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SYNTHESIS AND ANTIBACTERIAL PROPERTIES OF PYRIMIDINE DERIVATIVES

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Abstract: The paper presents the synthesis of 1,2,3,7-tetraaryl-1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidines. The structures of the obtained compounds were confirmed by crystallographic and spectroscopic analyses, and their antibacterial activity was tested on 9 selected strains, comparing chemical structure changes with increased microbiological activity. It was confirmed that aromatic residues in the hydrogenated pyrimidine ring constitute a significant element influencing antibacterial activity. Electronegative radicals increase microbiological activity, but decrease solubility of the compounds. Therefore, substituents should be selected in a manner ensuring a balanced effect. The presented crystal structure of **6f** includes two stereoisomers, which we decided to isolate and compare the microbiological properties in further studies.

Keywords: pyrimidine derivatives, antibacterial effect, pyrimido[4,5-d]pyrimidines

Our earlier studies on the synthesis and biological properties of pyrimidine derivatives demonstrated that these systems reveal extremely potent biological activity. Pyrimidine derivatives have been found to exhibit cytostatic (1–3), immunomodulating (4, 5) and antibacterial properties (6–12). In particular, studies concerning the synthesis of pyrimido[4,5-d]1,3-oxazines (13) showing considerable similarity to quinoline chemotherapeutic agents with an antibacterial effect already in the use, e.g., piromidic and pipemidic acids, encouraged us to further work leading to replacement of the pyrimido[4,5-d]1,3-oxazine system with the pyrimido[4,5-d]pyrimidine system (Fig. 1). In the continuation of our search for more potent antibacterial agents, we have documented that 4-arylamine-6-methyl-2-phenyl-5-aminomethylpyrimidine and 1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidine derivatives, especially possessing three strongly electronegative substituents attached to phenyl rings, exhibited good antibacterial activity (14). With this background in mind, in the present study, we report the synthesis and antibacterial properties of the new derivatives (**6a**-**6l**) and the crystal structure of 1-(4-chlorophenyl)-3-(4-ethoxyphenyl)-5-methyl-2-(4-nitrophenyl)-7-phenyl-1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidine (**6f**).

RESULTS and DISCUSSION

The starting substrates in our investigations were 4-arylamino-6-methyl-2-phenyl-5-pyrimidinecarboxylic acids **1**, which were reduced with $LiAlH_4$ [4]. The obtained 4-arylamino-6-methyl-2-phenyl-5-hydroxymethylpyrimidines **2** were reacted with $SOCl_2$ to give 2-arylamino-6-methyl-2-phenyl-5-chloromethylpyrimidines **3**. The reaction of **3** with various aromatic amines yielded 4-arylamino-6-methyl-2-phenyl-5-aminomethylpyrimidines **4** and 4-arylamino-5-arylaminomethyl-6-methyl-2-phenylpyrimidines **5**. The obtained 5-aminomethylpyrimidines **4** and **5** were cyclized in a Mannich reaction to give 1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidines **6** and **7**. It is notable that Mannich reaction **8** could not be completed while acting with aromatic aldehydes on primary amines, and Schiff bases **9** could not be obtained (Scheme 1).

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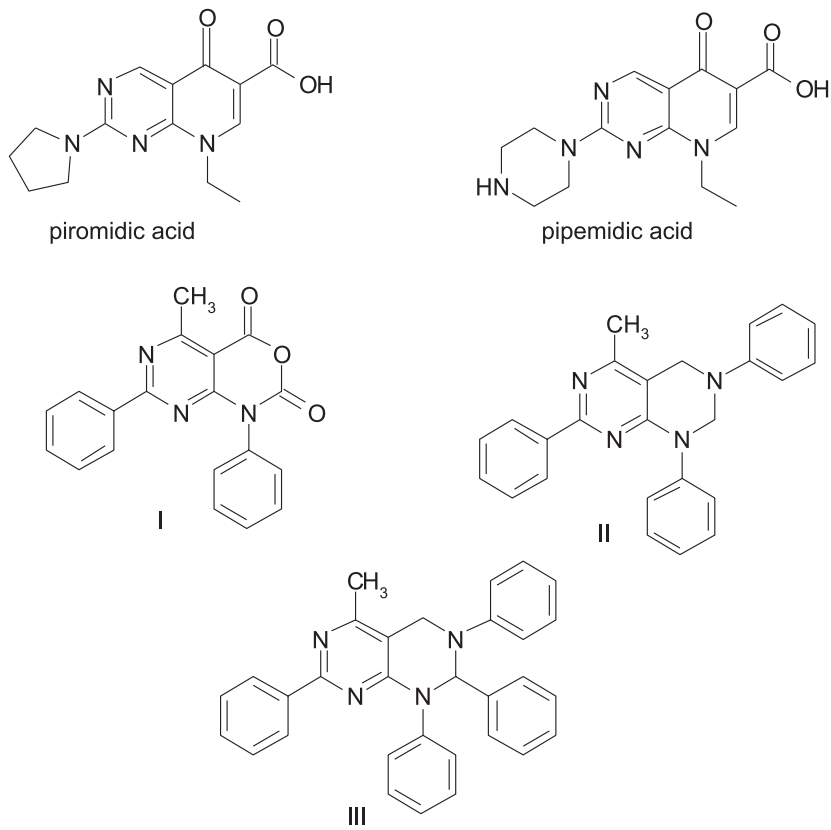


Figure 1. Chemical structures of piromidic acid, pipemidic acid, pyrimido[4,5-d]1,3-oxazine system (I and II) and pyrimido[4,5-d]pyrimidine system (III)

The newly obtained derivatives of **7** were evaluated for their antibacterial and antifungal activity. The physical properties of these compounds are shown in Table 1. The X-ray crystal structure of compound **6f** confirms the cyclization reaction. The molecular structure of **6f** with atom-labelling scheme is shown in Figure 2. The compound **6f** crystallizes from acetone-methanol with two independent molecules (denoted as ***A*** and ***B***) in the asymmetric unit. The conformation of these molecules is rather similar.

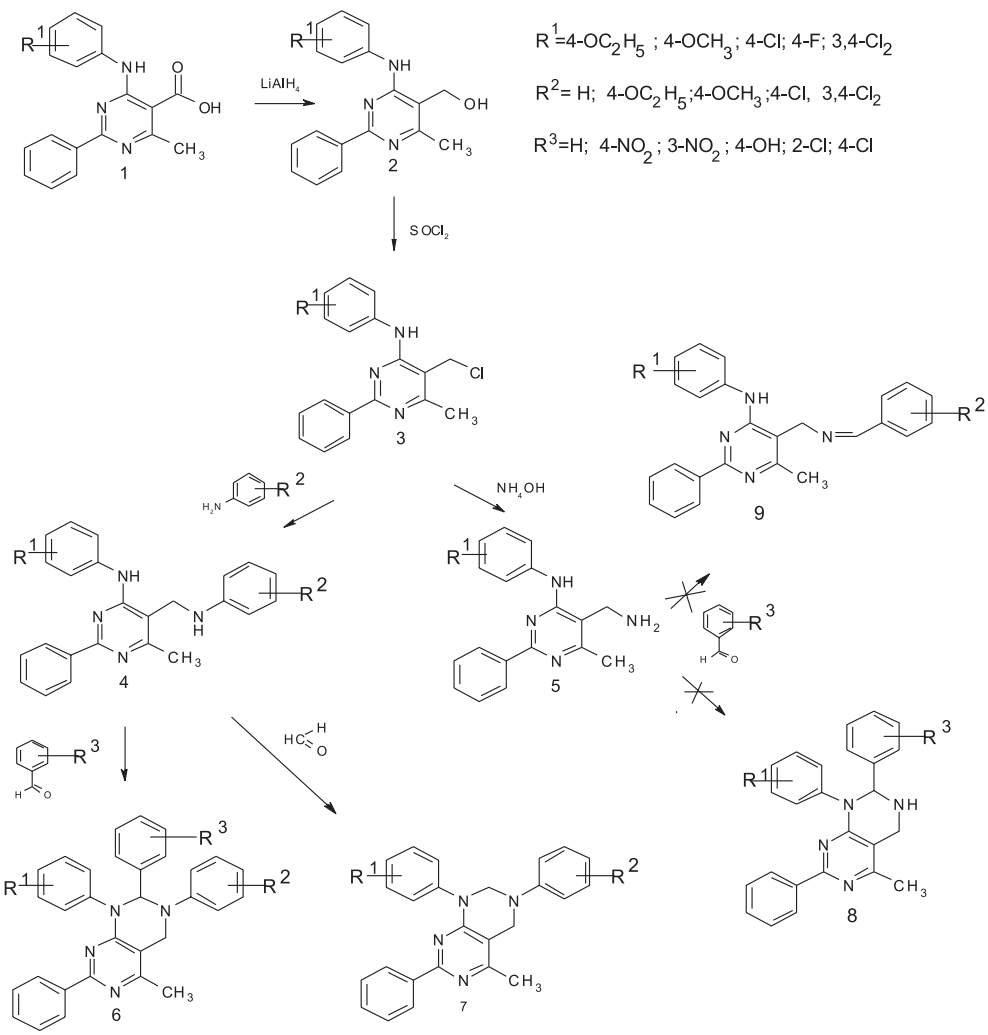
X-ray crystallography for 6f

The recrystallization from acetone-methanol (1:1, v/v) resulted in the formation of needle-shaped crystals of **6f** that were submitted for X-ray analysis. X-ray data were collected from a single crystal, 0.4 × 0.07 × 0.04 mm, at 100 K using graphite-monochromated Mo-K_α (λ = 0.71073 Å) radiation on a Kuma KM4CCD k-geometry diffractometer (w-scan). The instrument was equipped with Oxford Cryosystems low-temperature devices. The structure was solved by direct methods using the SHELXS-97 program and refined by full-matrix least-squares calculations on F^2 with SHELXL-97 (15). Non-hydrogen atoms were refined with anisotropically thermal parameters. The H atoms bound to C atoms were included in geometrically calculated positions, with C-H distances in the range 0.95–1.0 Å, and refined using a riding model, with $U_{iso}(H) = 1.5\ U_{eq}(C)$ for methyl and $1.2\ U_{eq}(C)$ for the remainder. The asymmetric unit of the crystal contains two independent molecules: $C_{33}H_{28}ClN_5O_3$, M_r = 578.05, triclinic, space group P 1, a = 13.033(4), b = 13.783(4), c = 16.707(5) Å, α = 73.79(3)°, β = 87.64(3)°, γ = 85.07(3)°, V = 2871(2) Å^3, d_{calc} = 1.337, $F(000)$ = 1208, m = 0.177 mm^{-1}, Z = 4, 42792 measured reflections (-16 = h = 15, -17 = k = 15, -21 = l = 21), 11901 independent and 5996 observed reflections with $I > 2\sigma(I)$ (R_{int} = 0.1111), 759 parameters, R = 0.2156, wR_2 = 0.1086 for all reflections, $w = 1/[\sigma^2(F^2) + (0.0155P)^2]$ where $P = (F_o^2 + 2F_c^2)/3$.

CCDC 736046 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223 336033; e-mail: deposit@ccdc.cam.uk).

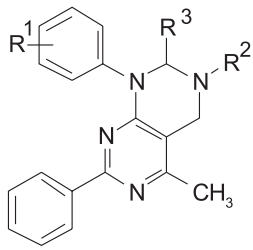
Fourteen newly obtained pyrimidine derivatives **6a**-**6l** were investigated microbiologically on one mycotic and eight bacterial strains: *Candida albicans, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Seratia marcesceus, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus epidermidis* and *Staphylococcus aureus*. The microbiological investigations were based on M-7 and A-5 standards (MIC Testing). The MIC (minimum inhibitory concentration) values were determined and compared to erytromycin as the reference drug. The most significant results are presented in Table 2.

The cyclization of 5-aminomethylpyrimidines **4, 5** to 1,2,3,4-tetrahydropyramido[4,5-d]pyrimidines **6,7** caused a considerable increase in antibacterial activity. These pyrimidine derivatives emerge as potent active antibacterial agents when one phenyl ring contains a strong electronegative substituent, such as a chlorine atom. Placement of an electron-withdrawing group at N-1, C-2 and N-3 aryl rings leads to a decrease in activity, but an increase in solubility. Compounds possessing three strongly electronegative substituents attached to phenyl rings, such as chlorine atoms (compounds **6**),



Scheme 1. Synthesis of 1,2,3-aryl-1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidine derivatives

Table 1. Physical data of compounds **6a-l**.



Comp.	R^1	R^2	R^3	Formula (m.w.)	M.p. (°C)	Yield (%)	Analysis calc. found			
							C	H	N	Cl
6a	4-OC_2H_5	C_6H_5-4-OC_2H_5	C_6H_5-4-NO_2	$C_{35}H_{33}N_5O_4$ (587.68)	183–185	73.5	71.53 71.24	5.66 5.54	11.92 10.63	
6b	4-OC_2H_5	C_6H_5-4-OC_2H_5	C_6H_5-4-OH	$C_{35}H_{34}N_4O_3$ (558.68)	186–188	71.4	75.24 75.43	6.13 6.46	10.03 10.24	
6c	4-OCH_3	C_6H_5-4-OCH_3	C_6H_5-3-NO_2	$C_{33}H_{29}N_5O_4$ (559.63)	151–153	82.0	70.83 70.59	5.22 5.32	12.51 12.59	
6d	4-OCH_3	C_6H_5-4-OCH_3	C_6H_5-2-Cl	$C_{33}H_{29}N_4ClO_2$ (549.07)	217–219	75.7	72.19 72.53	5.32 5.66	10.20 10.44	6.46 6.52
6e	4-Cl	C_6H_5-4-OCH_3	C_6H_5-2-Cl	$C_{32}H_{26}N_4Cl_2O$ (553.49)	212–214	81.7	69.44 69.59	4.73 4.32	10.12 10.29	12.81 12.52
6f	4-Cl	C_6H_5-4-OC_2H_5	C_6H_5-4-NO_2	$C_{33}H_{28}N_5ClO_3$ (578.07)	188–190	80.0	68.57 68.94	4.86 4.53	12.11 12.33	6.13 6.01
6g	4-F	C_6H_5-4-Cl	C_6H_5-4-NO_2	$C_{31}H_{23}N_5ClFO_2$ (552.00)	213–215	72.1	67.45 67.71	4.20 4.11	12.69 12.31	6.42 6.08
6h	3,4-Cl_2	C_6H_5-4-OCH_3	C_6H_5-3-NO_2	$C_{32}H_{25}N_5Cl_2O_3$ (566.49)	165–167	72.0	64.22 64.51	4.21 4.25	11.70 11.41	11.85 12.11
6i	3,4-Cl_2	C_6H_5-4-OCH_3	C_6H_5-4-OH	$C_{32}H_{26}N_4Cl_2O_2$ (569.49)	175–177	76.5	67.49 67.31	4.60 4.44	9.83 9.53	12.30 12.22
6j	3,4-Cl_2	3,4-Cl_2	C_6H_5-4-OH	$C_{31}H_{22}N_4Cl_4O$ (608.36)	178–180	75.5	61.20 61.31	3.64 3.14	9.22 9.23	23.31 23.22
6k	3,4-Cl_2	3,4-Cl_2	C_6H_5-4-Cl	$C_{31}H_{21}N_4Cl_5$ (626.97)	220–222	78.5	59.38 59.31	3.40 3.14	8.94 8.23	28.27 28.22
6l	4-OC_2H_5	C_6H_5-4-OC_2H_5	H	$C_{29}H_{30}N_4O_2$ (466.58)	182–184	73.1	74.65 74.4	6.48 36.66	12.00 12.24	

exhibited significant anctibacterial activity (Table 2) but low solubility.

Microbiology

The obtained chemical compounds were investigated microbiologically on selected strains, in order to evaluate their bioactivity. The investigation was based on M-7, A-5 standards (MIC Testing) (16). The fungal strains also were cultivated on this standard recommended broth – Mueller Hinton Broth II.

Sample bacterial cultures were suspended in 3 mL of a sterile solution of PBS according to 0.5 Mc Farland's standard (corresponding to 1 to 2 × 10^8 CFU/mL), and then were diluted with a sterile 1:10 PBS solution (giving 1 × 10^7 CFU/mL).

Ten microliters of obtained inoculum was added to 0.2 mL of sterile final dilutions of the investigated substances according to Table 2, obtaining 5 × 10^4 concentration of bacteria in the investigated samples. Six trials were carried out for every dilution of the investigated substance – one control without the inoculum.

Based on the determined experimental data, the effect on mean MIC values of three parameters was evaluated:

– type of substituent of the examined chemical compound;

Table 2 Minimal inhibitory concentrations (MIC) (mg/mL) Testing M-7, A-5

	6a	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	Erytromycin
Bacillus subtilis (PCM – 2021)	128	256	256	64	32	8	128	128	64	128	256	256	2
Escherichia coli PCM 2057 (ATCC 25922)	256	128	64	128	32	16	64	64	32	64	128	256	4
Klebsiella pneumoniae (PCM – 1)	128	128	128	128	128	64	128	128	128	128	128	128	8
Proteus vulgaris PCM 542 (ATCC -13315)	256	64	128	256	64	32	256	64	128	64	128	128	32
Serratia marcescens PCM 549 (ATCC 274)	128	128	64	128	128	16	128	128	64	64	128	256	8
Pseudomonas aeruginosa (ATCC-27853)	128	64	64	128	16	16	128	128	128	128	256	128	64
Enterococcus aecalis (PCM-2673)	64	128	128	64	32	32	64	128	128	64	128	128	2
Staphylococcus epidermidis (PCM-2118)	16	32	128	32	8	64	16	32	32	128	64	128	2
Staphylococcus aureus (PCM-1932)	32	32	64	32	32	32	32	32	64	64	128	256	32
Candida albicans (PCM-2566)	16	32	16	8	32	16	16	32	16	128	64	128	8

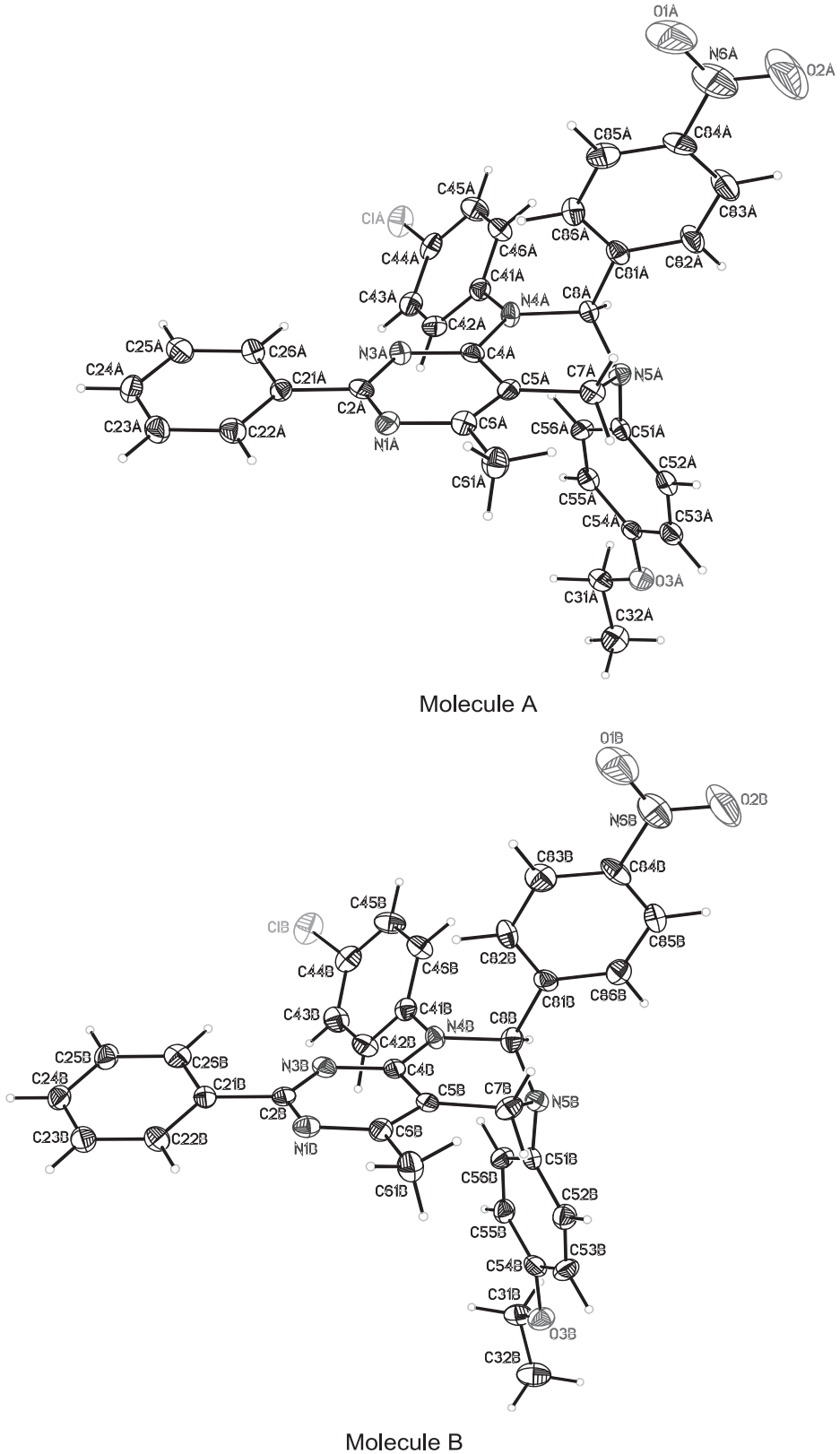


Figure 2. The molecular structure of stereoisomers of **6f**, showing the atom labeling scheme. Displacement ellipsoids are drawn at 50% probability level

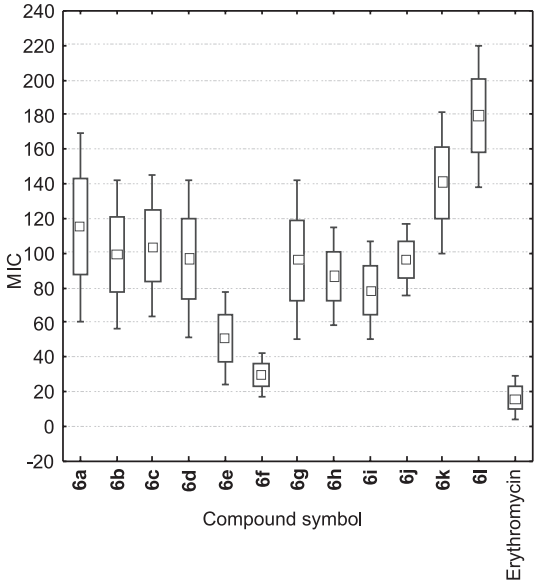


Figure 3. Effect of substituent type on mean MIC values. □ = mean

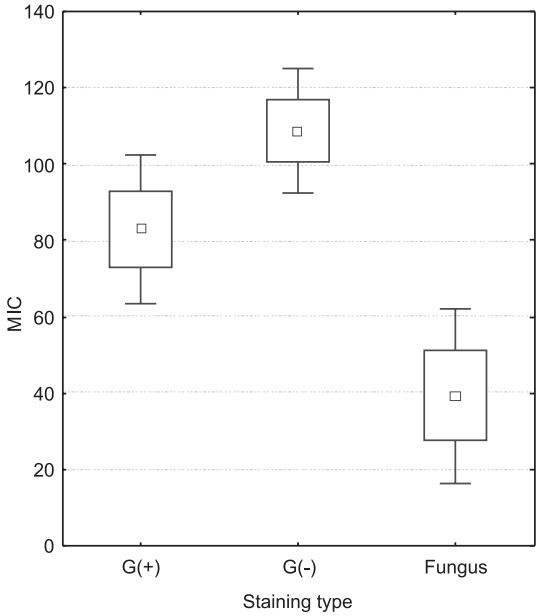


Figure 5. Effect of bacteria type depending on the color of stain using the Gram method on mean MIC values. □ = mean

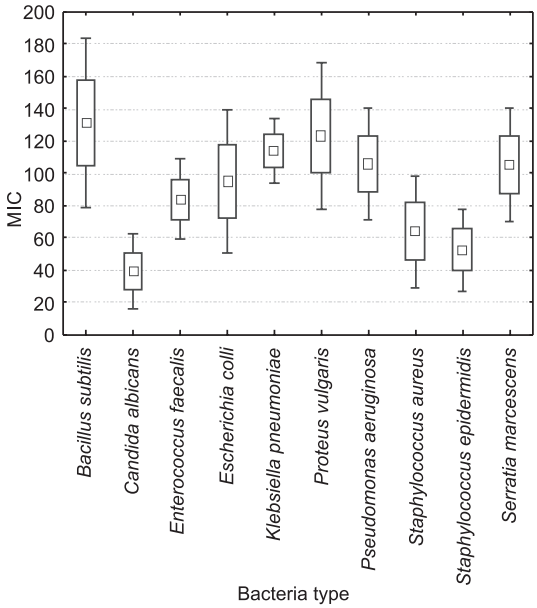


Figure 4. Effect of bacteria type on mean MIC values. □ = mean

– strain of bacterium/fungus;

– type of bacterium depending on the color of stain using the Gram method.

The obtained mean MIC values were compared with each other using parametric ANOVA analysis of variations, as well as nonparametric ANOVA by Kruskal-Wallis. The normality of the distributions of the analyzed random variables was checked using Shapiro-Wilk and Kolmogorow-Smirnow tests with Lillefors correction at the assumed confidence level $p = 0.05$. The uniformity of the variations was determined using Levene and Brown-Forsythe tests using the confidence level of $p = 0.05$.

Effect of substituent type on mean MIC values

The mean MIC values determined for 13 substituents (from **6a** to **6l**) were compared using the parametric ANOVA analysis of variations (Fig. 3) together with *post-hoc* NIR and Scheffe's tests and the nonparametric Kruskal-Wallis test.

Effect of bacteria type

In the completed microbiological tests, 9 strains of bacteria and one type of fungus – *Candida albicans* – were used. Using the same statistical methods as above, comparison of mean MIC values was determined for each of the tested strains (Fig. 4).

Effect of bacteria type depending on the color of stain using the Gram method on mean MIC values

Microbiological tests were conducted on 4 species of bacteria G(+), 5 species of bacteria G(-) and one species of fungus. Using the parametric and nonparametric ANOVA analysis of variations (as

above), the statistical importance of the differences between mean MIC values obtained in these three groups was evaluated and results are presented in Figure 5.

ANALYSIS OF THE RESULTS

The conducted ANOVA analysis of variations showed that with the assumed confidence level p = 0.05, type of substituent, strain of bacteria as well as bacteria depending on the color of stain using the Gram method all had a statistically significant influence on experimentally determined mean MIC values.

1. The highest mean MIC values were for compounds with substituents in **6k** and **6l**, and this difference was statistically significant.

2. The lowest statistically significant mean MIC values were obtained for compounds with substituent **6f**.

3. There is not any statistically significant difference between mean MIC values determined for the compound with substituent **6f** and the reference antibiotic erythromycin.

4. The lowest statistically significant mean MIC values were obtained for *Candida albicans* fungus and for *Staphylococcus epidermidis* bacteria strain.

5. The differences between mean MIC values determined within G(+), G(-) groups and funguses also turned out to be statistically significant. The highest MIC values were obtained within G(-) bacteria, medium for G(+), and the lowest for *Candida albicans* fungus.

EXPERIMENTAL

Chemistry

Melting points were determined in Köfler apparatus. ^{1}H-NMR spectra were recorded on BS-487-C-300 MHz Tesla spectrometer. Infrared (IR) spectra were recorded in nujol with a Specord spectrophotometer, at the Analytical Laboratory of Medical University in Wrocław. Elemental analyses indicated by the symbols were within ± 0.4 of the theoretical values.

1,3-Di(4-ethoxyphenyl)-5-methyl-2-(4-nitrophenyl)-7-phenyl-1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidine (**6a**)

4-(4-Ethoxyphenyl)amino-5-[(4-methoxyphenyl)amino]methyl-6-methyl-2-phenylpyrimidine **4,** (4 g, 0.00879 mol) was dissolved in THF (50 mL) and 36% HCl (1 mL) and 4-nitrobenzaldehyde (1 g) were added. The mixture was refluxed for 18 h with vigorous stirring, then it was cooled and poured into H_2O (300 mL). The solution was neutralized using 25% aqueous NH_3 and extracted three times with $CHCl_3$ (50 mL). The combined $CHCl_3$ extracts were dried over $MgSO_4$, filtered, and concentrated under vacuum. The oily residue was purified by column chromatography on silica gel (200–300 mesh) using $CHCl_3$ as the eluent and by crystallization from methanol to give **6a**.Yield: 73.5% (3.8 g), m.p. 183–185§C; IR (nujol, cm^{-1}): 1570 (NO_2), 1340 (NO_2), 1325 (C-N). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 0.80 (t, 3H, $OCH_2C\mathbf{H}_3$), 0.85 (t, 3H, $OCH_2C\mathbf{H}_3$), 1.40 (s, 3H, CH_3), 2.80 (q, 2H, $OC\mathbf{H}_2CH_3$), 2.90 (q, 2H, $OC\mathbf{H}_2CH_3$), 3.20 (s, 2H CH_2), 3.80 (s, 1H, CH), 7.50–8.40 (m, 17H, arom.).

Compounds **6b**, **6d**, **6e**, **6f**, **6g**, **6h**, **6i**, **6j** and **6k** were obtained by adopting the same procedure.

6b: IR (nujol, cm^{-1}): 1550 (OH), 1325 (C-N), 1170 (OH). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 0.80 (t, 3H, $OCH_2C\mathbf{H}_3$); 0.85 (t, 3H $OCH_2C\mathbf{H}_3$); 1.40 (s, 3H, CH_3) 2.80 (q, 2H, $OC\mathbf{H}_2CH_3$) 2.90 (q, 2H, $OC\mathbf{H}_2CH_3$); 3.20 (s, 2H CH_2); 3.80 (s, 1H CH); 5.20 (s, 1H, OH); 7.50–8.40 (m 17 H arom.).

6c: IR (nujol, cm^{-1}): 2966 OCH_3, 1575 (NO_2), 1330 (NO_2), 1320 (C-N). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.20 (t, 3H, OCH_3), 1.25 (t, 3H, OCH_3), 1.40 (s, 3H, CH_3), 3.30 (s, 2H, CH_2), 3.85 (s, 1H, CH), 7.50–8.40 (m, 17 H arom.).

6d: IR (nujol, cm^{-1}): 2960 (OCH_3), 1325 (C-N), 725 (C-Cl). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.25 (t, 3H, OCH_3), 1.30 (t, 3H OCH_3), 1.40 (s, 3H, CH_3), 3.30 (s, 2H CH_2), 3.85 (s, 1H CH), 7.50–8.50 (m, 17H, arom.).

6e: IR (nujol, cm^{-1}): 2960 (OCH_3), 1325 (C-N), 725 (C-Cl). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.30 (t, 3H OCH_3), 1.40 (s, 3H, CH_3), 3.30 (s, 2H CH_2), 3.85 (s, 1H CH), 7.20–8.40 (m, 17 H arom.).

6f: IR (nujol, cm^{-1}): 1340 (NO_2), 1325 (C-N), 750 (C-Cl). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 0.80 (t, 3H, $OCH_2C\mathbf{H}_3$), 1.40 (s, 3H, CH_3), 2.90 (q, 2H, $OC\mathbf{H}_2CH_3$), 3.20 (s, 2H, CH_2), 3.85 (s, 1H CH), 7.50–8.40 (m, 17H arom.).

6g: IR (nujol, cm^{-1}): 1570 (NO_2), 1340 (NO_2), 1325 (C-N), 1075 (C-F), 750 (C-Cl). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.40 (s, 3H, CH_3), 3.20 (s, 2H, CH_2), 3.80 (s, 1H, CH); 7.50–8.40 (m, 17H, arom.).

6h: IR (nujol, cm^{-1}): 1570 (NO_2), 1340 (NO_2), 1320 (C-N), 750 (C-Cl), 725 (C-Cl). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.30 (t, 3H, OCH_3), 1.40 (s, 3H, CH_3), 3.30 (s, 2H, CH_2), 3.85 (s, 1H, CH), 7.20–8.40 (m, 16 H arom.).

6i: IR (nujol, cm^{-1}): 2966 (OCH_3), 1235 (OH), 1170 (OH), 750 (C-Cl), 725 (C-Cl). ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.30 (t, 3H, OCH_3), 1.40 (s, 3H, CH_3), 3.30 (s, 2H, CH_2), 3.85 (s, 1H, CH), 7.20–8.40 (m, 16H, arom.).

6j: IR (nujol, cm^{-1}): 1320 (C-N), 1235 (OH), 1170 (OH), 750 (C-Cl), 725 (C-Cl), ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.40 (s, 3H, CH_3), 3.30 (s, 2H, CH_2), 3.85 (s, 1H, CH), 7.25–8.40 (m, 15H, arom.).

6k: IR (nujol, cm^{-1}): 750 (C-Cl); 725 (C-Cl); 1325 (C-N). ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 1.40 (s, 3H, CH_3) 3.30 (s, 2H CH_2); 3.85 (s, 1H CH); 7.25-8.40 (m 15 H aromat).

1,3-Di(4-ethoxyphenyl)-5-methyl-7-phenyl-1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidine (**6l**)

4-(4-Ethoxyphenyl)amino-5-[(4-methoxyphenyl)amino]methyl-6-methyl-2-phenylpyrimidine **4** (4 g, 0.00879 mol) was dissolved in THF (50 mL) and 36% HCl (1 mL) and 25% formaldehyde (25 mL) were added. The reaction mixture was refluxed for 20 h with vigorous stirring, then was cooled down and poured into water (300 mL). The solution was neutralized using 25% ammonia solution and extracted three times with chloroform (50 mL). The combined chloroform extracts were dried over $MgSO_4$, filtered and concentrated under vacuum. The oily residue was purified by column chromatography on silica gel 200–300 mesh using chloroform as an eluent, then by crystallization from methanol to give **6l**. Yield: 73.1% (3.0 g), m.p. 182–184°C; IR (nujol, cm^{-1}): 1470 (OCH_3), 1345 (C-N), 1270 (C-N). ^1H NMR (300 MHz, $CDCl_3$, δ, ppm): 0.80 (t, 3H, $OCH_2C\mathbf{H}_3$), 0.85 (t, 3H, $OCH_2C\mathbf{H}_3$), 1.40 (s, 3H, CH_3), 2.80 (q, 2H, $OC\mathbf{H}_2CH_3$), 2.90 (q, 2H, $OC\mathbf{H}_2CH_3$), 3.20 (s, 2H, CH_2), 3.30 (s, 2H, CH_2), 7.50–8.40 (m, 13H, arom.).

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