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SYNTHESIS AND ANTIBACTERIAL PROPERTIES OF PYRIMIDINE DERIVATIVES

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Abstract: In this study, a series of syntheses was conducted on the pyrimidine system, obtaining bisulfite carboxyl derivatives **4** and hydroxy derivatives **5**. In addition, a series of syntheses were carried out as a result of which both alkyl and aromatic amines were obtained. Then, the attempt was made to cyclize these amines in the Mannich reaction to pyrimido[4,5-d]pyrimidines **11**, **12**. After determination of chemical structure using physicochemical tests, also by means of crystallographic tests, all the newly obtained derivatives underwent microbiological tests on bacterial strains and fungi. The most interesting results of the microbiological tests are included later in the study.

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

Our earlier work on the synthesis and biological properties of the pyrimidine ring proved that this system is extremely active biologically. The derivatives obtained showed cytostatic (1, 2), immunomodulatory (3, 4), and most of all antibacterial (5–9) properties. Therefore, it was advisable to conduct a series of syntheses aimed at obtaining pyrimido-pyrimidine derivatives and subjecting them to microbiological tests. During synthesis of the new pyrimidine derivatives, it turned out completely unexpectedly that the pyrimido[4,5-d]pyrimidine system can be obtained using two methods fully independent of each other.

The substrate in our study was ethyl 4-methyl-2-phenyl-6-sulfanylpyrimidine-5-carboxylate (**1**), which, when heated in the presence of phenylhydrazine, condensed to bisulfite fusion of diethyl 4,4'-disulfanediylbis[6-(methyl-2-phenylpyrimidine-5-carboxlate) (**2**). Before that, similar fusions on ethyl ester were obtained by Brazilian scientists, Cunta and coworkers (10). Bisulfite ester **2** was subjected to $LiAlH_4$ a THF and CS_2 reduction, as a result of which tetrasulfite fusion with reduced carboxyl group down to hydroxyl group [tetrasulfane-1,4-diylbis-(methyl-2-phenylpyrimidine-4,5-diyl)]dimethanol (**3**) was obtained. Initial ester **1** was exposed to $SOCl_2$, thus yielding diethyl 4,4'-disulfanediylbis[6-(methoxycarbothionyl)-2-phenylpyrimidine-5-carboxylate] (**4**). Structures of **3** and **4** were confirmed using crystallographic tests. Initial substrate **1** melted with aromatic amines gave aminoesters **5** which, when hydrolyzed, changed into 5-carboxyl amino acids **6**. Both esters **5** and amino acids **6** were reduced with $LiAlH_4$ to 5-hydroxy derivatives **7**. Hydroxy derivatives **7**, under the influence of $SOCl_2$, gave 5-chloro derivatives **8** which, when condensed with aromatic amines, changed into 5-amino-substituted pyrimidines **10**.

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Fusions **10** were cyclized in the Mannich reaction to compounds **11** and **12** which showed strong antibacterial properties.

Ester **1** melted with aromatic amines yielded a series of derivatives ethyl-4-methyl-2-phenyl-6-aryloaminopyrimidine-5-carboxylate (**5**) which also hydrolyzed to 4-methyl-2-phenyl-6-arylaminopyrimidine-5-carboxylate acid (**6**). Ester **5** or acid **6** were reduced with $LiAlH_4$ to (4-methyl-2-phenyl)-6-aryloaminopyrimidin-5-yl-methanol (**7**). By exposing compound **7** to $SOCl_2$, pyrimidine chloroderivatives **8** were obtained. Compound **8** was treated with ammonia and aromatic amines, thus yielding compounds **9** and **10**, confirmed with crystallographic tests. The attempt to obtain compound **13** by means of the Mannich reaction was not successful.

EXPERIMENTAL

Chemistry

Melting points were determined in Köfler apparatus. ^{1}H NMR spectra were recorded on a BS-487-C-80 MHz Tesla spectrometer. Infrared (IR) spectra were recorded in nujol with a Specord spectrophotometer, at the Elemental Laboratory of the Medical University in Wrocław. Elemental analyses indicated by the symbols were within ± 0.4% of theoretical values.

[Tetrasulfane-1,4-diylbis-(6-methyl-2-phenylpyrimidine-4,5-diyl)]dimethanol (3)

Four grams (0.005 mol) of diethyl 4,4'-disulfanediylbis-(6-methyl-2-phenylpyrimidine)-5-carboxylate (**2**) was dissolved in anhydrous tetrahydrofuran (THF) and CS_2, and $LiAlH_4$ was added in small quantities until the solution stopped foaming. After this time, 100 mL of chloroform was added to the post-reaction mixture. This mixture was poured into 500 mL of cold water and extracted three times with 50 mL of chloroform. Chloroform extracts were combined and evaporated under vacuum; the oily reaction mixture was crystalized from methanol, yielding 4.5 g (47.5%) of crystals (**3**) with m.p. 145–147^{o}C.

The structure of compound **3** was confirmed with crystallographic tests and IR and ^{1}H NMR spectra. IR (KBr, cm^{-1}): 2560 (C-S), 650 (C-S). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.85 (s, 3H, CH_3), 3.5 (s, 1H, OH), 7.20–8.35 (m, 10 H, arom.).

Diethyl 4,4'-disulfanediylbis[6-(methoxycarbothioyl)-2-phenylpyrimidine-5-carboxylate] (4)

Four grams (0.014 mol) of ethyl 4-methyl-2-phenyl-6-sulfanylpyrimidine-5-carboxylate (**1**) was dissolved in 50.0 mL of benzene and 5.0 mL $SOCl_2$ (thionyl chloride) was added. The reaction mixture was refluxed for 3 h, Then, the excess of $SOCl_2$ was distilled off and the residue was recrystallized from methanol, yielding 6.1 g (56.5%) of crystals (**4**) with m.p. 165–166^{o}C.

The structure of compound **4** was confirmed with crystallographic tests and IR and ^{1}H NMR spectra. IR (KBr, cm^{-1}): 2560 (C-S), 650 (C-S). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.20 (t, 3H, $CH_2\underline{CH_3}$), 1.55 (s, 3H OCH_3), 2.50 (q, 3H, $\underline{CH_2}CH_3$), 1.85 (s, 3H, CH_3), 7.20–8.35 (m, 10 H, arom.).

Ethyl 4-methyl-2–phenyl-6{[3-(trifluoromethyl) phenyl]amino}pyrimidine-5-carboxylate (5a)

Four grams (0.014 mol) of ethyl 4-methyl-2-phenyl-6-sulfanylpyrimidine-5-carboxylate (**1**) was melted with 2.0 g of 3-trifluoroaniline for 4 h in a round-bottom flask under the reflux condenser. After this time, the reaction mixture was poured into 50.0 mL of methanol and cooled down. The precipitate was filtered and recrystalized from methanol, yielding 3.9 g (68%) of crystals (**5a**). M.p. 137–137^{o}C. IR (KBr, cm^{-1}): 3440 (NH), 940 (NH). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.20 (t. 3H, $CH_2\underline{CH_3}$), 2.50 (q, 3H, $\underline{CH_2}CH_3$), (s, 3H, CH_3), 3.85 (s, 1H NH), 7.20–8.35 (m, 9 H, arom.).

In a similar way, compound **5b** was obtained. IR (KBr, cm^{-1}): 3450 (NH), 9450 (NH). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.25 (t. 3H, $CH_2\underline{CH_3}$), 2.45 (q, 3H, $\underline{CH_2}CH_3$), 1.80 (s, 3H, CH_3); 7.20–8.35 (m, 9 H, arom.).

4-Methyl-2-phenyl-6-{[3-(trifluoromethyl) phenyl]amino}pyrimidine-5-carboxylic acid (6a).

Four grams (0.009 mol) of ethyl 4-methyl-2-phenyl-6-{[3-(trifluoromethyl)phenyl]amino}pyrimidine-5-carboxylate (**5a**) was dissolved in 10% methanol solution of NaOH. The mixture was refluxed in the round-bottom flask for 24 h. After this time, the solution was neutralized with HCl to pH = 7. The precipitate was filtered and recrystalized from methanol, yielding 3.4 g (92%) of crystals (**6a**). M.p. 205–206^{o}C. IR (KBr, cm^{-1}): 3440 (NH), 940 (NH). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.85 (s, 3H, CH_3), 3.85 (s, 1H NH), 7.20–8.35 (m, 9 H, arom.).
In the same way, compound **6b** was obtained. IR (KBr, cm^{-1}): 3445 (NH), 945 (NH). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.80 (s, 3H, CH_3), 3.80 (s, 1H, NH), 7.20–8.35 (m, 9 H, arom.).

4-Methyl-2-phenyl-6-{[3-(trifluoromethyl)phenyl]amino}(pyrimidin-5-yl)methanol (7)

Four grams (0.009 mol) of ethyl 4-methyl-2-phenyl-6-{[3-(trifluoromethyl)phenyl]amino}pyr-

imidine-5-carboxylate (**5a**) was dissolved in 50.0 mL THF and $LiAlH_4$ was added in small portions until the solution stopped foaming. After this time, 100.0 mL of chloroform was added to the reaction mixture and it was poured into water with ice in order to destroy any unreacted $LiAlH_4$. The solution was filtered and extracted three times with chloroform. Chloroform extracts were combined and dried with $MgSO_4$. The dried solution was concentrated under vacuum and the precipitade was filtered and crystalized from methanol yielding 2.3 g (65.5%) of crystals (**7**). M.p. 150–152°C. IR (KBr, cm^{-1}): 3445 (NH), 3450 (OH), 945 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.80 (s, 3H, CH_3), 2.50 (s, 1H, OH), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 9 H, arom.).

5-(Chloromethyl)-6-methyl-2-phenyl-N-[3-(trifluoromethyl)phenyl]pyrimidine (8)

Four grams (0.011 mol) of, (4-methyl-2-phenyl)-6-{[3-(trifluoromethyl)phenyl]amino}(pyrimidin-5-yl)methanol (**7**) was dissolved in 50.0 mL of benzene and 3.0 mL $SOCl_2$ was added. The reaction mixture was left for 24 h at room temperature. After this time, the excess of thionyl chlorine was distilled off and the oily residue was crystalized from chloroform, yielding 3.1 (74%) of crystals (**8**). M.p. 196–198°C. IR (KBr, cm^{-1}): 3445 (NH), 945 (NH), 725 (C-Cl). ^1H NMR ($CDCl_3$, δ, ppm): 1.80 (s, 3H, CH_3), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 9 H, arom.).

5-(Aminomethyl)-6-methyl-2-phenyl-N-[3-(trifluoromethyl)phenylpyrimidine-4-amine (9)

Four grams (0.010 mol) of (**8**) was dissolved in 50.0 mL of THF and 10.0 mL of 25% of ammonia solution was added. The mixture was intensively mixed in the round-bottom flask under the reflux condenser at 80°C for 8 h. After this time, the reaction mixture was poured into 500.0 mL of water and extracted three times with 50.0 mL of chloroform. Chloroform extracts were combined and dried with $MgSO_4$. The solution was filtered and the filtrate was concentrated under vacuum. The oily residue was cleaned by passing it through the chromatographic column filled with silica gel (200–300 mesh). Chloroform was distilled off from the eluate and the residue was recrystalized from methanol, yielding 3.1 g (82.0 %) of crystals (**9**) with m.p. 158–160°C. IR (KBr, cm^{-1}): 3445 (NH), 945 (NH), 725 (C-Cl). ^1H NMR ($CDCl_3$, δ, ppm): 1.80 (s, 3H, CH_3), 2.12 (s, 2H, NH_2), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 9 H, arom.).

5-{[(4-Ethoxyphenyl)amino]metyl}-N-(3-trifluoromethyl)phenyl-6-methyl-2-phenylpyrimidine-4-amine (10a)

Four grams (0.010 mol) of **(8)** was dissolved in 50.0 mL of THF and 2.0 g of p-phenetidine was added. The reaction mixture was refluxed at 80°C for 8 h. After this time, the solution was poured into 500.0 mL of cold water and extracted three times with 50.0 mL of chloroform. Chloroform extracts were combined and dried with $MgSO_4$. The solution was filtered and the filtrate was concentrated and cleaned by passing it through the chromatographic column filled with silica gel (200–300 mesh). The eluate was concentrated again and the precipitate was recrystalized from methanol, yelding 3.9 g (78.5%) of crystals (**10a**) with m.p. 120–121°C. IR (KBr, cm^{-1}): 3445 (NH), 945 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, $CH_2\underline{CH_3}$), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH), 2.45 (q, 3H, $\underline{CH_2}CH_3$), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 13 H, arom.).

Similarly, compounds: **10b–e** were obtained.

10b: IR (KBr, cm^{-1}): 3450 (NH), 940 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, $CH_2\underline{CH_3}$), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH), 2.45 (q, 3H, $\underline{CH_2}CH_3$), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 13 H, arom.).

10c: IR (KBr, cm^{-1}): 3445 (NH), 945 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, CH_3), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 13 H, arom.).

10d: IR (KBr, cm^{-1}): 3445 (NH), 945 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, $CH_2\underline{CH_3}$), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH), 2.45 (q, 3H, $\underline{CH_2}CH_3$), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 13 H, arom.).

10e: IR (KBr, cm^{-1}): 3440 (NH), 940 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t. 3H, $CH_2\underline{CH_3}$), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH), 2.45 (q, 3H, $\underline{CH_2}CH_3$), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 7.20–8.35 (m, 13 H, arom.).

3-(4-Ethoxyphenyl)-2-(4-nitrophenyl)-1[(3-trifluorometyl)phenyl]-5-methyl-7-phenyl-1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidine (11a)

Four grams (0.010 mol) of **(10a)** was dissolved in 50.0 mL of THF and 2.0 g of p-nitrobenzaldehyde was added. The reaction mixture was refluxed with intensive mixing at 80°C for 8 h. Then, the reaction mixture was poured into 500 mL of cold water and extracted three times with chloroform. Chloroform extracts were combined and dried with $MgSO_4$. The solution was filtered, concentrated under vacuum and cleaned by passing it through the chromatographic column filled with silica gel (200–300

mesh). The chloroform eluate was concentrated again and the precipitate was filtered and crystallized from methanol, yielding 5.2 g (81.5%) of crystals (**11a**) with m.p. 162–164°C. IR (KBr, cm^{-1}), 1325 (N). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, $CH_2\underline{CH_3}$), 1.80 (s, 3H, CH_3), 2.45 (q, 3H, $\underline{CH_2}CH_3$), 4.30 (s, 2H CH_2), 7.20–8.55 (m, 17 H, arom.).

In similar way, compounds **11b** and **11c** were obtained.

11b: IR (KBr, cm^{-1}): 1335 (N). ^{1}H NMR ($CDCl_3$, δ, ppm): 1.30 (t, 3H, $CH_2\underline{CH_3}$), 1.85 (s, 3H, CH_3), 2.55 (q, 3H, $\underline{CH_2}CH_3$), 4.40 (s, 2H CH_2), 7.30–8.65 (m, 17 H, arom.).

Table 1. Physical properties of the tested compounds.

Compound	R^1*	R^2*	Formula (m.w.)	M.p. [°C]	Yield [%]	Analysis			
						C	H	N	Cl
3			$C_{24}H_{22}N_4O_2S_4$ (526.71)	145–147	47.5	54.73 54.55	4.21 4.33	10.64 10.72	
4			$C_{36}H_{32}N_4O_6S_4$ (744.92)	165–166	56.5	58.04 58.22	4.33 4.38	7.52 7.62	
5a	3-CF_3		$C_{21}H_{18}F_3N_3O_2$ (401.38)	137–139	68.0	62.84 62.66	4.52 4.68	10.47 10.73	
5b	2-CF_3		$C_{21}H_{18}F_3N_3O_2$ (401.38)	145–147	69.5	62.84 63.02	4.52 4.33	11.26 11.51	
6a	3-CF_3		$C_{19}H_{14}F_3N_3O_2$ (373.32)	205–207	86.0	61.13 61.44	3.78 3.84	11.26 11.37	
6b	2-CF_3		$C_{19}H_{14}F_3N_3O_2$ (373.32)	200–202	82.5	61.13 61.24	3.78 3.41	11.26 1135	
7	3-CF_3		$C_{19}H_{16}F_3N_3O$ (359.34)	150–152	65.5	63.51 63.67	4.49 4.55	11.69 11.72	
8	3-CF_3		$C_{19}H_{15}ClF_3N_4$ (377.79)	196–198	74.0	60.40 60.42	4.00 4.12	11.12 11.31	
9	3-CF_3		$C_{19}H_{17}F_3N_4$ (358.36)	158–160	82.0	63.68 63.72	4.78 4.82	15.63 15.73	9.38 9.43
10a	3-CF_3	4-OC_2H_5	$C_{27}H_{25}F_3N_4O$ (478.50)	120–121	78.5	67.77 67.88	5.27 5.33	11.71 11.82	
10b	4-OC_2H_5	3-CF_3	$C_{27}H_{25}F_3N_4O$ (478.51)	116–117	72.0	67.77 67.42	5.27 5.12	11.71 12.02	
10c	4-OCH_3	4-CF_3	$C_{26}H_{23}F_3N_4O$ (464.48)	196–198	77.5	67.23 67.42	4.99 5.12	12.06 11.88	
10d	4-OC_2H_5	2-CF_3	$C_{27}H_{25}F_3N_4O$ (411.49)	128–130	79.0	67.77 67.62	5.27 5.22	11.71 11.62	
10e	4-OC_2H_5	C_6H_5N	$C_{25}H_{25}N_5O$ (411.49)	182–184	79.0	72.97 72.84	6.12 6.22	17.02 17.25	
11a	3-CF_3	4-OC_2H_5	$C_{34}H_{28}F_3N_5O_3$ (611.61)	162–165	81.5	66.77 66.45	4.61 4.72	11.45 11.55	
11b	4-OCH_3	4-CF_3	$C_{33}H_{26}F_3N_5O_3$ (597.58)	115–117	82.5	66.33 66.14	4.39 4.42	11.72 11.83	
11c	4-OC_2H_5	3-CF_3	$C_{34}H_{28}F_3N_5O_3$ (611.61)	127–128	77.5	66.77 66.53	4.61 4.58	11.45 11.32	
12a	3-CF_3	4-OC_2H_5	$C_{28}H_{25}F_3N_4O$ (490.52)	172–174	79.0	68.56 68.43	5.14 5.21	11.42 11.53	
12b	4-OCH_3	4-CF_3	$C_{27}H_{23}F_3N_4O$ (476.49)	156–157	83.5	68.06 67.93	4.87 4.92	11.76 11.83	
12c	4-OC_2H_5	3-CF_3	$C_{28}H_{25}F_3N_4O$ (490.52)	177–178	78.5	68.56 68.22	5.14 5.43	11.42 11.53	

* R^1 and R^2 see Scheme 1.

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

Microorganism	Compound			
	3	**4**	**10a**	Erythromycin
Bacillus subtilis PCM 2021 (ATCC 6633)	4	8	16	0.25
Escherichia coli PCM 2057 (ATCC 25922)	16	16	8	32
Klebsiella pneumoniae PCM 1 (ATCC 13886)	32	32	8	0.5
Proteus vulgaris PCM 542 (ATCC 13315)	32	32	4	128
Serratia marcescens PCM 549 (ATCC 274)	16	16	32	64
Pseudomonas aeruginosa PCM 2058 (ATCC 27853)	16	16	32	128
Enterococcus faecalis PMC 2673 (ATCC 29212)	8	8	16	4
Staphylococcus epidermidis PCM 2118 (ATC 14990)	4	4	8	8
Staphylococcus aureus PCM 1932 (ATCC 6538 P)	8	8	4	0.5
Candida albicans PCM 2566 (ATCC 10231)	4	16	16	256

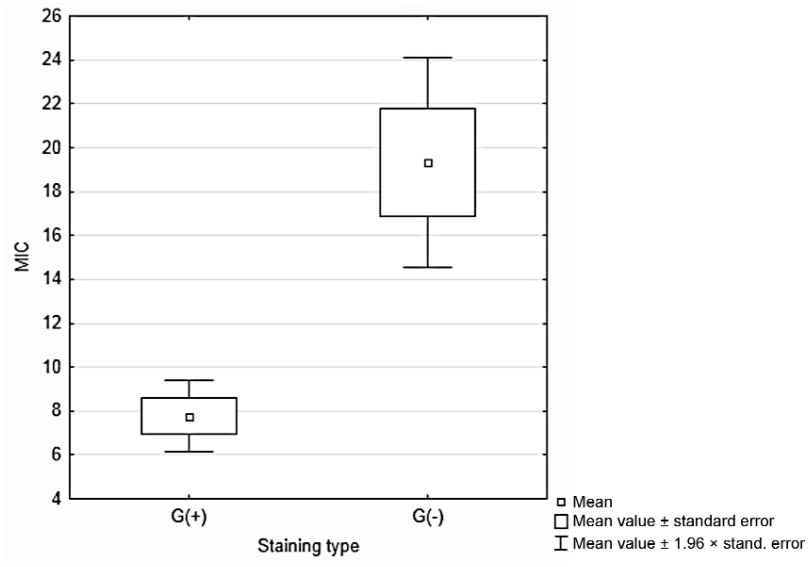


Figure 1. Effect of bacteria type depending on the color stain using the Gram method on MIC values

Table 3. Minimal inhibitory concentrations (MIC) (mg/mL), Testing M-7, A-5.

Microorganism	Compound			
	10d	**11a**	**12b**	Erythromycin
Bacillus subtilis PCM 2021 (ATCC 6633)	8	4	8	0.25
Escherichia coli PCM 2057 (ATCC 25922)	16	8	8	32
Klebsiella pneumoniae PCM 1 (ATCC 13886)	4	4	16	0.5
Proteus vulgaris PCM 542 (ATCC 13315)	8	8	32	128
Serratia marcescens PCM 549 (ATCC 274)	16	16	64	64
Pseudomonas aeruginosa PCM 2058 (ATCC 27853)	32	8	32	128
Enterococcus faecalis PMC 2673 (ATCC 29212)	16	2	8	4
Staphylococcus epidermidis PCM 2118 (ATC 14990)	8	4	8	8
Staphylococcus aureus PCM 1932 (ATCC 6538 P)	4	4	16	0.5
Candida albicans PCM 2566 (ATCC 10231)	8	2	4	256

Table 4. *t*-Tests; Grouping: Dyeing Group 1: G(+) Group 2 G(–).

	Mean – G(+)	Mean – G(–)	t	df	p	N valid – G(+)	N valid – G(–)	Std. var. – G(+)	Std. var. – G(–)	quotient F variance	p variances
MIC	7.76000	19.33333	4.167	53	0.000114	25	30	4.176123	13.33	10.191	1.74E-07

Table 5. Mann-Whitney U test; *Versus* variable: Dyeing Marked results are valid with $p < 0.05$.

	Sum. rang. – G(+)	Sum. rang. – G(–)	U	Z	p	Z – correct.	p	N valid – G(+)	N valid – G(–)	2 ×1 side – ccur. p
MIC	475.00	1065.	150.00	3.79	0.00014	-3.93455	0.000083	25	30	0.000082

Table 6. Wald-Wolfowitz (runs) test; *Versus* variable: Dyeing Marked results are valid with $p < 0.05$.

	N valid – G(+)	N valid – G(–)	Mean – G(+)	Mean – G(–)	Z	p	Z correct.	p	Number – runs	Number – connected
MIC	25	30	7.760000	19.33333	–2.820	0.0048	2.68284	0.0073	18	15

Table 7. Analysis of variances. Marked effects are valid with $p < .05$ Inclusion requirement: grouping variable = G(+).

	SS – Effect	df – Effect	MS – Effect	SS – Error	df – Error	MS – Error	F	p
MIC	226.8705	6	37.81176	302.6719	21	14.41295	2.623458	0.046649

11c: IR (KBr, cm^{-1}): 1325 (N). ^1H NMR ($CDCl_3$, δ, ppm): 1.30 (t, 3H, CH_3), 1.85 (s, 3H, CH_3), 4.40 (s, 2H CH_2), 7.30–8.65 (m, 17 H, arom.).

1-(4-Methoxyphenyl)-3-(4-trifluoromethylphenyl)-5-methyl-7-phenyl-1,2,3,4-tetrahydropyrimido[4,5-d]pyrimidine(1,4-d]pyrimidine (12c).

Four grams (0.008 mol) of (**10c)** was dissolved in 50.0 mL THF and 20.0 mL of formaldehyde was added. The reaction mixture was refluxed with intensive mixing for 8 h. After this time, the reaction mixture was poured into 500.0 mL of cold water and extracted three times with 50.0 mL of chloroform. The chloroform extracts were combined and dried with $MgSO_4$. The solution was filtered, concentrated under vacuum and cleaned preliminarily in the chromatographic column filled with silica gel (200–300 mesh). The obtained chloroform eluate was concentrated and crystalized from methanol, yielding 3.4 g (83.5%) of crystals (**12c**) with m.p. 156–157^oC.

IR (KBr, cm^{-1}): 3445 (NH), 945 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, CH_3), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 4.45 (s, 2H CH_2), 7.20–8.40 (m, 13 H, arom.).

In similar way, compounds **12a** and **12b** were obtained.

12a: IR (KBr, cm^{-1}): 3445 (NH), 945 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, $CH_2\underline{CH_3}$), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH), 2.45 (q, 3H, $\underline{CH_2}CH_3$), 3.80 (s, 1H NH), 4.35 (s, 2H CH_2), 4.50 (s, 2H CH_2), 7.20–8.45 (m, 13 H, arom.).

12b: IR (KBr, cm^{-1}): 3450 (NH), 940 (NH). ^1H NMR ($CDCl_3$, δ, ppm): 1.25 (t, 3H, $CH_2\underline{CH_3}$), 1.80 (s, 3H, CH_3), 2.12 (s, 1H, NH);, 2.45 (q, 3H, $\underline{CH_2}CH_3$), 3.80 (s, 1H NH), 4.30 (s, 2H CH_2), 4.45 (s, 2H CH_2), 7.20–8.50 (m, 13 H, arom.).

Microbiological methods

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) (11). 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 next were diluted with a sterile 1 : 10 PBS solution (giving 1×10^7 CFU / mL).

The obtained inoculum (0.01 mL) was added to 0.2 mL of sterile final dilutions of the investigated substances according to Table 1, 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.

Statistical methods used in microbiological tests

Based on the determined experimental data, the effect of two parameters was evaluated: the type of substituent of the tested chemical compound and type of bacteria depending on dyeing color using the Gram method

The obtained mean MIC values for seven tested compounds (6 compounds + erythromycin as a reference) were compared with each other using parametric analysis of ANOVA variances and non-parametric Kruskal-Wallis ANOVA analysis. Normality of distributions for analyzed random variables was checked using the Shapiro-Wilk and Kolmogorov-Smirnov tests with Lillefors correction at the assumed confidence level of $p = 0.05$. Uniformity of variances was determined using the Levene and Brown-Forsythe tests at the confidence level of $p = 0.05$. In order to establish differences between two mean values, parametric Student t-test was used for independent samples and its non-parametric equivalents: the Mann-Whitney U test and the Wald-Wolfowitz (runs) test. For all completed statistical tests, confidence level of $p = 0.05$ was assumed.

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

Microbiological tests were conducted on 4 bacteria species G(+) and 5 bacteria species G(–). Using parametric t test for independent samples and non-parametric tests: the Mann-Withney U and Wald-Wolfowitz tests, the statistical validity of differences between mean MIC values obtained in both groups was evaluated, and results were summarized in Tables 1–3 and in Figure 1.

Effect of the substituent type on mean MIC values

Mean MIC values determined for six tested compounds were compared using parametric analysis of variances together with *post-hoc* NIR tests. Normality of distributions for analyzed random variables was confirmed with the Shapiro-Wilk and Kolmogorov-Smirnov tests with Lillefors correction at the assumed confidence level of $p = 0.05$, and uniformity of variances using the Brown-Forsythe test at the confidence level of $p = 0.05$.

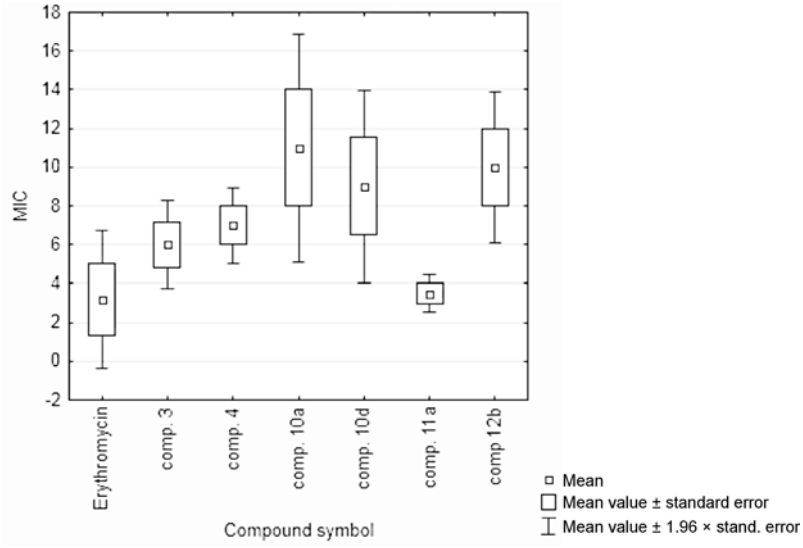


Figure 2. Effect of substituent type on mean MIC values of bacteria G(+)

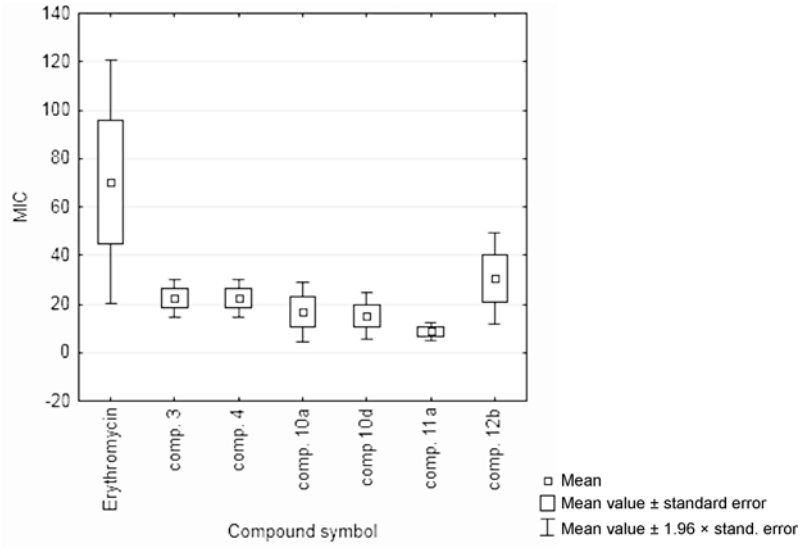


Figure 3. Effect of substituent type on mean MIC values for bacteria G(–)

The analysis was carried out separately for bacteria G(+) and G(–), and results were summarized in tables and figures

The completed statistical analysis showed that with the assumed confidence level of p = 0.05 both the type of substituent and type of bacteria depending on the dyeing color using the Gram method had a statistically valid effect on the experimentally determined mean MIC values.

1. The tested chemical compounds showed higher, statistically valid antibacterial effectiveness for bacteria G(+) compared to bacteria G(–) (Fig. 1 and Tables 1–3).

2. All tested compounds showed higher, statistically valid antibacterial effectiveness for bacterial G(–) compared to the standard substance which was erythromycin, and not statistically valid differences were observed in antibacterial effectiveness for bacteria G(–) between them (Fig. 3, Tables 6, 7).

3. The highest antibacterial effectiveness for bacteria G(–) was shown by compound **11a**.

4. No statistically valid difference was found between antibacterial effectiveness for bacteria G(–) for erythromycin and compound: **3**, **4** and **11a** (Table 7).

5. Out of all tested compounds, the strongest antibacterial properties for the analyzed bacteria were shown by compound **11a**.

CONCLUSIONS

Seventeen newly obtained pyrimidine derivatives were tested microbiologically on 9 bacterial strains and one fungal strain: *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Serratia marcescens, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus epider-*

Table 8. NIR test. Marked differences are valid with $p < 0.05$ Inclusion requirement: grouping variable = G(+).

Compound	{1} – M = 3.1875	{2} – M = 6.0000	{3} – M = 7.0000	{4} – M = 11.000	{5} – M = 9.0000	{6} – M = 3.5000	{7} – M = 10.000
Erythr. {1}		–	–	+	+	–	+
Compd. **3** {2}	–		–	-	–	–	–
Compd. **4** 3}	–	–		–	–	–	–
Compd. **10a** {4}	+	–	–		–	+	–
Compd. **10d** {5}	+	–	–	–		–	–
Compd. **11a** {6}	–	–	–	+	–		+
Compd. **12b** {7}	+	–	–	-	–	+	

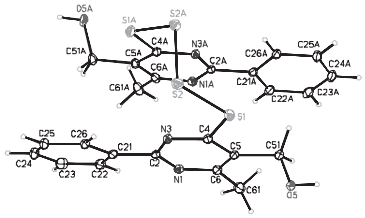
Erythr. – erythromycin

Table 9. Analysis of variances; Marked effects are valid with $p < .05$; Inclusion requirement: grouping variable = G(–).

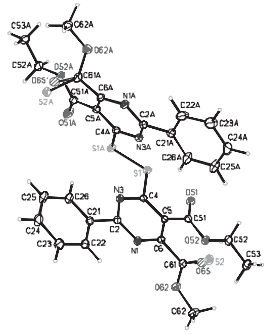
	SS – Effect	df – Effect	MS – Effect	SS – Error	df – Error	MS – Error	F	p
MIC	12598.79	6	2099.798	16813.00	28	600.4643	3.496957	0.010449

Table 10. NIR test; Marked differences are valid with $p < 0.05$; Inclusion requirement: grouping variables = G(–).

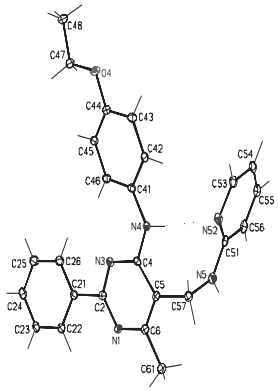
Compound	{1} – M = 70.500	{2} – M = 22.400	{3} – M = 22.400	{4} – M = 16.800	{5} – M = 15.200	{6} – M = 8.8000	{7} – M = 30.400
Erythromycin {1}		+	+	+	+	+	+
3 {2}	+		–	–	–	–	–
4 {3}	+	–		–	–	–	–
10a {4}	+	–	–		–	–	–
10d {5}	+	–	–	–		–	–
11a {6}	+	–	–	–	–		–
12b {7}	+	–	–	–	–	–	



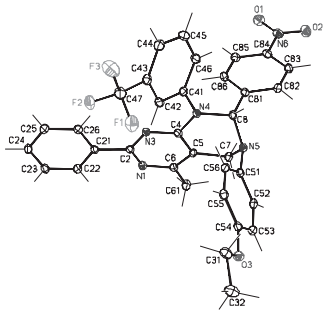
compound **3**



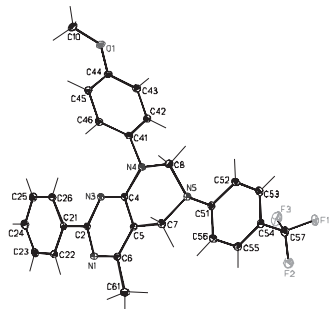
compound **4**



compound **10e**



compound **11a**

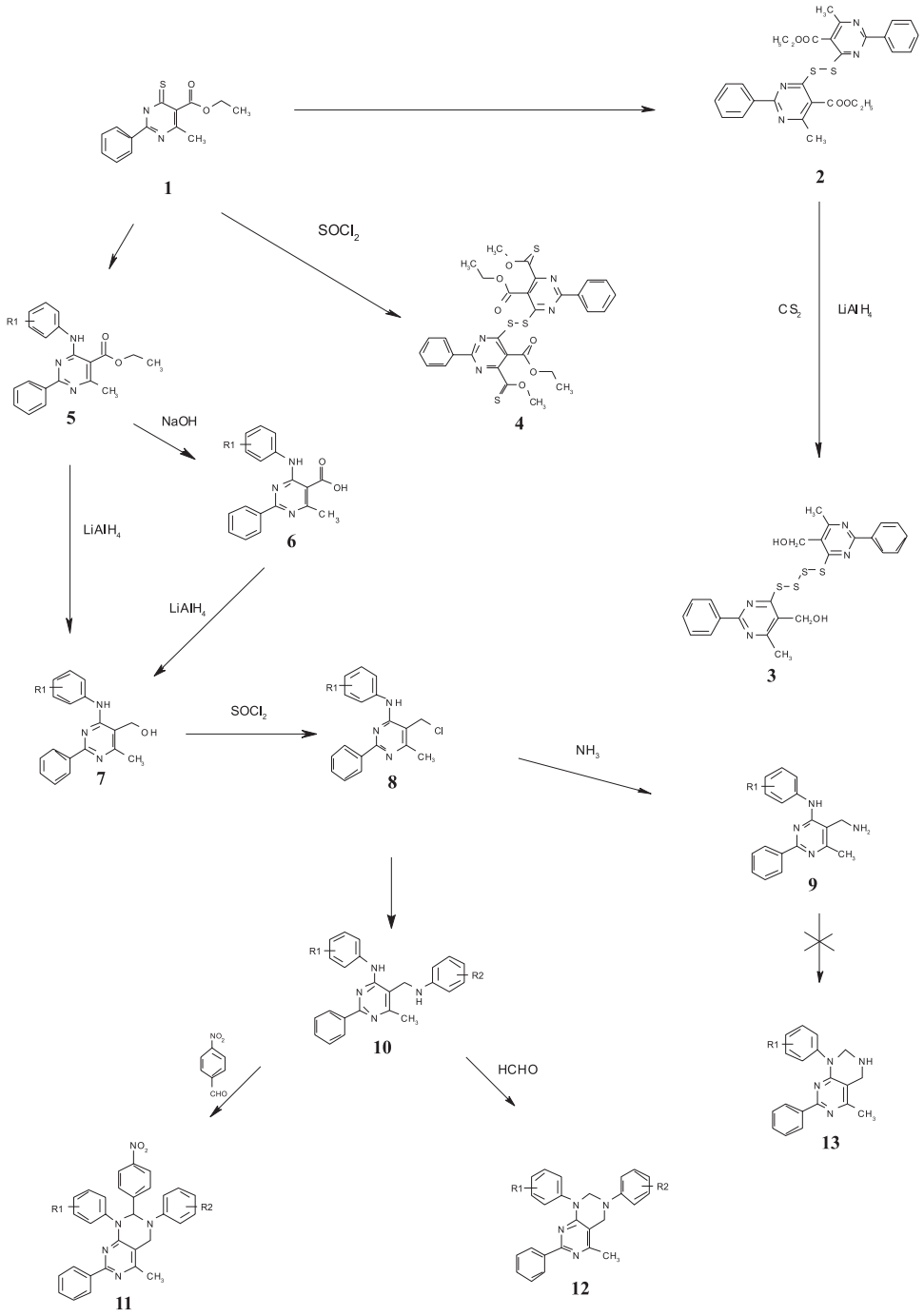


compound **12b**

Figure 4. Crystallographic structures of selected synthesized compounds

midis, Staphylococcus aureus and Candida albicans. Some of the obtained compounds showed extremely interesting antibacterial activity.

Pyrimidine bibonding with multiple sulfide bridge showed interesting microbiological activity which was affected also by the functional group in position 5. Higher microbiological activity was shown by sulfide compounds (**3**, **4**). Antibacterial activity was shown also by 5-amine pyrimidine derivatives (compounds **10a** and **10d**), and their activity to a considerable extent depends on substituted amine in position 5, which undoubtedly causes better solubili-



Scheme 1. Synthesis of pyrimidine derivatives

ty of the given compound. Cyclization of amines **10** in the Mannich reaction to pyrimido[4,5-d]pyrimidine moieties (**11**) increases their microbiological activity which becomes slightly weaker, if nitrophenyl substituent (**12**) is missing in the pyrimido[4,5-d]pyrimidine system in position 2 .

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