Full Paper

New Amides of 5-(4-Chlorobenzoyl)aminoorotic Acid: Their Synthesis and Biological Activity

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The synthesis and *in-vitro* biological evaluation of the amide series **4** of 5-(4-chlorobenzoyl)aminoorotic acid **2** are presented. The biological properties of a few 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-*N*-substituted-4-pyrimidinecarboxamide derivatives **4** tested here were compared with those of the isosteric isothiazole derivative **MR-2/94** (5-(4-chlorobenzoyl)amino-*N*-(4-chlorophenyl)-3-methyl-4-isothiazolecarboxamide), which possesses a strong immunosuppressive and anti-inflammatory activity [1, 2], It must be suggested that replacement of the isothiazole by a pyrimidine core ring system resulted in considerable lowering of the anti-inflammatory and immunotropic actions of the obtained amides. Physicochemical properties of 2-(4-chlorophenyl)-6,8-dihydroxy-4*H*-pyrimido[5,4-*d*]-1,3-oxazin-4-on **3** are also briefly described.

Keywords: 5-Aminoorotic acid / Immunotropic activity / Nitric oxide / Proinflammatory cytokines / Pyrimido[5,4-*d*]-4*H*-1,3-oxazine

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Introduction

The different previously described derivatives of 5-amino-3-methyl-4-isothiazolecarboxylic acid **5** (Fig: 1) displayed remarkable antiviral, anti-inflammatory, cytostatic, analgesic, and a recently discovered immunotropic activity [1, 3 – 8]. Of this group of compounds, 5-benzoylamine-*N*-(4-chlorophenyl)-3-methyl-4-isothiazolecarboxamide, *i. e.* Denotivir (ITCL, Vratizolin) [9, 10] (see Fig. 1), an antiviral drug with anti-inflammatory activity, has been put into medical practice. Currently, it is mainly used against herpes virus infections [3, 11, 12]. It also has significant immunotropic properties [10].

Based on the structure of Denotivir, a number of isothiazole derivatives have been synthesized and biologically tested recently [1, 4, 8]. Some new amides have shown *in-vitro* and *in-vivo* immunosuppressive and anti-inflammatory activities stronger than those of cyclospor-

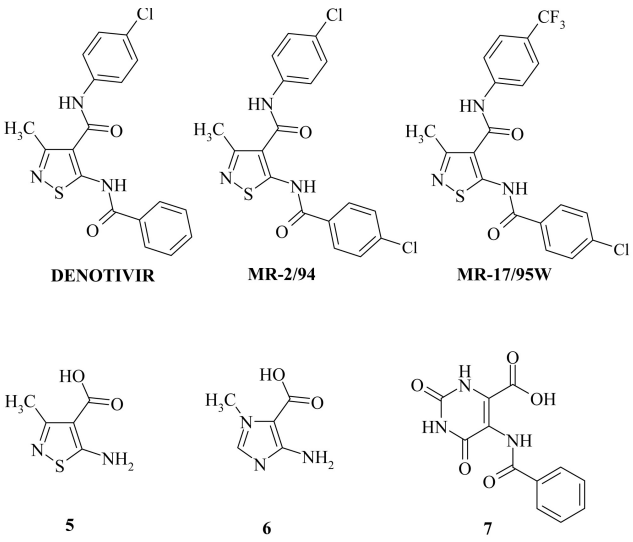

Figure 1. 5-amino-3-methyl-4-isothiazole carboxylic acid derivatives and related compounds described previously.

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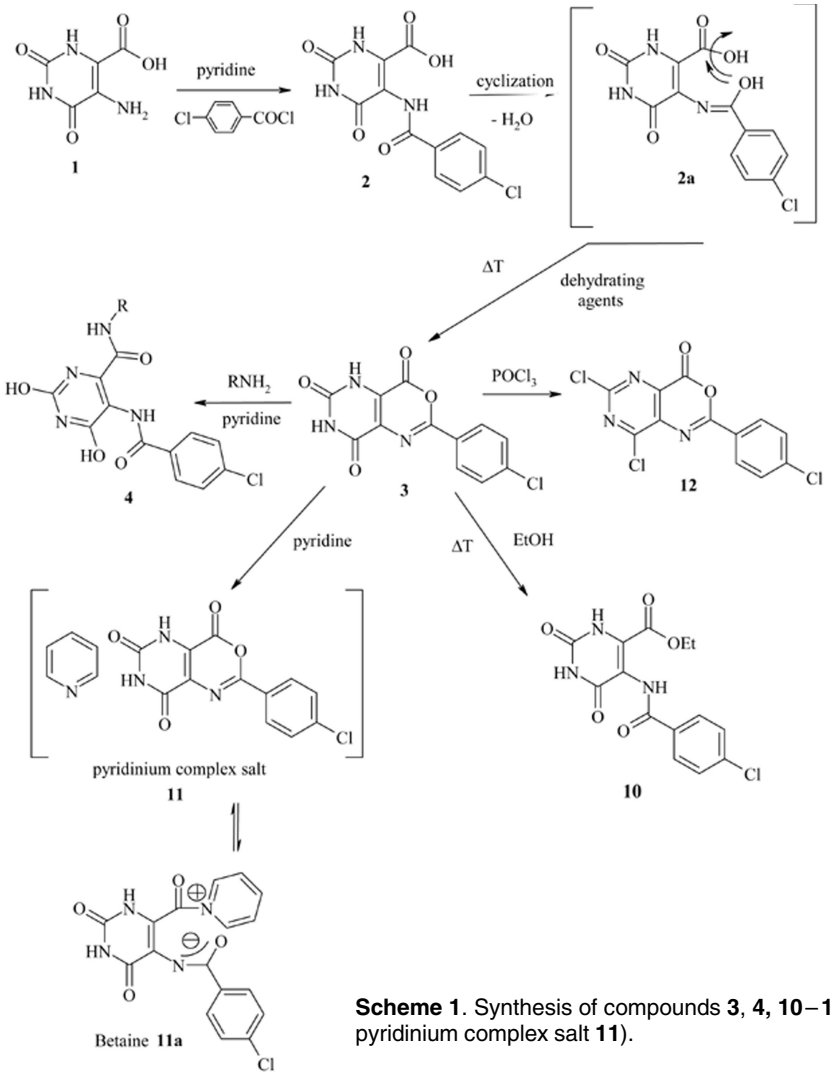
Abbreviations: peripheral blood mononuclear cells (PBMCs); phytohemagglutinin (PHA)

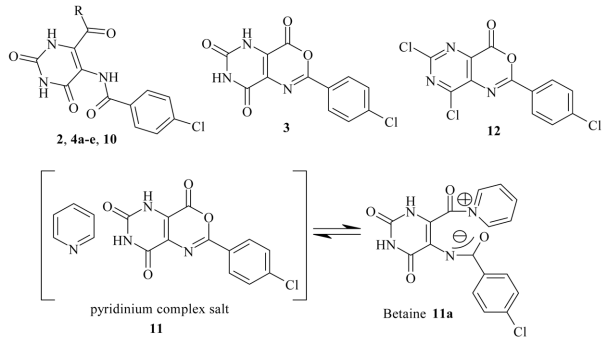


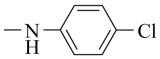
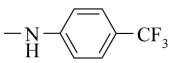
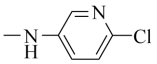
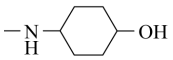
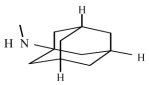
Scheme 1. Synthesis of compounds **3**, **4, 10**–**12**. (oxazinones **3**, **12**, amides **4a**–**e,** ester **10** and pyridinium complex salt **11**).

ine A, leflunomide, and ibuprofen, for example the compounds designated as **MR-2/94** and **MR-17/95W**, *i. e.* 5-(4-chlorobenzoyl)amino-*N*-(4-chlorophenyl)-3-methyl-4-isothiazolecarboxamide and 5-(4-chlorobenzoyl)amino-*N*-(4-trifluoromethylphenyl)-3-methyl-4-isothiazolecarboxamide, respectively [1, 2, 4, 7, 13] (see Fig. 1). It has been shown that the amides of the 5-(4-chloro)benzoyl series of derivatives of 5-amino-3-methyl-4-isothiazolecarboxylic acid **5** should be better anti-inflammatory agents than the non-substituted 5-benzoyl series [1]. In a previous study [13], the influence of exchanging the isothiazole for an imidazole core ring system on biological activity was dealt with on the example of 4-amino-1-methyl-5-imidazolecarboxylic acid **6** derivatives (Fig. 1). On the basis of biological tests carried out previously for **MR-2/94** [1, 2, 4, 7] and presented in [13], it was suggested that the isothiazolecarboxamide derivatives are only slightly more active than the structurally related imidazolecarboxamide derivatives of 4-amino-1-methyl-5-imidazolecarboxylic acid **6**, despite the heteroaromatic core ring replacement. The results also indicated a considerable lowering of toxicity while maintaining anti-inflammatory and immunotropic properties. This strongly suggests that the presence of the isothiazole ring system may not be a crucial factor for the anti-inflammatory and immunotropic activity of *N*-(4-chorobenzoyl)amino-*N*-substituted heterocyclecarboxamides.

Because of the considerations described above and data that some of the previously described and biologically tested amides of 5-benzoylaminoorotic acid **7** (Fig. 1) [14–16] showed anti-inflammatory and analgesic activity, the main aim of this study was to investigate the influence of replacing the five-membered heterocycle-isothiazole by a six-membered pyrimidine core ring on the biological activity of heterocyclic carboxamides (5-(4-chlorobenzoyl)amino-2,6-dihydroxy-*N*-substituted-4-pyri-

Table 1. Physical constants and analytical data of compounds **2** – **4**, and **10** – **12**.



Compound	R	Formula M. wt. (g/mol)	Elemental analysis (calculated / found) (%)		
			C	H	N
2	– OH	$C_{12}H_8N_3O_5Cl$	43.99[a)]	3.08[a)]	12.82[a)]
		309.67 (327.69[a])	44.06	3.24	12.61
3	–	$C_{12}H_6N_3O_4Cl$	49.42	2.07	14.41
		291.66	49.03	2.13	14.28
4a		$C_{18}H_{12}N_4O_4Cl_2$	51.57	2.89	13.36
		419.24	51.36	3.11	13.75
4b		$C_{19}H_{12}N_4O_4ClF_3$	50.40	2.67	12.37
		452.78	50.10	2.46	12.41
4c		$C_{17}H_{11}N_5O_4Cl_2$	48.59	2.64	16.67
		420.22	48.38	2.40	16.97
4d		$C_{18}H_{19}N_4O_5Cl$	53.14	4.71	13.77
		406.83	53.31	5.01	13.40
4e		$C_{22}H_{23}N_4O_4Cl$	59.66	5.23	12.65
		442.91	59.27	5.35	12.69
10	– O – CH_2CH_3	$C_{14}H_{12}N_3O_5Cl$	49.79	3.58	12.44
		337.73	49.86	3.80	12.24
11, **11a**	–	$C_{17}H_{11}N_4O_4Cl$	55.07	2.99	15.11
		370.76	54.76	2.67	15.15
12	–	$C_{12}H_4N_3O_2Cl_3$	43.87	1.23	12.79
		328.56	43.79	1.41	12.90

[a)] Data for monohydrate $C_{12}H_8ClN_3O_5 \cdot H_2O$.

midinecarboxamides **4**) under consideration possessing those substituents, which have been proven to be advantageous in the 5-amino-3-methyl-4-isothiazolecarboxylic acid **5** derivatives series.

Results and discussion

Chemistry

The new amides **4a** – **e** (Table 1) were prepared using the methods outlined in Scheme 1. The starting material used in the synthesis was the 5-aminoorotic acid, *i. e.* 5-amino-2,6-dioxo-1,2,3,6-tetrahydro-4-pyrimidinecarboxylic acid **1** [17], which had given the previously described 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid **2** in reaction with 4-chlorobenzoyl chloride in pyridine solution [14]. Compound **2** was transformed into a semianhydride-type compound, 2-(4-chlorophenyl)-6,8-dihydroxypyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **3**, under the influence of dehydrating reagents such as $SOCl_2$ in solvents such as benzene, toluene, and carbon tetrachloride. The single, very strong band of the stretching oscillations of the carbonyl group at 1770 cm^{-1} and the medium sharp band of the stretching oscillations of

Table 2. IR (KBr tablets) data of the compounds **2**–**4**, and **10**–**12**.

Compound	ν (cm^{-1})
2	3450 (ν_{-NH}, ν_{-OH} medium sharp band, intramolecular hydrogen bond), 3330 (ν_{-NH}, ν_{-OH} medium sharp band, intramolecular hydrogen bond), 3220–3140 (ν_{-OH}, ν_{-NH}), 3500–2400 (ν_{-COO-H}), 1710–1660 ($\nu_{-C=O}$), 1595, 1585 ($\nu_{-C=N-, -C=C-}$)
3	3600–3340, 3180, 3100 (ν_{-OH}, ν_{-NH}), 1770 ($_{-C=O}$, very strong, α,β,δ,γ-unsaturated lactone carbonyl), 1720–1710, 1675 (ν_{-CO-}, strong, pyrimidinon ring carbonyls), 1615, 1585 ($\nu_{-C=N-, -C=C-}$, strong)
4a	3650–3400, 3280, 3200 ($\nu_{-OH,}\nu_{-NH}$),1730, 1660 ($\nu_{-C=O}$), 1600, 1590 ($\nu_{-C=N-, -C=C-}$)
4b	3600–3350, 3270, 3200 ($\nu_{-OH,}\nu_{-NH}$), 1730, 1670, 1650 ($\nu_{-C=O}$), 1605, 1590 ($\nu_{-C=N-, -C=C-}$)
4c	3650–3390, 3260, 3180 ($\nu_{-OH,}\nu_{-NH}$), 1727, 1700, 1680, 1660 ($\nu_{-C=O}$), 1605–1590 ($\nu_{-C=N-, -C=C-}$)
4d	3650–3100 ($\nu_{-OH,}\nu_{-NH}$, medium strong, very broad), 2950 (ν_{-C-H}, medium), 2865 (ν_{-C-H}, weak to medium), 1730 ($\nu_{-C=O}$, very strong), 1700–1650 ($\nu_{-C=O}$, very strong, broad), 1600, 1590 ($\nu_{-C=N-, -C=C-}$ medium)
4e	3650–3100 ($\nu_{-OH,}\nu_{-NH}$, strong, very broad), 2925 (ν_{-C-H2-}, strong), 2865 (ν_{-C-H}, strong), 2820 (ν_{-C-H}, medium), 1745 ($\nu_{-C=O}$, very strong), 1700–1650 ($\nu_{-C=O}$, very strong, broad), 1600, 1590 ($\nu_{-C=N-, -C=C-}$ medium)
10	3650–3400 ($\nu_{-OH,}\nu_{-NH}$, strong broad band), 3300 ($\nu_{-OH,}\nu_{-NH}$,sharp and very strong), 3260, 3180($\nu_{-OH,}\nu_{-NH}$), 2820 ($\nu_{-CH3, -CH2}$, weak), 1730 ($\nu_{-C=O}$ very strong), 1700 ($\nu_{-C=O}$ very strong), 1675 ($\nu_{-C=O}$ very strong), 1650 ($\nu_{-C=O}$, medium), 1600 ($\nu_{-C=N-, -C=C-}$), 1190 ($\nu_{-C-O-C=O}$, asymmetric, strong) 1050, 1010 (ν_{-C-O-C} symmetric, very strong)
11, **11a**	3600–3350 ($\nu_{-OH,}\nu_{-NH}$ weak), 3195 ($\nu_{-OH,}\nu_{-NH}$ medium), 3060 ($\nu_{=C-H}$ medium), 2830, 2700-2300($\nu_{-HN^{+}=C-H}$ weak to medium, very broad), 1750, 1690 ($\nu_{-C=O}$ very strong), 1630–1590 (strong), 1530, 1485 ($\nu_{-C=N^{+}-, -C=C-}$ medium)
12	3080–3020 ($\nu_{=C-H}$ aromatic ring, weak), 1775 ($\nu_{-C=O}$, strong, α,β,δ,γ-unsaturated lactone carbonyl), 1615, 1585 ($\nu_{-C=N-, -C=C-}$, very strong)

the ring imine bond at 1615 cm^{-1} appeared in the IR spectrum (see Table 2). Two medium sharp signals at 3450 and 3330 cm^{-1} of the stretching oscillations of the intramolecular hydrogen bonds (NH, OH) completely vanished and the broad band in the range of 2400–3300 cm^{-1} of carboxyl group disappeared. The strong stretching oscillation at 1660 cm^{-1} of the carbonyl 4-chlorobenzoylamino group at pyrimidine ring position 5 also vanished. However, the valence stretching oscillations of the pyrimidinone ring carbonyls in the 1720–1675 cm^{-1} range remained unchanged. Moreover, these valence stretching oscillations of the pyrimidinone ring carbonyls in the 1720–1675 cm^{-1} range in IR spectrum completely vanished after replacing of carbonyl oxygen groups of the pyrimidinone ring with chlorine substituents (see compound **12** in Scheme 1, Table 2, and Experimental, Section 4). After chlorination of oxazinonone **3**, the single, very strong band of the stretching oscillations of the oxazinon carbonyl group at 1770 cm^{-1} remain practically unchanged except the vibration intensity that slightly lowered. The chlorination also caused the sharp band of the stretching vibrations of the imine bond (-C=N-) and aromatic (-C=C-) with maximum at 1615 cm^{-1} amplified itself (from medium to strongest one in the spectrum) and broadened in the 1575–1620 cm^{-1} range. There is the lack of other bands of vibrations between 1620–2800 cm^{-1} and above 3080 cm^{-1} in the IR spectrum of the chloroderivative **12** (Table 2). The 6,8-dihydroxypyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **3** has been easily transformed into 6,8-dichloropyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **12** under phosphoryl oxychloride ($POCl_3$) influence. These spectral parameters support a semianhydride structure of compound **3**. Oxazinone **3**

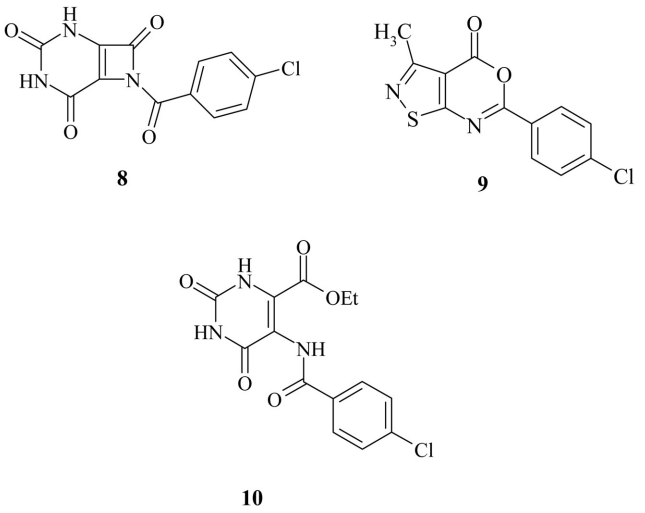


Figure 2. Substances **8**, **9**, and **10**.

was created from **2** as a result of intramolecular dehydration of the imide form **2a** under the influence of dehydrating reagents. The mechanism of intramolecular dehydration of the imide form **2a** of 5-(4-chlorobenzoyl)amino acid **2** is probably similar to that initially described for acyl antranilic acid cyclodehydration [18, 19] as well as that for the corresponding 5-acylamino-4-isothiazolecarboxylic acid derivatives [1, 8]. 2-(4-Chlorophenyl)-6,8-dihydroxypyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on has been obtained in [14], but the interpretation of its structure there was incorrect. At that time, it was incorrectly interpreted and presented as 5-(4-chlorophenyl)-2,4-dihydoxy-6-oxoazetino[3,2-*d*]pyrimidine **8** (lactam) [14] (Fig. 2). Its

Table 3. ^{1}H-NMR and ^{13}C-NMR data of the compounds **2** – **4**, and **10** – **12**.

Compound	^{1}H-NMR, chemical shift: [ppm]	^{13}C-NMR, chemical shift: [ppm]
2	(DMSO-d_6, Bruker, 300.15 MHz): δ = 7.57 (d, J = 8.6 Hz, 2H, 3,5 – H, 4-chlorobenzoyl), 7.91 (d, J = 8.6 Hz, 2H, 2,6-H, 4-chlorobenzoyl), 9.65 (s, 1H, N*H* of 4-chlorobenzamide group), 11.05 (s, 1H, N*H* or O*H*, sharp henol-lactamic), 11.62 (s, 1H, N*H* or O*H*, sharp phenol-lactamic) ppm.	(DMSO-d_6, Bruker, 75.47 MHz): = 111.07 (quaternary carbon C5 of pyrimidine ring), 128.48 (C3, C5 of 4- chlorobenzoyl ring), 129.57 (C2, C6 of – chlorobenzoyl ring), 132.39 (quaternary carbon atom C1 of 4- chlorobenzoyl ring), 136.57 (quaternary carbon atom C4 of 4- chlorobenzoyl ring), 139.20 (quaternary carbon C4 of pyrimidine ring), 149.60 (carbonyl carbon atom C2 of pyrimidine ring), 161.75(carbonyl carbon atom C6 of pyrimidine ring), 161.84 (carbonyl carbon atom of 4-chlorobenzoyl group), 164.84 (carbonyl carbon atom of carboxylic group) ppm.
3	(DMSO- d_6, Bruker, 300.15 MHz): δ = 7.65 (d, J = 8.7 Hz, 2H, 3,5-H of 4-chlorophenyl ring), 8.07 (d, J = 8.7 Hz, 2H, 2,6-H of 4-chlorophenyl ring), 11.80 (s, 1H, N*H* or O*H*, phenollactamic), 11.83 (s, 1H, N*H* or O*H*, sharp and high phenollactamic) ppm.	(DMSO-d_6, Bruker, 75.47 MHz): = 126.43 (quaternary carbon C5 of pyrimidine ring), 128.22 (quaternary carbon atom C1 or C4 of 4-chlorophenyl ring), 128.80 (quaternary carbon atom C4 or C1 of 4-chlorophenyl ring), 129.05 (C2, C6 of 4-chlorophenyl ring), 129.27 (C3, C5 of-chlorophenyl ring), 137.34 (quaternary carbon C4 of pyrimidine ring), 149.32 (carbonyl carbon atom C2 of pyrimidine ring), 153.36 (quaternary carbon atom C2 of oxazinon ring), 154.15 (carbonyl carbon atom C4 of oxazinon ring), 160.34 (carbonyl carbon atom C6 of pyrimidine ring) ppm.
4a	(DMSO- d_6, Bruker, 300.13 MHz): δ = 7.35(d, J = 8.7 Hz, 2H, 3,5 -H 4-chlorophenyl), 7.51(d, J = 8.7 Hz, 2H, 3,5 - H 4-chlorobenzoyl), 7.55 (d, J = 8.7 Hz, 2H, 2,6-H 4-chlorophenyl), 7.82 (d, J = 8.4 Hz, 2H, 2,6-H 4-chlorobenzoyl), 8.53 (s, 1H, NH, carboxamide group at pyrimidine position 4), 9.55 (s, 1H, -NH, amide group at pyrimidine position 5), 10.74 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring), 11.6 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring) ppm.	(DMSO-d_6, Bruker, 75.47 MHz): = 108.47 (quaternary carbon C5 of pyrimidine ring), 121.34 (C2, C6 of 4-chlorophenyl ring), 127.90 (quaternary carbon atom C1 of 4-chlorobenzoyl ring), 128.42 (C2, C6 of 4- chlorobenzoyl ring), 128.709 (C3,C5 of 4-chlorophenyl ring), 129.49 (C3, C5 of 4-chlorobenzoyl ring), 132.27 (quaternary carbon atom C4 of 4- chlorophenyl ring), 136.52 (quaternary carbon atom C1 of 4- chlorophenyl ring) 136.87 (quaternary carbon atom C4 of 4- chlorobenzoyl ring), 144.36 (quaternary carbon C4 of pyrimidine ring), 150.34 (carbonyl carbon atom C2 of pyrimidine ring), 158.54 (carbonyl carbon atom C6 of pyrimidine ring), 161.85 (carbonyl carbon atom of 4-chlorobenzoyl group), 165.43 (carbonyl carbon atom of carboxamide group at 4th pyrimidine ring position) ppm.
4b	(DMSO-d_6, Bruker, 300.13 MHz): δ = 7.50 (d, J = 7.8 Hz, 2H, 3,5 -H 4-chlorobenzoyl), 7.66 (d, J = 8.4 Hz, 2H, 3,5 -H 4-trifluoromethylphenyl), 7.74 (d, J = 8.4 Hz, 2H, 2,6-H 4-trifluoromethylphenyl), 7.82 (d, J = 7.8 Hz, 2H, 2,6-H 4-chlorobenzoyl), 9.59 (s, 1H, -NH, amide group at pyrimidine position 5), 10.99 (s, 1H, NH, carboxamide group at pyrimidine position 4), 11.60 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring), 11.66 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring) ppm.	(DMSO-d_6, Bruker, 75.47 MHz): = 108.64 (quaternary carbon C5 of pyrimidine ring), 119.78 (C2, C6 of 4-chlorophenyl ring), 126.07, 126.12 (C3,C5 of 4-chlorophenyl ring), 128.41 (C2, C6 of 4- chlorobenzoyl ring),129.49 (C3, C5 of 4-chlorobenzoyl ring), 132.16 (quaternary carbon atom C4 of 4- chlorophenyl ring), 136.54 (quaternary carbon atom C4 of 4- chlorobenzoyl ring), 141.47 (quaternary carbon atom C1 of 4-chlorophenyl ring), 143.61 (quaternary carbon C4 of pyrimidine ring), 149.97 (carbonyl carbon atom C2 of pyrimidine ring), 158.74 (carbonyl carbon atom C6 of pyrimidine ring), 161.70 (carbonyl carbon atom of 4-chlorobenzoyl group), 165.41 (carbonyl carbon atom of carboxamide group at 4th pyrimidine ring position) ppm.
4c	(DMSO- d_6, Bruker, 300.13 MHz): δ = 7.49 (d, J = 8.8 Hz, 1H, H3' pyridyl), 7.53 (d, J = 8.2 Hz, 2H, 3,5-H 4-chlorobenzoyl), 7.85 (d, J = 8.2 Hz, 2H, 2,6-H p-chlorobenzoyl), 8.02 (dd, J_1 = 8.7 Hz, J_2 = 2.0 Hz, 1H, H4' pyridyl), 8.56 (d, J = 2.0 Hz, 1H, H6' pyridyl), 9.62 (s, 1H, -NH, amide group at pyrimidine position 5), 11.03 (s, 1H, NH, carboxamide group at pyrimidine position 4), 11.55 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring), 11.64 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring) ppm.	(DMSO-d_6, Bruker, 75.47 MHz): = 108.85 (quaternary carbon C5 of pyrimidine ring), 124.39 (ß-C of pyridine fragment), 128.42 (C2, C6 of 4-chlorobenzoyl ring), 129.51 (C3, C5 of 4-chlorobenzoyl ring), 130.44 (-C of pyridinium fragment), 132.16, 134.29, 136.57, 140.88 (quaternary carbon atoms of pyridine and 4-chlorobenzoyl groups), 143.03 (carbonyl carbon atom C2 of pyrimidine ring), 144.64 (quaternary carbon C4 of pyrimidine ring), 149.89 (a-C of pyridine fragment), 158.81 (carbonyl carbon atom C6 of pyrimidine ring), 161.61 (carbonyl carbon atom of 4-chlorobenzoyl group), 165.32 (carbonyl carbon atom of carboxamide group at 4th pyrimidine ring position) ppm.

Table 3. Continued.

Compound	^{1}H-NMR, chemical shift: [ppm]	^{13}C-NMR, chemical shift: [ppm]
4d	(DMSO-d_6, Bruker, 500.13 MHz): δ = 1.10-1.70 (m, 9H, protons at position 2,3,4,5,6 of 4-hydroxycyclohexyl ring), 4.35 (m, 1H, N-CH<, proton at position 1 of 4-hydroxycyclohexyl ring), 7.57 (d, J = 8.1 Hz, 2H, 3,5-H of 4-chlorobenzoyl), 7.89 (d, J = 8.1 Hz, 2H, 2,6-H of 4-chlorobenzoyl group), 8.16 (2s, 2 6 0.5H, -NH of both *cis* and *trans* isomers, carboxamide group at pyrimidine position 4), 9.40 (s, 1H, -NH, amide group at pyrimidine position 5), 11.25 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring), 11.46 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring) ppm.	DMSO-d_6, Bruker, 75.47 MHz): = 26.53, 29.50, 30.76, 33.36 (methylene carbons of 4-hydroxycyclohexyl ring, equimolar *cis* and *trans* isomers), 47.607, 46.772, (-NH-C1 carbon of 4-hydroxycyclohexyl ring), 64.281, 67.746 (HO-C4 carbon of 4-hydroxycyclohexyl ring), 107.70, 107.77 (quaternary carbon C5 of pyrimidine ring), 128.60 (C2, C6 of 4- chlorobenzoyl ring), 129.56 (C3, C5 of 4-chlorobenzoyl ring), 132.17, 132.20 (quaternary carbon atom C1 of 4-chlorobenzoyl ring), 136.77 (quaternary carbon atom C4 of 4- chlorobenzoyl ring), 145.24, 145.41 (quaternary carbon C4 of pyrimidine ring), 150.214 (carbonyl carbon atom C2 of pyrimidine ring), 158.81, 158.91 (carbonyl carbon atom C6 of pyrimidine ring), 162.00, 162.05 (carbonyl carbon atom of 4-chlorobenzoyl group), 165.77, 165.96 (carbonyl carbon atom of carboxamide group at 4th pyrimidine ring position) ppm.
4e	(DMSO-d_6, Bruker, 500.13 MHz): δ = 1.47 (m, 12H, protons of adamantyl ring), 1.92 (m, 3H, protons of adamantyl ring), 7.58 (s, 1H, -NH, carboxamide group at pyrimidine position 4), 7.60 (d, J = 8.5 Hz, 2H, 3,5-H of 4-chlorobenzoyl), 7.92 (d, J = 8.5 Hz, 2H, 2,6-H of 4-chlorobenzoyl group), 9.48 (s, 1H, -NH, amide group at pyrimidine position 5), 11.30 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring), 11.44 (s, 1H, N*H* or O*H*, phenol-lactamic of 2,6-dihydroxypyrimidine ring) ppm.	DMSO-d_6, Bruker, 75.47 MHz): = 28.61 (bridging carbon atoms of adamantyl moiety), 35.77 (rear methylene carbons of adamantyl moiety), 40.52 (front methylene carbons of adamantyl moiety), 51.98 (quaternary carbon C1 of adamantyl moiety) 107.19 (quaternary carbon C5 of pyrimidine ring), 128.64 (C2, C6 of 4-chlorobenzoyl ring), 129.52 (C3, C5 of 4-chlorobenzoyl ring), 132.15 (quaternary carbon atom C1 of 4-chlorobenzoyl ring), 136.83 (quaternary carbon atom C4 of 4- chlorobenzoyl ring), 145.84 (quaternary carbon C4 of pyrimidine ring), 150.24 (carbonyl carbon atom C2 of pyrimidine ring), 158.62 (carbonyl carbon atom C6 of pyrimidine ring), 162.18 (carbonyl carbon atom of 4-chlorobenzoyl group), 166.08 (carbonyl carbon atom of carboxamide group at 4th pyrimidine ring position) ppm.
10	(DMSO-d_6, Bruker, 300.15 MHz): δ = 1.07 (t, J = 7.1 Hz, 3H, methyl group protons of C*H*$_3$CH$_2$-), 4.15 (q, J = 7.1 Hz, 2H, methylen group protons of CH$_3$C*H*$_2$-), 7.58 (d, J = 8.5 Hz, 2H, 3,5 -H 4-chlorobenzoyl), 7.92 (d, J = 8.5 Hz, 2H, 2,6-H 4-chlorobenzoyl), 9.73 (s, 1H, -N*H*, high and sharp, 4-chlorobenzamide group at pyrimidine position 5), 11.70 (s, 1H, N*H* or O*H*, low and broad, phenol-lactamic of 2,6-dihydroxypyrimidine ring) ppm.	(DMSO-d_6, Bruker, 75.47 MHz): = 13.67 (methyl carbon of ester group), 63.38 (methylen carbon of ester group), 111.64 (quaternary carbon C5 of pyrimidine ring), 128.64 (C3, C5 of 4- chlorobenzoyl ring), 129.65 (C2, C6 of – chlorobenzoyl ring), 132.25 (quaternary carbon atom C1 of 4- chlorobenzoyl ring), 136.83 (quaternary carbon atom C4 of 4- chlorobenzoyl ring), 137.53 (quaternary carbon C4 of pyrimidine ring), 149.64 (carbonyl carbon atom C2 of pyrimidine ring), 160.50 (carbonyl carbon atom C6 of pyrimidine ring), 161.78 (carbonyl carbon atom of 4-chlorobenzoyl group), 165.02 (carbonyl carbon atom of ester group) ppm.
11, **11a**	(DMSO- d_6, Bruker, 300.15 MHz): δ = 7.38 (dd, J_1 = 5.7 Hz, J_2 = 7.7 Hz, 2H, ß-H of pyridinium fragment), 7.64 (d, J = 8.6 Hz, 2H, 3,5-H 4-chlorophenyl), 7.78 (m, J_1 = 7.7 Hz, J_2 = 1.8 Hz, 1H, -H of pyridinium fragment), 8.06 (d, J = 8.6 Hz, 2H, 2,6-H 4-chlorophenyl), 8.56 (d, J = 5.7 Hz, 2H, a-H of pyridinium fragment), 11.84 (s, 2H, N*H* or O*H*, sharp with broad base, phenol-lactamic of 2,6-dihydroxypyrimidine ring) ppm.	(DMSO-d_6, Bruker, 75.47 MHz): = 123.91 (ß-C of pyridinium fragment), 126.48 (quaternary carbon C5 of pyrimidine ring), 128.26 (quaternary carbon atom C4 of 4-chlorophenyl ring), 128.85 (quaternary carbon atom C1 of 4-chlorophenyl ring), 129.11 (C2, C6 of 4-chlorophenyl ring), 129.31 (C3, C5 of -chlorophenyl ring), 136.20 (-C of pyridinium fragment), 137.40 (quaternary carbon C4 of pyrimidine ring), 149.39 (carbonyl carbon atom C2 of pyrimidine ring), 149.51 (a-C of pyridinium fragment), 153.43 (quaternary carbon atom C2 of oxazinon ring), 154.21 (carbonyl carbon atom C4 of oxazinon ring), 160.42 (carbonyl carbon atom C6 of pyrimidine ring) ppm.
12	($CDCl_3$, Bruker, 300.15 MHz): δ = 7.54 (d, J = 8.7 Hz, 2H, 3,5-H of 4-chlorophenyl ring), 8.27 (d, J = 8.7 Hz, 2H, 2,6-H 4-chlorophenyl ring)	

physicochemical properties have been very poorly researched and documented to date.

Pyrimidooxazinone **3** was used to obtain the diamides **4a**–**e** as a result of a nucleophilic attack of the appropriate amine at the carbon atom of the carbonyl group in the oxazinone ring (Scheme 1). The reactions were conducted mainly in anhydrous pyridine solutions. Pyridine, as in the case of 6-(4-chlorophenyl)-3-methylisothia-

Table 4. MS-ESI (Mass Spectrum-Electrospray Ionization) data of the compounds **3–4, 10–12**.

Compound	Calculated value of the parent peak mass (a.m.u.)[a]	m/z [Th][b] (%)[c]
3	291.004685 (for the formula $C_{12}H_6ClN_3O_4$)	(negative ionization, −Q1MS in methanol): 322.0(100) $[M+MeOH-H]^-$- base peak, 292.0 (0.13) $[M+2-H]^-$ – isotope peak, 290.0 (3) $[M-H]^-$ – quasi-molecular ion peak, 279.0 $[M+MeOH, -CO_2]^-$, 228.0 (5) $[M-CO, -Cl]^-$
4a	418.023562 (for the formula $C_{18}H_{12}N_4O_4Cl_2$)	(negative ionization, −Q1MS in methanol): 419.9 (18) $[M+4-H_2]^{-\bullet}$ – isotope peak, 417.8(86) $[M+2-H_2]^{-\bullet}$ – isotope peak, 416.1(100) $[M-H_2]^{-\bullet}$ – quasi-molecular ion and base peak.
4b	452.049925 (for the formula $C_{19}H_{12}N_4O_4ClF_3$)	(negative ionization, −Q1MS in methanol): 452.1 (38) $[M+2-H_2]^{-\bullet}$ – isotope peak, 451.2(36) $[M+1-H_2]^{-\bullet}$ – isotope peak, 450.1(100) $[M-H_2]^{-\bullet}$ – quasi-molecular ion and base peak.
4c	419.018811 (for the formula $C_{17}H_{11}N_5O_4Cl_2$)	(negative ionization, −Q1MS in methanol): 421.8 (15) $[M+4-H]^-$ – isotope peak, 419.8 (73) $[M+2-H]^-$ – isotope peak, 417.8 (100) $[M-H]^-$ – quasi-molecular ion and base peak.
4d	406.104402 (for the formula $C_{18}H_{19}N_4O_5Cl$)	(negative ionization, −Q1MS in methanol): 408.1 (2) $[M+4-H]^-$ – isotope peak, 407.1 (31) $[M+3-H]^-$ – isotope peak, 406.1 (12) $[M+1-H]^-$ – isotope peak, 405.1 (100) $[M-H]^-$ – quasi-molecular ion and base peak
4e	442.140788 (for the formula $C_{22}H_{23}N_4O_4Cl$)	(negative ionization, −Q1MS in methanol): 444.1 (3) $[M+4-H]^-$ – isotope peak, 443.1 (36) $[M+3-H]^-$ – isotope peak, 442.1 (15.5) $[M+1-H]^-$ – isotope peak, 441.1 (100) $[M-H]^-$ – quasi-molecular ion and base peak.
10	337.046551 (for the formula $C_{14}H_{12}ClN_3O_5$)	(negative ionization, −Q1MS in methanol): 339(1.5) $[M+3-H]^-$ isotope peak, 338(27) $[M+2-H]^-$ isotope peak, 337(13) $[M+1-H]^-$ isotope peak, 336(100) $[M-H]^-$ – quasi-molecular ion and base peak
11, 11a	370.046886 (for the formula $C_{17}H_{11}N_4O_4Cl$)	(positive ionization, +Q1MS in methanol): 370.0(3) $[M+2-H_2]^{+\bullet}$ – isotope peak, 368.0 (21) $[M-H_2]^{+\bullet}$ – quasi-molecular ion peak, 346 (100)- base peak (negative ionization, −Q1MS in methanol): 324.0(21) $[M+2+MeOH-H-C_5H_5N$ (pyridine moiety)$]^-$, 322.0(100) $[M+MeOH-H-C_5H_5N$ (pyridine moiety)$]^-$, 292.0 (0.13) $[M+2-H]^-$ – isotope peak, 290.0 (3) $[M-H-C_5H_5N$ (pyridine moiety)$]^-$, 279.0 $[M+MeOH,-CO_2]^-$, 228.0 (5) $[M-CO, -Cl]^-$
12	326.936907 (for the formula $C_{12}H_4Cl_3N_3O_2$)	(negative ionization, -Q1MS in methanol/water mixture): = 362(7) $[M+4+MeOH-H]^-$ – isotope peak, 359.9 (46) $[M+2+MeOH-H]^-$ – isotope peak, 358(44) $[M+MeOH-H]^-$, 348(12) $[M+4+H_2O-H]^-$ isotope peak, 345.9 (100) $[M+2+H_2O-H]^-$ isotope and base peak, 344 (90) $[M+H_2O-H]^-$, 326 (2) $[M-H]^-$ – quasi-molecular ion peak

[a] a.m.u. is atom mass unit., [b] mass ion (m) to ion charge (z) ratio expressed in Th (Thomson) unit, [c] value in parenthesis means the relative intensities of peaks expressed in percents.

zolo[5,4-*d*]-4*H*-1,3-oxazin-4-one **9** [1], proved to be a much better solvent for this type of nucleophilic reaction because of its catalytic properties [20], particularly for the much less reactive aromatic amines such as 4-chloroaniline, 4-trifluoromethylaniline, and 5-amino-2-chloropyridine. Application of pyridine significantly increased the reaction yield, and in the case of weak aromatic amines such as 4-trifluoromethylaniline and 5-amino-2-chloropyridine, it made the reaction occur at all. Ethanol, used previously as a solvent for reactions with amines, appeared useless in these cases because the previously reported and described ethyl ester of 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid **10** (Fig. 2) was also created as a side product [14]. Long heating (10 h) of pyrimidooxazinone **3** with nothing but anhydrous ethanol caused the creation of ethyl ester of 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid **10** (Scheme 1) with a very high yield (see Experimental, Section 4). This is in contrary to the earlier described 6-(4-chlorophenyl)-3-methylisothiazolo[5,4-*d*]-4*H*-1,3-oxazin-4-one **9**, in which case even long heating of isothiazolooxazinone **9** with anhydrous ethanol did not cause any transformation of substrate **9** [1]. This is probably connected with the different electron-density set of the compared oxazines **3** and **9** on oxazinon carbonyl carbon. Pyrimidooxazine **3** is also more reactive because there is no steric hindrance close to the carbonyl group under consideration, in contrast to isothiazolooxazine [1], where the methyl group could repulse approaching nucleophilic agents. This huge difference of the reac-

tivity and chemical durability of both these oxazines **3** and **9** is also supported by electrospray MS spectra (see Table 4) recorded in methanolic (or methanol/water mixture) solution conditions. The quasi-molecular parent peak was observed with 100% relative (base peak) intensity in case of isothiazolooxazine **9** [1], while in case of pyrimidooxazines **3** and **12** no quasi-molecular peak was observed or this peak had very, very low intensity (2–3%) (see Table 4). It is also interesting that the phenolo-lactamic protons of the pyrimidine ring of 2-(4-chlorophenyl)-6,8-dihydroxy-4*H*-pyrimido[5,4-*d*]-1,3-oxazin-4-on **3** are acidic enough to give a pyridinium complex salt-type compound **11** (this could be a charge-transfer type compound, donor-acceptor complex) (Scheme 1). The compound **11** forms yellow needle crystals (see Experimental, Section 4) which are stable enough to separate but which are sensitive to water and water vapor, which cause hydrolyzation after some time, and then decompose to the starting 2-(4-chlorophenyl)-6,8-dihydroxy-4*H*-pyrimido[5,4-*d*]-1,3-oxazin-4-on **3** and 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid **2**. The structure of **11** can also be explained as a betaine-type compound **11a** (Scheme 1). In the case of isothiazolooxazinon, the betaine intermediate was too reactive and has not been liberated [1]. More results of research on the reactivity of the 2-(4-chlorophenyl)-6,8-dihydroxy-4*H*-pyrimido[5,4-*d*]-1,3-oxazin-4-on **3** will be presented in subsequent papers.

The structures of the compounds **2**–**4**, **11**, and **12** have been proven by IR (Table 2), ^{1}H-NMR and ^{13}C-NMR (Table 3), and MS (Table 4) spectroscopic techniques and elemental analysis (see Table 1).

Pharmacology

The biological activities of 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxamides **2** were tested in preliminary biological studies *in vitro* for their influence on the secretion of two pro-inflammatory cytokines, tumor necrosis factor α (TNF-α), and interleukin-6 (IL-6), by human peripheral blood mononuclear cells (PBMCs), the production of nitric oxide by rat peritoneal macrophages, erythrocyte membrane stabilization, and lymphocyte proliferation.

MR-2/94, *i. e.* 5-(4-chlorobenzoyl)amino-*N*-(4-chlorophenyl)-3-methyl-4-isothiazolecarboxamide, was chosen as the reference isothiazole for comparison because this compound proved to be one of the most active isothiazoles (in immunosuppressive and anti-inflammatory tests) and is structurally similar to the tested 4-pyrimidinecarboxamides **2** [1, 2], and leflunomide, a drug that is known for its immunosuppressive, and anti-inflammatory activity, was also used as a reference in these tests [21].

Table 5. Lymphocytes proliferation inhibition by tested amides **4a**–**e** and reference **MR-2/94**.

Compound	Inhibition of PMBCs proliferation IC_{50} (μM)[a]		
	Non-stimulated	Stimulated with PHA[b]	Stimulated with ConA[c]
4a	n.e.[d]	79.1 ± 5.2	408.8 ± 150.8
4b	n.e.[d]	57.6 ± 8.5	88.1 ± 11.3
4c	n.e.[d]	n.e.[d]	n.e.[d]
4d	n.e.[d]	n.e.[d]	n.e.[d]
4e	139.8 ± 65.4	49.1 ± 10.6	50.8 ± 15.4
MR-2/94	22.7 ± 1.8	1.6 ± 0.4	0.6 ± 0.2

[a] 50% inhibition concentration, ± SE.
[b] PHA – phytohemagglutinin.
[c] ConA – concanavalin A.
[d] Value very high or difficult to estimate (not estimated).

Influence of the tested diamides 4a-e on inhibition of proinflammatory cytokine production by PBMCs

The activity of the new compounds **4a**–**e** were tested at concentrations of 0.3, 1, 3, 10, 30, and 100 μM. Their IC_{50} values (inhibition concentrations, the concentrations that cause 50% inhibition of production) were calculated on the basis of their biological response. The calculated IC_{50} values of compounds **4**, which were higher than 1000 μM, were acknowledged to be inactive. Only one of the tested compounds, *i. e.* **4e** with an adamantyl moiety, inhibited TNF-α (IC_{50} = 14.0 ± 7.7 μM) and IL-6 (IC_{50} = 11.1 ± 3.0 μM) PBMC production at the levels of the reference isothiazole compound **MR-2/94** (31.4 ± 5.7 μM and 33.7 ± 4.9 μM, respectively [13]) and the reference drug leflunomide (50.0 ± 5.1 μM and 20.3 ± 7.3 μM, respectively [13]). Weak TNF-α-inhibiting activity was shown by amide **4a** with a *N*-4-chlorophenyl fragment (about 30% inhibition in the 1–100 μM concentration range) and very weak activity was shown by the *N*-(4-hydroxycyclohexyl) derivative **4d** (20% inhibition in the concentration range of 30–100 μM), but both amides **4a** and **4d** were inactive as IL-6 production inhibitors (IC_{50} > 1000 μM). The *N*-(6-chloro-3-pyridyl) amide derivative **4c** inhibited the production of IL-6 slightly at a level of 20% in the concentration range of 30-100 μM, but did not inhibit the production of TNF-α at all (IC_{50}>1000 μM). It is intriguing that amide **4b** with the *N*-4-trifluoromethylphenyl substituent did not inhibit the production of either pro-inflammatory cytokine (IC_{50} > 1000 μM). This is in contrast to the structurally similar isothiazole analogue **MR-17/95W** (see Fig. 1), which inhibits pro-inflammatory cytokine production exceptionally strongly (TNF-α (IC_{50} = 6.5 ± 1.2 μM) and IL-6 (IC_{50} = 8.2 ± 0.9 μM) [7]) and is one of the most active isothiazole derivatives [2, 4] It is interesting that, as in the case of leflunomide (CC_{50}, *i. e.* 50% cyto-

toxic concentration > 1000 μM) [13], no toxic effect of any of the new amides **4a – e** (CC_{50} > 1000 μM) was observed on PBMCs stimulated with LPS. The CC_{50}s of the reference isothiazole **MR-2/94** and the analogue **MR-17/95W** estimated previously for PMBCs were 454 ± 335 μM [13] and 1473 ± 596 μM [7], respectively.

Influence of the tested amides 4a – e on the NO production by macrophages

Inhibition of nitric oxide (NO) secretion by LPS-stimulated macrophages was estimated indirectly by measuring the concentration of the nitrite ion, which is a stable metabolite of NO and has been previously used as a marker of NO production in different fluids [22]. None of the tested diamides **4a – e** had the capability of specifically inhibiting NO secretion at the tested concentrations (0.3, 1, 3, 10, 30, and 100 μM). Compound **4e** showed nitric oxide secretion inhibition at a concentration (IC_{50} = 101.9 ± 6.0 μM), at which a remarkable toxic effect (CC_{50} = 33.7 ± 5.6 μM) appeared. There was no NO production inhibitory activity beyond the toxic concentrations of compounds **4** against macrophages. The corresponding values for the isothiazole reference **MR-2/94** are: IC_{50} = 15.4 ± 3.0 μM and CC_{50} = 35.2 ± 3.3 μM [13], for isothiazole **MR-17/95W** IC_{50} = 7.8 ± 0.2 μM and CC_{50} = 17.2 ± 1.6 μM [7], and for leflunomide IC_{50} = 280.3 ± 108.4 μM, CC_{50} = 125.8 ± 31.2 μM [13].

Membrane stabilization

Stabilization of the erythrocyte membrane is a characteristic of some non-steroidal anti-inflammatory agents (NSAIDs) [23]. The activities of the new compounds **4a – e** were tested at the concentrations of 0.001, 0.01, 0.1, 1, and 10 μg/mL. None of the tested pyrimidinecarboxamide derivatives **4a – e** rendered a stabilizing effect on the erythrocyte membrane. The **MR-2/94** reference showed strong membrane stabilization activity at concentrations of 1 and 10 μg/mL, with an estimated EC_{50} (50% effective concentration, *i. e.* the concentration at which relative hemolysis equals half of the control value) of 8.1 ± 0.98 μM.

Influence of the tested amides 4a-e on lymphocyte proliferation

The new derivatives **4a – e** were tested *in vitro* for their influence on the proliferation of human peripheral blood mononuclear cells (PBMCs), both unstimulated and stimulated with phytohemagglutinin (PHA) and concanavalin A (ConA), at the concentrations of 0.3, 1, 3, 10, 30, and 100 μM. The IC_{50}s (concentrations that caused 50% proliferation inhibition) of amides **4a – e** were calculated on the basis of biological response and compared with that of the reference isothiazole **MR-2/94**. The resulting IC_{50}s are collected in Table 5. Inhibition of lymphocyte production by unstimulated PMBCs at the level of the reference isothiazole **MR-2/94** (IC_{50} = 22.7 ± 1.8 μM) was shown only by amide **4e** with the adamantyl substituent (IC_{50} = 139.8 ± 65.4 μM). Compound **4b** (*N*-4-trifluoromethylphenyl amide) inhibited proliferation by 25% at concentrations of 10 and 30 μM. Derivative **4c** with the *N*-(6-chloro-3-pyridyl) substituent inhibited proliferation by 28% at a concentration of 10 μM, but concentrations above 30 μM slightly stimulated proliferation. The inhibitory effects of the compounds *N*-4-chlorophenyl amide **4a**, *N*-4-trifluoromethylphenyl amide **4b**, and *N*-adamantyl amide **4e** on the proliferation of PBMCs stimulated with PHA were comparable, but much weaker than that of the reference **MR-2/94** (IC_{50} = 1.6 ± 0.4 μM). The *N*-(4-hydroxycyclohexyl) derivative **4d** inhibited PHA-stimulated PBMCs by 33% at a concentration of 1 μM, but 30 and 100 μM increased the number of cells. The *N*-(6-chloro-3-pyridyl) derivative **4c** did not affect the proliferation PBMCs up to a concentration of 3 μM, but at higher concentrations compound **4c** stimulated it. In case of the influence of the tested compounds on the lymphocyte proliferation of PBMCs stimulated with concanavalin A (ConA), comparable inhibitory activities were revealed by the adamantyl derivative **4e** and the *N*-4-trifluoromethylphenyl amide **4b.** The *N*-4-chlorophenyl amide **4a** displayed a weaker activity than both **4e** and **4b**, but all these compounds, **4a**, **b**, and **e**, showed significantly less activity of this type than the reference **MR-2/94** (IC_{50} = 0.6 ± 0.2 μM). However, amides **4c** and **4d** inhibited proliferation in the concentration range of 0.3 to 3 μM by about 20%. But at higher concentrations, amide **4c** slightly stimulated proliferation and derivative **4d** did not influence the proliferation of PBMCs stimulated with concanavalin A (ConA). Such a profile of this type of activity could suggest immunomodulating properties of diamides **4c** and **4d**.

Conclusion

All the new tested amides **4**, apart from **4e**, showed very low or no activity in the biological *in-vitro* tests presented in this study. These results thus indicate that the distinguishing activity of derivative **4e** in these tests is rather connected with the presence of the adamantyl moiety in the molecular structure of **4e** and the pyrimidine core fragment is an unnecessary ballast in this case. It is known that 1-aminoadamantane (amantadine) has anti-parkinsonian activity and is used in Parkinson's disease therapy [24]. In addition, amantadine is an effective anti-

viral agent [25]. Its derivatives also show a broad scope of activity, namely the antiviral [25], anti-inflammatory [26], immunosuppressive [27], and antimicrobial [28] activities already mentioned. In summary, on the basis of the biological tests conducted, exchanging the isothiazole for a pyrimidine core ring caused a decrease in the anti-inflammatory and immunotropic profile of biological activity.

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The authors have declared no conflict of interest.

Experimental

Chemistry

The melting points of all the new compounds were measured on a Büchi 510 apparatus (Büchi Laboratoriums-Technik AG, Flawil, Switzerland) and were uncorrected. Elemental analyses were conducted by the Microlaboratory of the Department of Pharmacy, Medical Academy of Wroclaw with Carlo Erba series NA 1500 elemental analyzer (Carlo Erba, Milan, Italy). IR spectra: Pye-Unicam SP-1000 apparatus (Pye Unicam Ltd. Cambridge, England), KBr tablets. ^{1}H-NMR spectra (300.14 MHz and 500.13 MHz), ^{13}C-NMR spectra (broadband full decoupling method: 75.47 MHz), 5 mm tubes, concentration 20 – 30 mg of compound in 0.6 mL of solvent (≈ 0.1 M solution), TMS (tetramethylsilane) as internal reference: Bruker Fourier spectrometer (Bruker, Rheinstetten, Germany), MS, Electrospray Ionization (ESI): mass spectrometer micrOTOF-Q Bruker Daltonics. Thin-layer chromatography method (TLC) was applied to monitor the reaction course as well as to confirm the purity of the synthesized compounds. Polygram SIL G/UV$_{254}$ plates (Macherey-Nagel, Düren, Germany) for TLC were used, eluting medium was chloroform / methanol (9 : 1), detection of the compounds on the chromatograms was done with UV light and / or by treatment with iodine vapors.

*Synthesis and physicochemical properties of compounds 5-(4-Chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid **5***

5-(4-Chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid was obtained with the method described in [14].

Modification of the procedure described in [14]

3.5 g (0.0205 mol) of 5-aminoorotic acid (5-amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid) was suspended (partly solved) in 50 mL of anhydrous pyridine and was continuously stirred. The mixture was cooled to 5 – 10°C, then, while cooling was sustained, 3.9 mL (0.030 mol) of 4-chlorobenzoyl chloride were dropped in. Afterwards, the mixture was heated for 6 h at about 80°C. After cooling, the precipitate was filtered off, pressed, and the crude solid was suspended in a 10% water solution of HCl to liberate 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid. The mixture was stirred for 30 minutes, free acid was filtered off (92%), and the crude solid was washed with distilled water and was solvated in sodium hydroxide solution, filtered off, and then the free acid **5** was precipitated from basic solution with 10% water solution of HCl, then the precipitate was dried, and a sample for elemental and spectral analysis was crystallized from DMF (dimethylformamide). M.p. = 330°C (dec.), (m.p. reported in [14] = 327 – 330°C (dec.)).

*2-(4-Chlorophenyl)-6,8-dihydroxypyrimido[5,4-d]-4H-1,3-oxazin-4-on **3***

A mixture of 5.0 g (0.016 mol) of 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylic acid and 100 mL of toluene was heated to 80°C, then, ten drops of DMF (dimethylformamide) were added and 10 mL thionyl chloride ($SOCl_2$) was dropped in. The whole mixture was stirred and heated at 80°C for 8 h. After cooling, the crude solid was filtered off and dried, giving 4.65 g (100%) of crude compound. A sample for elemental and spectral analysis was crystallized from a toluene / DMF (dimethylformamide) 9 : 1 mixture. M.p. = 330 – 340°C (dec.).

*General methods for amides 4 synthesis on the example of 5-(4-chlorobenzoyl)amino-N-(4-chlorophenyl)-2,6-dihydroxy-4-pyrimidinecarboxamide **4a***

870 mg (6.82 mmol) of 4-chloroaniline was solved in 15 mL of anhydrous pyridine, then the solution was heated to boiling and 1.0 g (3.43 mmol) of 2-(4-chlorophenyl)-6,8-dihydroxypyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **3** was added. The whole mixture was refluxed and stirred for 10 h, then cooled, and the yellow needle crystals of the pyridinium complex salt of 2-(4-chlorophenyl)-6,8-dihydroxypyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **11** was filtered out (in case it formed). The filtration was evaporated to dryness *in vacuo*. 200 mL of a 10% water solution of sulphuric acid was added to the crude residue, the mixture was stirred for 30 minutes to remove excess amine that did not react and remaining residue of the pyridine solvent. Then, the crude compound was filtered off and washed with distilled water to neutral pH. 1.32 g (92%) of the crude solid was obtained that was crystallized from methanol to give 880 mg (61%) of pure compound **4a**. M.p. = 248 – 250°C.

*5-(4-Chlorobenzoyl)amino-2,6-dihydroxy-N-(4-trifluoromethylphenyl)-4-pyrimidinecarboxamide **4b***

The crude solid (yield = 34%) was crystallized from methanol to give pure **4b** with a yield of 17%. M.p. = 297 – 298°C. Pyridinium complex salt of 2-(4-chlorophenyl)-6,8-dihydroxy-4*H*-pyrimido[5,4-d]-4*H*-1,3-oxazin-4-on **11** was also formed (39%).

*5-(4-Chlorobenzoyl)amino-N-(6-chloro-3-pyridyl)-2,6-dihydroxy- 4-pyrimidinecarboxamide **4c***

The crude solid (yield = 56%) was crystallized from a methanol / water mixture of in a ratio of 2.3 / 1 to give pure **4b** with a yield of 26%. M.p. = 302.5 – 303°C (dec.). Pyridinium complex salt of 2-(4-chlorophenyl)-6,8-dihydroxy-4*H*-pyrimido[5,4-d]-4*H*-1,3-oxazin-4-on **11** was also created (19%).

5-(4-Chlorobenzoyl)amino-2,6-dihydroxy-N-(4-hydroxycyclohexyl)-4-pyrimidinecarboxamide 4d (as equimolar mixture of isomer cis and trans)

The crude product (87%) was crystallized from methanol. M.p. = 248 – 252°C (dec.).

*5-(4-Chlorobenzoyl)amino-N-(1-adamantyl)-2,6-dihydroxy-4-pyrimidinecarboxamide **4e***

The crude solid (95%) was crystallized from methanol. M.p. = 239 – 241°C (dec.).

Ethyl 5-(4-chlorobenzoyl)amino-2,6-dihydroxy-4-pyrimidinecarboxylate 10 [14]

200 mg (685.8 μmol) of 2-(4-chlorophenyl)-6,8-dihydroxypyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **3** was heated with 20 mL of anhydrous ethanol with molecular sieves for 10 h. After the mixture had cooled, the small white needle crystals were filtered off and dried, giving 217 mg (94%) of pure ester **10**. M.p. = 271°C (dec.) (m.p. reported in [14] = 264 – 267°C).

*Pyridinium complex salt of 2-(4-chlorophenyl)-6,8-dihydroxypyrimido[5,4-d]-4H-1,3-oxazin-4-on **11 (11a)***

Yellow needle crystals, m.p. >285°C (dec.).

2-(4-Chlorophenyl)-6,8-dichloropyrimido[5,4-d]-4H-1,3-oxazin-4-on 12 [16]

The mixture of 250 mg (857.2 μmol) of 2-(4-chlorophenyl)-6,8-dihydroxypyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **3**, 10 mL of phosphoryl chloride ($POCl_3$) and a few drops of *N*,*N*,*N*-triethylamine was heated in reflux for 5 h. Afterwards $POCl_3$ was evaporated. The obtained crude solid was treated with boiling benzene and then, the benzene solution was filtered off from solid insoluble impurities. The filtrate was evaporated *in vacuo* to give a dry solid that was crystallized from cyclohexane to give 153 mg (54%) of pure compound **12**. M.p. = 223 – 225°C. 2-(4-Chlorophenyl)-6,8-dichloropyrimido[5,4-*d*]-4*H*-1,3-oxazin-4-on **12** creates very durable yellow complex with *N*,*N*-dimethylaniline, m.p. = 235 – 237°C.

Pharmacology

Dilution of the compounds for in-vitro tests

For the *in-vitro* tests, the compounds were dissolved initially in dimethyl sulphoxide (DMSO, ICN) and then in culture medium, *i. e.* RPMI 1640 supplemented with 25 mM HEPES buffer, 10% fetal calf serum (Life Technologies, St. Paul, MN, USA), 2 mM L-glutamine (Sigma, Poznan, Poland), 100 U/mL penicillin, and 100 mg/mL streptomycin (both from Polfa Tarchomin; Warsaw, Poland) supplemented with DMSO to maintain a constant concentration. The final concentration of the compounds ranged from 0.001 to 100 μM and the DMSO dilution was 1 : 1000. At this dilution, DMSO in culture medium was used as a control.

Cell isolation and stimulation for cytokine production

Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats (enriched with 2500 UI/mL heparin) of volunteer blood donors according to Bøyum using Lymphoprep (Nycodenz, Aachen, Germany) and resuspended (10^6 cells/mL) in culture medium [29]. The cells (2×10^5 per well) were incubated (5% CO_2 in air, 37°C) with compounds at the tested concentrations (0.3, 1, 3, 10, 30, 100 μM) in 2 – 3 replicates on 96-well plates (Costar, Vitaris AG, Baar, Switzerland) and simultaneously stimulated with 1 μg/mL of *E. coli* O1 lipopolysaccharide. After 18 h, the plates were centrifuged and the supernatants for cytokine determination were collected and kept at −70°C until assay. Cell viability was determined immediately as described below.

Measurement of tumor necrosis factor α activity

Tumor necrosis factor α (TNF-α) activity in the supernatants was determined according to Espevik and Nissen – Meyer [30]. Briefly, WEHI-164.13 cells were cultured in 96-well plates (Nunc, Roskilde, Denmark) at a density of 2×10^4 cells/well with serial dilutions of the tested supernatants. Cell proliferation was evaluated after 20 h of culture using the 3-(4,5-dimethyl-2-tiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma) colorimetric method. Absorbance was measured at 550 nm (reference wavelength: 630 nm) with a colorimetric microplate reader (Dynex Opsys MR, Dynex Technologies GmbH, Berlin, Germany) [31].

Measurement of interleukin-6 concentration

Interleukin-6 (IL-6) concentration in the supernatants was determined by enzyme-linked immunosorbent assay according to [32] and the Cytokine ELISA Protocol (May 29, 1998, 2nd Edition, Pharmingen). Shortly, 100 μL per well of the ten times diluted supernatants or serial dilutions of recombinant human IL-6 (Pharmingen, BD Bioscience, USA) were incubated in duplicate for 18 h at 4°C on 96-well plates (Nunc-Immuno Plates, MaxiSorp Surface, Nunc, Denmark) previously covered with rat anti-human IL-6 antibody (1 μg/mL, Pharmingen). After washings, biotinylated rat anti-human IL-6 antibody (5 μg/mL, Pharmingen) was incubated on the plate for 60 min at room temperature. Then avidine conjugated with horse radish peroxidase (1 : 4000, Dako) was added for 30 min at room temperature. The colorimetric reaction was elicited by addition of ABTS substrate solution (0.03% 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citric acid, pH = 4.35, with addition of 0.03% hydrogen peroxide). Absorbance was measured at 405 nm (reference wavelength: 630 nm) after 30 min of incubation at 20°C. The IL-6 concentration was calculated from an IL-6 standard curve.

Nitrite measurement

Macrophages elicited with thioglycolate according to [33] were derived from the peritoneal cavity of Wistar rats and suspended after washing in culture medium (RPMI 1640 without phenol red, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin). Then, they were incubated (1.2×10^6 cells per well, 5% CO_2 in air, 37°C) for 24 h with the tested compounds at the specified concentrations (0.3, 1, 3, 10, 30, 100 μM) in duplicate in 96-well plates and simultaneously stimulated with 10 μg/mL of *E. coli* O1 LPS. Then, the plates were centrifuged and supernatants for cytokine determination were collected. The cells were detached for viability assessment as described below immediately after centrifugation.

For an indirect determination of NO secretion by the stimulated macrophages, 100 μL of the supernatant was incubated at room temperature with 100 μL of Griess reagent (1% sulfanilamide / 0.1% naphthylenediamine dihydrochloride, both from Sigma, in 2.5% H_3PO_4) [34]. After 10 min, absorbance at 550 nm

was measured. The concentration of nitrite ion was calculated from a $NaNO_2$ standard curve.

Membrane stabilization

Stabilization of erythrocyte membranes was determined by hemoglobin release according to Seeman and Weinstein [35]. A suspension of rabbit erythrocytes was used (4×10^6 cells/mL in PBS without Ca^{2+} and Mg^{2+}, pH = 7.0). The compounds were dissolved in DMSO and further diluted with hypotonic phosphate buffer to a concentration ranging from 0.001 to 10 μg/mL. Five mL of dissolved compound was incubated with 0.1 mL of the erythrocyte suspension for 5 min at room temperature and then the absorbance of the supernatant at 543 nm (corresponding to the release of hemoglobin) was measured. Inhibition of hemoglobin release by more than 20% compared with controls (control hemolysis was set at around 50%) was considered a positive effect.

Lymphocyte proliferation assay

PBMCs were isolated according to Bøyum as described earlier [29]. One hundred five cells per well were not stimulated or stimulated with 10 μg/mL PHA or ConA (in 2–3 replicates) and incubated with the tested compounds at concentrations of 0.3, 1, 3, 10, 30, or 100 μM in culture medium in 96-well plates. Cell proliferation was evaluated after 72 h using the 3-(4,5-dimethyl-2-tiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma) colorimetric method as described earlier [29]. Control cells were incubated with DMSO at the corresponding concentrations.

Cytotoxic activity

The cytotoxic activities of the compounds on PBMCs and macrophages were determined using the 3-(4,5-dimethyl-2-tiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma) colorimetric method as described earlier in methods. Control cells were incubated with DMSO at the appropriate concentration. In the proliferative tests, cell viability of a total of 100 or 200 cells counted from two wells for each compound dilution was assessed with an acridine orange / ethidium bromide mixture solution with a fluorescence microscope (viable cells appear green, dead cells orange) [36].

Data analysis

IC_{50} (inhibition concentration that caused 50% inhibition of inflammatory marker production), EC_{50} (50% effective concentration), and CC_{50} (50% cytotoxic concentration) were calculated using a model of a sigmoid curve with a Hill slope in Microsoft Office Excel 2000 (Microsoft) and LSW Data Analysis Toolbox ver. 1.1.1 (MDL1 Information Systems, Inc.).

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