-3-methyl-4-isoxazolecarboxylic acid 4-phenylamides, dissolved in 100 mL of EtOH, were added (0.02 mol) amine in 45 mL. of ethanol and refluxed for 2 h. After cooling and filtration product **IV** was obtained. The analytical samples were prepared by recrystallization from EtOH.

5-Morpholinomethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chloro-phenylamide) ***4a***

From **3b** and morpholine; as colorless crystals.

5-Morpholinomethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-ethoxy-phenylamide) ***4b***

From **3a** and morpholine; as colorless plates.

5-(2-Hydroxyethyl)piperazinomethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chlorophenylamide) ***4c***

From **3b** and 2-hydroxyethylpiperazine; as colorless crystals.

5-(2-Hydroxyethyl)piperazinomethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-ethoxyphenylamide) ***4d.***

From **3a** and 2-hydroxyethylpiperazine; as colorless rhomboidal crystals. MS = M^+ at m/e 401

5-Piperidinomethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chloro-phenylamide) ***4e***

From **3b** and piperidine; as colorless crystals.

5-Methylpiperazinomethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chlorophenylamide) ***4f***

From **3b** and methylpiperazine; as colorless plates.

5-(4-methyl-2-pirydylamino)methinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chlorophenylamide) ***4g***

From **3b** and 2-amino-5-methylpyridine; as colorless crystals.

5-Benzylaminomethinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chloro-phenylamide) ***4h***

From **3b** and 2-hydroxyethylpiperazine; as colorless needles.

5-(2-hydroxyethylaminoethylamino)methinimino-3-methyl-4-isoxazole-carbo-xylic acid 4-(4-chlorophenylamide) ***4i***

From **3b** and 2-hydroxyethylaminoethylamine; as colorless crystals.

5-(2-Hydroxyethylamino)methinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chlorophenylamide) ***4k***

From **3b** and 2-hydroxyethylamine; as colorless crystals.

5-(3-Hydroxypropylamino)methinimino-3-methyl-4-isoxazole-carboxylic acid 4-(4-chlorophenylamide) ***4l***

From **3b** and 3-hydroxypropylamine; as colorless needles.

5-(2-Propenyloamino)methinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chlorophenylamide) ***4m***

From **3b** and 2-propenylamine; as colorless crystals.

5-(2-Diethylaminoethylamino)methinimino-3-methyl-4-isoxazolecarboxylic acid 4-(4-chlorophenylamide) ***4n***

From **3b** and 2-diethylaminoethylamine ; as colorless crystals.

5-Benzylidene-3-methyl-4-isoxazolecarboxylic acid 4-phenylamides ***5****; General Procedure*

A mixture of **1** (0.01 mol), the appropriate benzaldehyde (0.01 mol), and toluene (40 mL) was heated under reflux for 30 min. The mixture was cooled to room temperature to give the corresponding Schiff base **5**. The pure products were prepared by recrystallization from EtOH.

5-(4-methoxybenzylidene)-3-methyl-4-isoxazolecarboxylic acid 4-chloro-phenylamide ***5a***

From **1b** and 4-methoxybenzaldehyde; as yellow crystals.

5-(2-methylbenzylidene)-3-methyl-4-isoxazolecarboxylic acid 4-chloro-phenylamide ***5b***

From **1b** and 2-methylbenzaldehyde; as yellow-orange crystals.

5-Aminomethyleneamino-3-methyl-4-isoxazolecarboxylic acid 4-phenyl-amides 6; General Procedure

To a stirred suspension of lithium aluminium hydride (0.6 g. 0.015 mol) in dry THF (200 mL) was slowly added a solution of **5** (0.01 mol) in dry THF (200 mL). The mixture was stirred and heated under reflux for 3 h, then cooled and carefully treated with absolute ethanol. 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.

5-(4-hydroxyethylpiperazinylmethyleneamine)-3-methyl-4-isoxazole-carboxylic acid 4-chlorophenylamide ***6a***

From **4c;** as colorless crystals.

5-(4-hydroxyethylpiperazinylmethyleneamine)-3-methyl-4-isoxazole-carboxylic acid 4-ethoxyphenylamide ***6b***

From **4d;** as colorless crystals.

5-(4-methyl-2-pirydylaminomethyleneamine)-3-methyl-4-isoxazole-carboxylic acid 4-chlorophenylamide ***6c***

From **4f;** as colorless crystals.

Immunological Activity

Animals

CBA/liw mice were used for plaque forming cells *in vivo* and *in vitro* experiments, 129/liw mice were used for delayed type hypersensitivity experiments, Balb/c/liw mice were used for acute toxicity. Antigen: sheep red blood cells.

Humoral immune response

Effect of the compounds on the humoral immune response to SRBC was tested by the PFC test performed both *in vitro* and *in vivo*. The number of plaque forming cells was determined and compared to the control. The details of PFC number determination were described previously [6].

Cellular immune response

The influence of compounds **I–V** on the cellular immune response to SRBC was examined *in vivo* by delayed type hypersensitivity test, using the methodological approach of Lagrange et al. [7]. The results are expressed in units (1 unit = 10^{-2} cm of the increase of foot pad test thickness). The details of this procedure were presented elsewhere [8].

Cyclosporin A was used as a reference substance in both the PFC and the DTH tests. Only the inductive phase of DTH was examined in the DTH test. Statistical analysis of these data was performed using t Student's test.

Crystallographic Investigation

The crystal structure of 5-β-hydroxyethylpiperazino-3-methyl-4-isoxazolecarboxylic acid 4-(4-ethoxyphenylamide) **4d** has been studied.

The crystal system and space group were determined from rotation and Weissenberg photographs. All measurements were made on a Kuma KM-4 computer-controlled κ-axis diffractometer with graphite - monochromated CuKα radiation (λ = 1.5418 Å) using a crystal of 0.25 × 0.25 × 0.35 mm. The ω -2θ scan technique was applied for $3.92\theta < 150.06^{\circ}$. Two reflections were used as standard and measured during the data collection: crystal decomposition was not observed: 3785 reflections measured ($0 \le h \le 8$; $-14 \le k \le 14$; $-15 \le l \le 14$) and 2909 were classified as observed with $I > 2.0\sigma(I)$ The intensities were corrected for Lorentz and polarization effects, but no corrections were made for extinction or absorption.The cell dimensions were obtained and refined by the least-square method on the basis of the diffractometric measurement for 25 reflections ($19.8 \le \theta \le 28.7^{\circ}$). The structure was solved by the direct methods with SHELXS – 86 [9] and refined by full-matrix least-square methods using SHELXL – 93 [10] with anisotropic thermal parameters for non-H atoms. Positions of all hydrogen atoms were determined from the difference Fourier synthesis. In the final cycles of the refinement, H-atom parameters with isotropic thermal parameters were included. Scattering factors were those incorporated in SHELXL – 93.

The *R* factors at the end of the refinement were $R = 0.0510$ and $R_w = 0.1593$.

Crystal data for **4d** : $C_{20}H_{27}N_5O_4$; mol. mass: 401.47; triclinic, P-1 space group; $a = 8.210(2)$, $b = 11.315(2)$, $c = 12.091(2)$Å; $\alpha = 110.30(3)$, $\beta = 94.47(3)$, $\gamma = 93.37(3)^{\circ}$; $V = 1045.8(4)$ Å^3; $d_x = 1.275$ Mg/m^3; $z = 2$; F(000) = 428; μ(Cuk α) = 7.45 cm^{-1}; Goodness-of-fit on $F^2 = 1.092$.

Supplementary Material Available

IR and NMR data for all compounds, as well as crystallographic data are available on request.

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Received: February 7, 1997 [FP184]