Synthesis and dihydrofolate reductase inhibitory activity of 2-amino-6-(arylaminomethyl)thieno[2,3-d]pyrimidin-4(3H)-ones

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The present work deals with synthesis and biological evaluation of novel compounds as potential inhibitors of dihydrofolate reductase (DHFR) from opportunistic microorganisms – *Pneumocystis carinii* (pc), *Toxoplasma gondii* (tg) and *Mycobacterium avium* (ma). A set of 6 lipophilic and 1 classical antifolates of thieno[2,3-d]pyrimidine series were designed and synthesised with key pharmacophoric features of DHFR inhibitors and aminomethylene bridge joining the aromatic part and 2-amino-4-oxothieno[2,3-d]pyrimidine nucleus. Investigation of the DHFR inhibitory activity of the synthesised compounds revealed that 2-amino-6-[(4-methoxyphenylamino)-, 6--[(4-chlorophenylamino)- and 6-[(2,5-dichlorophenylamino)methyl]thieno[2,3-d]pyrimidin-4(3H)-ones had a moderate activity (IC$_{50}$ 56.5, 49.9 and 23 μM, respectively) against pcDHFR. Activity of those compounds against tgDHFR (IC$_{50}$ 6.0, 2.8 and 2.3 μM, respectively) was comparable to that of trimethoprim (IC$_{50}$ 2.7 μM). No synthesised lipophilic antifolates showed activity towards maDHFR. The classical antifolate 2-{4-[(2-amino-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-6-yl)methylamino]benzamido}-L-glutamic acid inhibited DHFRs from all microorganisms (IC$_{50}$ 6.6 μM for pcDHFR, 1.06 μM for tgDHFR and 16.9 μM for maDHFR).

Keywords: thieno[2,3-d]pyrimidines, synthesis, dihydrofolate reductase inhibitors, lipophilic antifolates, classical lipofolates

INTRODUCTION

Dihydrofolate reductase (DHFR) and thymidylate synthetase (TS) are enzymes, which play an essential role in nucleic acid synthesis as take part in the biosynthesis of pyrimidine and purine nucleotides. DHFR catalyses the reduction reaction of dihydrofolate to tetrahydrofolate by using NADPH, while TS catalyses the synthesis of deoxythymidine monophosphate from deoxyuridine monophosphate using N^5, N^{10}-methylenetetrahydrofolate, and thus initiates DNA synthesis and cell proliferation [1, 2]. Inhibition of these enzymatic reactions by folate antagonists, the so-called antifolates, leads to an intracellular depletion of nucleotides, disturbance of DNA biosynthesis, and thus apoptotic cell death. The essential role of DHFR in the control of intracellular concentrations of nucleotides makes DHFR a perfect target to achieve antitumor, antimicrobial and antiprotozoal therapeutic effects. Structurally, antifolates are divided into two groups according to whether they have a glutamic acid moiety as in folic acid or not. Those with a glutamic acid moiety are called classical antifolates, while antifolates without glutamic acid residue are called lipophilic

antifolates. The main pharmacophore of classical and lipophilic antifolates is pyrimidine or fused pyrimidine ring system bearing an amino group in the position 2 and oxo or amino groups in the position 4 of the pyrimidine moiety. Of course, inhibitory activity also depends on the origin and structure of a linker between aromatic and heterocyclic parts of a molecule and substituents at the aromatic moiety. Classical antifolates such as methotrexate, lometrexol and pemetrexed (Fig. 1) provide a utility for cancer treatment.

Lipophilic antifolates, for example, trimethoprim, pyrimethamine, trimetrexate and piritrexim are used to treat microbial, protozoal and fungal infections caused by opportunistic microorganisms *Pneumocystis carinii* (pc), *Toxoplasma gondii* (tg) and *Micobacterium avium* (ma) which pose a great threat to individuals with the compromised immunosystem such as immunosupressed organ transplant recipients, AIDS patients, and patients receiving cancer chemotherapy [3].

Some of the known DHFR and TS inhibitors have several drawbacks, such as the dose-limiting toxicities, low solubility, low specificity, short plasma half-life, low absorption and drug resistance development [2–5]. Therefore, development of novel antifolates remains relevant and has interest for various research groups [2, 5, 6–11]. Thieno[2,3-*d*]pyrimidine heterocycle also attracts attention in that respect [12–15]. Thieno[2,3-*d*]pyrimidine scaffold with a linker attached to the position 6 of heterocycle provides a geometry similar but slightly different to that of folic acid as a sulfur atom is isosteric to two methine groups. Taking this into account and as a continuation of our program aimed for the development of synthesis methods of pyrimidine nucleous containing heterocycles [16–20], we present herein the synthesis of novel 2-amino-6-(arylaminomethyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-ones and results of their inhibitory activity on DHFR isolated from *Pneumocystis carinii*, *Toxoplasma gondii* and *Micobacterium avium*. Considering the structure of the known antifolates with good activities, the substitution pattern of the benzene ring with substituents such as 4-methoxy, 2,5-dimethoxy, 3,4,5-trimethoxy, 4-chloro, 2,5-dichloro, 2-naphthyl and 4-carbonyl-L-glutamic acid has been chosen (Fig. 3).
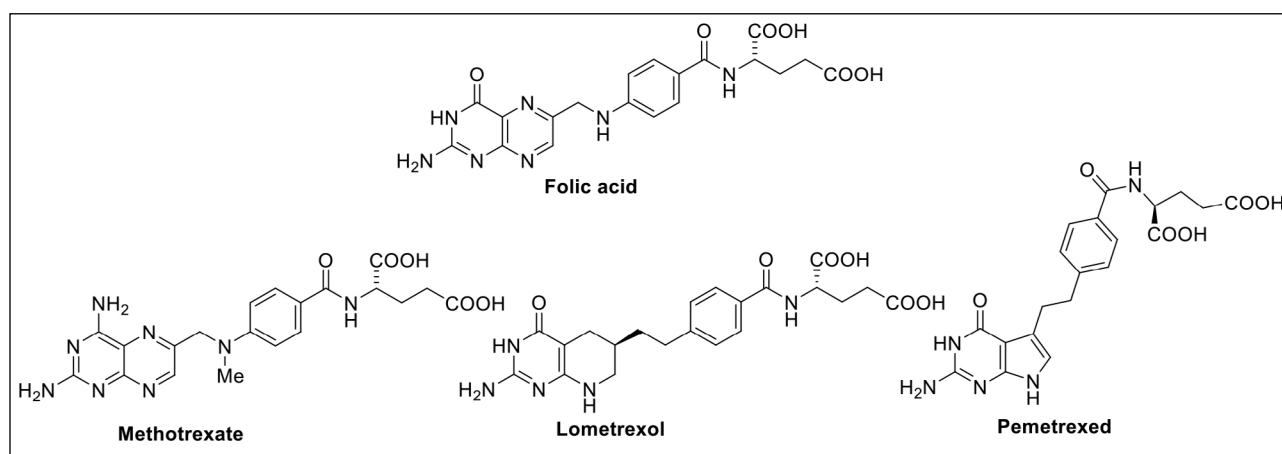

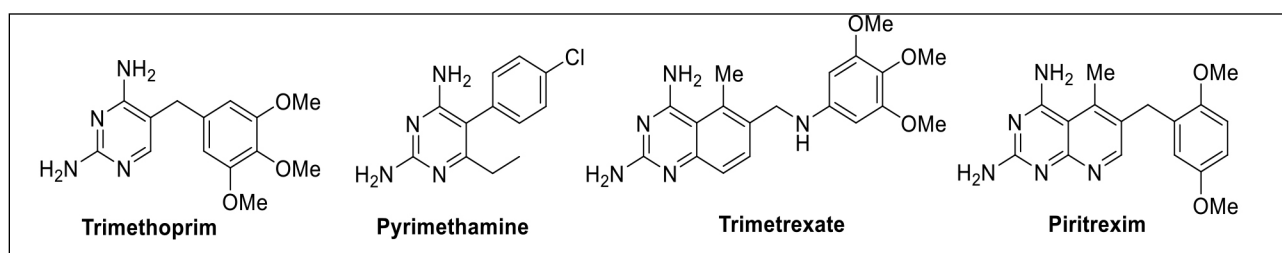**Fig. 1.** Structures of folic acid and some classical antifolates

Fig. 2. Structures of some lipophilic antifolates

R = 4-OMe, 2,5-(OMe)₂, 3,4,5-(OMe)₃, 4-Cl, 2,5-(Cl)₂, 3,4-(CH=CH)₂, 4-CO-L-Glu
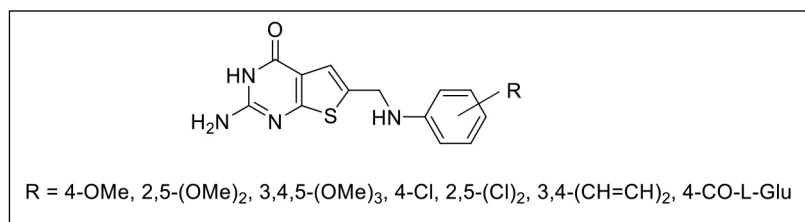
Fig. 3. General structure of thieno[2,3-*d*]pyrimidine antifolates

EXPERIMENTAL

Melting points were determined in open capillaries with a digital melting point IA9100 series apparatus (ThermoFischer Scientific). All the reactions and purity of the synthesised compounds were monitored by TLC using Silica gel 60 F$_{254}$ aluminum plates (Merck). Visualisation was accomplished by UV light. Column chromatography was performed using Silica gel 60 (0.040–0.063 mm) (Merck). IR spectra were run on a Perkin-Elmer FT-IR spectrophotometer Spectrum BX II and are reported in cm^{-1}. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA spectrometer (300 MHz) in dimethylsulfoxide-D$_{6}$ (unless stated otherwise). Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane using tresidual solvent peaks as internal standards. Elemental analyses were performed at the Elemental Analysis Laboratory of the Department of Organic Chemistry of Vilnius University.

Spectrophotometric assays used to determine the DHFR potency were kindly performed by prof. Sherry F. Queener (Indiana University School of Medicine) and have been reported in detail previously [21–23].

(2-Amino-4-methoxythieno[2,3-d]pyrimidin-6-yl)metanol (**1**) and 2-amino-6-(hydroxymethyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one (**5**) were synthesised following the procedures described in Ref. [20].

2-Amino-4-methoxythieno[2,3-*d*]pyrimidine-6-carbaldehyde (2). Method A. Cerium ammonium nitrate (0.47 g; 0.86 mmol) was added to a solution of (2-amino-4-methoxythieno-[2,3-*d*]pyrimidin-6-yl)methanol (**1**) (80 mg; 0.39 mmol) in a mixture of acetonitrile (4.5 mL) and water (0.5 mL). The suspension was stirred at room temperature for 1.5 h. Then conc. ammonia (10 mL) was added. Brown precipitate was filtered off and

the filtrate was concentrated under reduced pressure to dryness. Recrystallisation of the obtained solid afforded 10 mg (13%) of compound **2** as off-white solid; m.p. 204–207°C (from 1,4-dioxane); IR (Nujol): 3314, 3172 (NH₂), 1654 (CO); ^{1}H NMR: 4.01 (s, 3H, OCH₃), 7.42 (br.s, 1H, NH₂), 8.13 (s, 1H, C₅-H), 9.86 (s, 1H, CHO); ^{13}C NMR: 54.6, 111.3, 132.8, 133.3, 163.3, 166.5, 174.1, 185.4.

Anal. calcd. for C₈H₇N₃O₂S: C, 45.92; H, 3.37; N, 20.08. Found: C, 45.77; H, 3.47; N, 19.81.

Method B. To a solution of 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one (DMP) (1.04 g, 2.47 mmol) in dimethylformamide (11 mL) a drop of water and (2-amino-4-methoxythieno[2,3-*d*]pyrimidin-6-yl)methanol (**1**) (0.47 g, 2.33 mmol) was added. The mixture was stirred for 1 h at room temperature and quenched with a solution of sodium thiosulfate (0.391 g, 2.47 mmol) and sodium hydrocarbonate (0.934 g, 11.1 mmol) in water (*ca.* 50 mL). The resulted precipitate was filtered off, washed with water (3 × 10 mL) and methanol (3 × 5 mL) to give 0.43 g (91%) of compound **2** whose properties were identical to those of the product obtained by method A. The substance was pure enough to use further without purification.

4-Methoxy-6-(arylaminomethyl)thieno[2,3-*d*]pyrimidin-2-amines (3a–c). General procedure. To a stirred suspension of 2-amino-4-methoxythieno[2,3-*d*]pyrimidine-6-carbaldehyde (**2**) (0.200 g, 0.956 mmoles), sodium cyanoborohydride (66.1 mg, 1.05 mmol) and a solution of the corresponding aniline (1.05 mmol) in methanol (5 mL) was added. The flask was capped with rubber septum and flushed with argon. The reaction mixture was adjusted to pH 6 with conc. hydrochloric acid and stirred at room temperature for 1–1.5 h. Then, the reaction mixture was adjusted to pH 8 with a conc. aqueous ammonia solution or a saturated sodium hydrocarbonate

solution. Volatiles were removed under reduced pressure. The resulted solid was washed with water (3 × 5 mL) and methanol (1 mL), purified, dried in a Fisher drying pistol and stored under argon.

4-Methoxy-6-[(4-methoxyphenylamino)methyl]thieno[2,3-*d*]pyrimidin-2-amine (3a). The reaction time was 1 h 15 min. Compound **3a** was purified by column chromatography (R_f = 0.50; diethylether:chloroform, 1:1); colourless solid, yield 0.263 g (87%); m.p. 139–140°C (dec.); IR (KBr): 3490, 3315 (NH_2, NH); ^{1}H NMR: 3.63 (s, 3H, $C_{4'}$-OCH_3), 3.95 (s, 3H, C_4-OCH_3), 4.37 (d, *J* = 5.7 Hz, 2H, CH_2), 5.88 (t, *J* = 5.7 Hz, 1H, NH), 6.61 (d, *J* = 9.0 Hz, 2H, $C_{2';6'}$-H), 6.63 (br.s, 2H, NH_2), 6.72 (d, *J* = 9.0 Hz, 2H, $C_{3';5'}$-H), 7.03 (s, 1H, C_5-H); ^{13}C NMR: 44.0, 53.9, 56.0, 110.6, 114.4, 115.2, 115.6, 136.9, 143.0, 151.8, 161.1, 164.1, 170.9.

Anal. calcd. for $C_{15}H_{16}N_4O_2S$: C, 56.94; H, 5.10; N, 17.71. Found: C, 56.64; H, 5.28; N, 17.48.

6-[(2,5-Dimethoxyphenylamino)methyl]-4-methoxythieno[2,3-*d*]pyrimidin-2-amine (3b). Reaction time 1.5 h, column chromatography (R_f = 0.59; diethylether:chloroform, 1:1); colourless solid, yield 0.189 g (57%); m.p. 160–162°C (dec.); IR (KBr): 3510, 3379, 3306 (NH_2, NH); ^{1}H NMR: 3.60 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.95 (s, 3H, C_4-OCH_3), 4.46 (d, *J* = 6.0 Hz, 2H, CH_2), 5.74 (t, *J* = 6.0 Hz, 1H, NH), 6.09 (dd, *J* = 8.6 Hz, *J* = 2.9 Hz, 1H, $C_{4'}$-H), 6.17 (d, *J* = 2.9 Hz, 1H, $C_{6'}$-H), 6.66 (br.s, 2H, NH_2), 6.71 (d, *J* = 8.6 Hz, 1H, $C_{3'}$-H), 7.04 (s, 1H, C_5-H); ^{13}C NMR: 43.0, 53.9, 55.6, 56.6, 98.8, 99.1, 110.6, 111.2, 115.8, 136.6, 139.0, 141.9, 154.8, 161.1, 164.1, 170.9.

Anal. calcd. for $C_{16}H_{18}N_4O_3S$: C, 55.48; H, 5.24; N, 16.17. Found: C, 55.38; H, 5.38; N, 16.27.

4-Methoxy-6-[(3,4,5-trimethoxyphenylamino)methyl]thieno[2,3-*d*]pyrimidin-2-amine (3c). The reaction time was 1 h. Compound **3c** was purified by column chromatography (R_f = 0.59; diethylether:chloroform, 1:1); grey solid, yield 0.288 g (80%). Physical characteristics, IR and NMR spectra of compound **3e** were identical to those reported by us earlier [24].

6-[(4-Chlorophenylamino)methyl]-4-methoxythieno[2,3-*d*]pyrimidin-2-amine (3d). Reaction time 2 days, yellowish solid, yield 0.175 g (57%); m.p. 161–165°C (from 2-propanol); IR (KBr) 3471, 3350, 3320 (NH_2, NH); ^{1}H NMR: 3.96 (s, 3H, C_4-OCH_3), 4.42 (d, *J* = 6.0 Hz, 2H, CH_2), 6.51 (t, *J* = 6.0 Hz, 1H, NH), 6.67 (br. s, 2H, NH_2),

6.66 (d, *J* = 9.0 Hz, 2H, $C_{2';6'}$-H), 7.05 (s, 1H, C_5-H), 7.11 (d, *J* = 9.0 Hz, 2H, $C_{3';5'}$-H); ^{13}C NMR: 43.1, 54.0, 110.5, 114.6, 116.1, 120.3, 129.3, 135.9, 147.8, 161.2, 164.1, 170.9.

Anal. calcd. for $C_{14}H_{13}ClN_4OS$: C, 52.42; H, 4.08; N, 17.46. Found: C, 52.37; H, 4.12; N, 17.28.

6-{[(2,5-Dichlorophenyl)amino]methyl}-4-methoxythieno[2,3-*d*]pyrimidin-2-amine (3e). A stirred suspension of 2-amino-4-methoxythieno[2,3-*d*]pyrimidine-6-carbaldehyde (**2**) (0.30 g, 1.43 mmol) and 2,5-dichloroaniline (0.70 g, 4.32 mmol) in dry benzene (10 mL) was flushed with argon and through rubber septum titanium tetrachloride (0.19 g, 1.0 mmol) was syringed. After stirring overnight at room temperature, the reaction mixture was quenched with methanol (0.1 mL) and volatiles were removed under reduced pressure. The remaining solid was suspended in methanol (10 mL) and sodium cyanoborohydride (0.45 g, 7.17 mmoles) was added. Stirring was continued at room temperature overnight. Then the mixture was adjusted to pH 8 by dropwise addition of a conc. aqueous ammonia solution. Then volatiles were removed under reduced pressure. The remaining solid was well washed with water, purified on a short silica gel plug (benzene:acetone 10:1) and dried in a Fisher drying pistol to give 0.20 g (41%) of **3e** as a yellowish amorphous solid, m.p. 159–161°C; R_f = 0.51 (benzene:acetone, 10:1); IR (KBr) 3491, 3382, 3299 (NH_2, NH); ^{1}H NMR: 3.95 (s, 3H, C_4-OCH_3), 4.58 (d, *J* = 6.0 Hz, 2H, CH_2), 6.53 (t, *J* = 6.0 Hz, 1H, NH), 6.62 (dd, *J* = 8.4 Hz, 2.4 Hz, 1H, $C_{4'}$-H), 6.71 (br.s, 2H, NH_2), 6.79 (d, *J* = 2.4 Hz, 1H, $C_{2'}$-H), 7.08 (s, 1H, C_5-H), 7.28 (d, *J* = 8.4 Hz, 1H, $C_{3'}$-H); ^{13}C NMR: 42.5, 54.0, 110.4, 111.8, 116.4, 117.0, 117.4, 130.9, 133.2, 135.0, 145.3, 161.2, 164.1, 170.9.

Anal. calcd. for $C_{14}H_{12}Cl_2N_4OS$: C, 47.33; H, 3.40; N, 15.77. Found: C, 47.58; H, 3.40; N, 15.55.

2-Amino-6-[(arylamino)methyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-ones (4a, b, d, e). General procedure. A mixture of the corresponding compound **3a–d** (0.3 mmol) and conc. hydrochloric acid (5 mL) was refluxed until completion of the reaction. The resulted solid was filtered off, dissolved in water and then neutralised with a saturated sodium hydrocarbonate solution. The precipitate was collected by filtration, washed with water (2 × 5 mL) and diethyl ether (2 × 5 mL), purified, dried in a Fisher drying pistol and stored under argon.

2-Amino-6-[(4-methoxyphenylamino)methyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (4a). The reaction time 1.5 h, column chromatography (R*f* = 0.59; chloroform:acetone, 1:1); grey solid, yield 79 mg (87%), m.p. 214–215°C (dec.); IR (KBr): 3364, 3325, 3239 (NH$_2$, NH), 1666 (CO); ^{1}H NMR: 3.63 (s, 3H, C$_{4'}$-OCH$_3$), 4.29 (d, *J* = 5.7 Hz, 2H, CH$_2$), 5.83 (t, *J* = 5.7 Hz, 1H, NH), 6.49 (br.s, 2H, NH$_2$), 6.59 (d, *J* = 8.4 Hz, 2H, C$_{2';6'}$-H), 6.71 (d, *J* = 9.0 Hz, 2H, C$_{3';5'}$-H), 7.00 (s, 1H, C$_5$-H); 10.86 (br.s, 1H, NH); ^{13}C NMR: 43.8, 55.9, 114.4, 115.2, 115.9, 118.6, 135.4, 143.0, 151.7, 153.6, 158.6, 168.2.

Anal. calcd. for C$_{14}$H$_{14}$N$_4$O$_2$S: C, 55.61; H, 4.67; N, 18.53. Found: C, 55.52; H, 4.69; N, 18.33.

2-Amino-6-[(2,5-dimethoxyphenylamino)methyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (4b).

The reaction time 1.5 h, grey solid, 58 mg (58%), m.p. 232–233°C (dec.) (from a mixture DMF-H$_2$O); IR (KBr): 3398, 3330, (NH$_2$, NH), 1665 (CO); ^{1}H NMR: 3.62 (s, 3H, OCH$_3$), 3.75 (s, 3H, OCH$_3$), 4.41 (d, *J* = 5.7 Hz, 2H, CH$_2$), 5.69 (t, *J* = 5.7 Hz, 1H, NH), 6.09 (dd, *J* = 8.9 Hz, *J* = 2.7 Hz, 1H, C$_{4'}$-H), 6.18 (d, *J* = 2.7 Hz, C$_{6'}$-H), 6.51 (br.s, 2H, NH$_2$), 6.71 (d, *J* = 8.9 Hz, 1H, C$_{3'}$-H), 7.03 (s, 1H, C$_5$-H), 10.88 (br.s, 1H, NH); ^{13}C NMR: 42.7, 55.6, 56.6, 98.8, 99.1, 111.2, 115.9, 118.8, 135.1, 139.0, 141.9, 153.7, 154.8, 158.6, 168.3.

Anal. calcd. for C$_{15}$H$_{16}$N$_4$O$_3$S: C, 54.20; H, 4.85; N, 16.86. Found: C, 54.39; H, 4.76; N, 16.66.

2-Amino-6-[(4-chlorophenylamino)methyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (4d).

The reaction time 1.5 h, column chromatography (R*f* = 0.20; chloroform:acetone, 4:1); yellowish solid, 66 mg (72%), m.p. 254–256°C (dec.); IR (Nujol): 3461, 3372 (NH$_2$), 3314 (NH), 1664 (CO); ^{1}H NMR: 4.38 (s, 2H, CH$_2$), 6.53 (br.s, 3H, NH, NH$_2$), 6.66 (d, *J* = 7.4 Hz, 2H, C$_{2';6'}$-H), 7.04 (s, 1H, C$_5$-H), 7.11 (d, *J* = 7.4 Hz, 2H, C$_{3';5'}$-H), 10.88 (br.s, 1H, NH); ^{13}C NMR: 42.9, 114.7, 115.9, 119.1, 120.3, 129.3, 134.4, 147.8, 153.7, 158.6, 168.4.

Anal. calcd. for C$_{13}$H$_{11}$ClN$_4$OS: H, 3.61; N, 18.26. Found: H, 3.97; N, 18.06.

2-Amino-6-[(2,5-dichlorophenylamino)methyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (4e). The reaction time 4 h, yellowish solid, 62 mg (61%), m.p. 250°C (dec) (from a mixture DMF-H$_2$O); IR (Nujol) 3456, 3367 (NH$_2$), 3310 (NH), 1663 (CO); ^{1}H NMR: 3.96 (br.s, 2H, CH$_2$), 5.61 (br.s, 1H, NH), 6.53 (br.s, 3H, NH$_2$, C$_{4'}$-H), 6.78 (s, 1H, C$_{3'}$-H); 6.86 (s, 1H, C$_5$-H), 7.27 (s, 1H, C$_{6'}$-H), 10.89 (br.s, 1H,

NH); ^{13}C NMR: 33.0, 115.6, 115.9, 116.5, 119.1, 125.6, 131.5, 132.4, 133.7, 145.5, 153.7, 158.6, 168.3.

Anal. calcd. for C$_{13}$H$_{10}$Cl$_2$N$_4$OS: C, 45.76; H, 2.95; N, 16.42. Found: C, 45.78; H, 3.25; N, 16.31.

2-Amino-6-formylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (6). 2-Amino-6-(hydroxymethyl)thieno-[2,3-*d*]pyrimidin-4(3*H*)-one (**5**) (1.12 g, 5.62 mmol) was added to a solution of 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one (DMP) (2.63 g, 6.20 mmol) in dimethylformamide (43 mL). A drop of water was added and the mixture was stirred for 3 h at room temperature and quenched with a solution of sodium thiosulfate (0.980 g, 6.20 mmol) and sodium hydrocarbonate (2.34 g, 27.9 mmol) in water (*ca.* 100 mL). The resulted solid was collected by filtration, washed with water (3 × 15 mL) and methanol (3 × 5 mL) to give 0.90 g, (81%) of **17** as a colourless solid. The substance was pure enough to use further without purification. The analytical sample was prepared by recrystallisation from a mixture of DMF and water. Physical characteristics, IR and NMR spectra of compound **6** were identical to those reported by us earlier [20].

2-Amino-6-[(3,4,5-trimethoxyphenylamino)methyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (4c). Sodium cyanoborohydride (37.7 mg, 0.60 mmol) was added to a stirred suspension of 2-amino-6-formylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**6**) (0.10 mg, 0.512 mmol) and 3,4,5-trimethoxyaniline (0.137 g, 0.750 mmol) in methanol (5 mL). The flask was capped with rubber septum and flushed with argon. The reaction mixture was adjusted to pH 6 with conc. hydrochloric acid and stirred for 2 h at room temperature. Then the reaction mixture was neutralised with saturated sodium hydrocarbonate solution. The resulted precipitate was collected by filtration, washed with water (2 × 5 mL) and methanol (2 × 5 mL), purified by column chromatography (R*f* = 0.44; chloroform:acetone, 1:2) and dried in a Fisher drying pistol to give 0.165 g (89%) of **4c** as a grey solid, m.p. 219–220°C (dec.); IR (KBr): 3355 (NH$_2$, 2NH), 1667 (CO); ^{1}H NMR: 3.53 (s, 3H, C$_{4'}$-OCH$_3$), 3.68 (s, 6H, C$_{3';5'}$-OCH$_3$), 4.35 (d, *J* = 5.7 Hz, 2H, CH$_2$), 5.97 (s, 2H, C$_{2';6'}$-H), 6.03 (t, *J* = 5.7 Hz, 1H, NH), 6.51 (br.s, 2H, NH$_2$), 7.06 (s, 1H, C$_5$-H), 10.89 (br.s, 1H, NH); ^{13}C NMR: 43.3, 56.2, 60.8, 91.2, 115.8, 119.0, 129.6, 134.9, 145.5, 153.7, 154.0, 158.7, 168.3.

Anal. calcd. for $C_{16}H_{18}N_4O_4S$: C, 53.03; H, 5.01; N, 15.46. Found: C, 52.88; H, 4.93; N, 15.41.

2-Amino-6-[(naphthalen-2-yl-amino)methyl] thieno[2,3-*d*]pyrimidin-4(3*H*)-one (4f). This compound was prepared analogously to **4c** from compound **6**. Grey solid, 142 mg (86%), m.p. >300°C (dec.); R_f = 0.50 (chloroform:acetone, 1:1); IR (KBr): 3415, 3352 (NH_2, NH), 1663 (CO); ¹H NMR: 4.49 (d, *J* = 6.0 Hz, 2H, CH_2), 6.52 (br.s, 2H, NH_2), 6.59 (t, *J* = 6.0 Hz, 1H, NH), 6.85 (d, *J* = 2.0 Hz, 1H, Ar-H), 7.05 (dd, *J* = 8.7 Hz, *J* = 2.0, 1H, Ar-H), 7.09–7.19 (m, 2H, C_5-H, Ar-H), 7.29–7.35 (m, 1H, Ar-H), 7.52–7.71 (m, 3H, Ar-H), 10.87 (br.s, 1H, NH); ¹³C NMR: 42.9, 104.2, 115.0, 119.0, 119.1, 122.0, 126.2, 126.7, 127.4, 128.1, 129.1, 134.4, 135.6, 146.7, 153.7, 158.6, 168.3.

Anal. calcd. for $C_{17}H_{14}N_4O_4S$: C, 63.33; H, 4.38; N, 17.38. Found: C, 63.38; H, 4.20; N, 17.28.

2-{4-[(2-Amino-4-oxo-3,4-dihydro-thieno[2,3-*d*]pyrimidin-6-yl)methylamino]-benzamido}-L-glutamic acid (4g). A round bottom flask equiped with a magnetic stirrer was loaded with 2-amino-6-formylthieno[2,3-*d*] pyrimidin-4(3*H*)-one (**6**) (0.300 g, 1.54 mmol) and *N*-(4-aminobenzoyl)-L-glutamic acid diethyl ester (0.644 g, 2.00 mmol), capped with rubber septum, flushed with argon and followed with dry methanol (40 mL). A solution of sodium cianoborohydride (0.314 g, 5.00 mmol) in methanol (10 mL) was syringed. The pH of the reaction mixture was adjusted to 5 with conc. hydrochloric acid. The mixture was stirred at room temperature for 18 h and neutralised with a saturated sodium hydrocarbonate solution. The precipitate was collected by filtration, washed with water (3 × 5 ml) and cold methanol (3 mL) and subjected to column chromatography. Fractions containing a single spot with R_f = 0.28 (chloroform:acetone 1:1) were collected and evaporated until dryness to give 0.260 g of yellowish solid, which was added to a 1 M sodium hydroxide solution and stirred for 15 min at room temperature. The reaction mixture was roughly acidified with concentrated hydrochloric acid to pH 7 and then adjusted to pH 4 with 5% hydrochloric acid. The precipitate was collected by filtration, washed with water, recrystallised from a mixture of dimethylformamide and water and dried in a Fisher drying pistol to give 0.307 g (45%) of **4g** as off-white solid, m.p. >300°C (dec.); IR (Nujol): 3330 (NH_2, NH, OH),

1643 (CO); ¹H NMR: 1.85–2.17 (m, 2H, CH_2), 2.32 (t, *J* = 7.4 Hz, 2H, CH_2), 4.29–4.40 (m, 1H, CH), 4.44 (d, *J* = 5.7 Hz, 2H, CH_2), 6.66 (br.s, 2H, NH_2), 6.68 (d, *J* = 8.7 Hz, 2H, Ar-H), 6.84 (t, *J* = 5.7 Hz, 1H, NH), 7.06 (s, 1H, C_5-H), 7.66 (d, *J* = 8.7 Hz, 2H, Ar-H), 8.26 (d, *J* = 7.5 Hz, 1H, CONH), 12.38 (br.s, 3H, CONH, CO_2H).

Anal. calcd. for $C_{19}H_{19}N_5O_6S$: C, 51.23; H, 4.30; N, 15.72. Found: C, 51.56; H, 4.61; N, 15.49.

RESULTS AND DISCUSSION

One of possible and short routes to 6-arylamino-methylthieno[2,3-*d*]pyrimidine derivatives is the reductive amination reaction of the appropriate thieno[2,3-*d*]pyrimidine-6-carbaldehydes with arylamines. For this purpose, 2-amino-4-methoxythieno[2,3-*d*]pyrimidine-6-carbalde-hyde (**2**) has been chosen as a key starting material. Previously, we obtained aldehyde **2** by oxidation of (thieno[2,3-d]pyrimidin-6-yl)methanol **1** with molecular iodine in 54% yield within 40 h [20]. A moderate reaction yield and a long reaction time prompted us to develop a more efficient method for the synthesis of compound **2**. In this connection, oxidation reactions of alcohol **1** with cerium ammonium nitrate and Dess-Martin periodinane (DMP) [25, 26] were attempted. The reaction with cerium ammonium nitrate proceeded with a full substrate conversion within 1.5 h as monitored by TLC. However, the desired carbaldehyde **2** was isolated only in 13% yield (Scheme 1). The low yield, probably, was caused by the formation of relatively difficult to break complexes between cerium ions and the product.

Oxidation of alcohol **1** with DMP in the presence of water proceeded smoothly at room temperature to give carbaldehyde **2** in the high 91% yield. Having the efficient method for the preparation of carbaldehyde **2**, we explored its reductive amination reaction with anilines using sodium cyanoborohydride. Aldehyde **2** reacted with mono-, di- or trimethoxyanilines under the reductive amination conditions at pH = 6 to give the desired 6-arylaminomethylthieno[2,3-*d*]pyrimidine derivatives **3a–d** in 57–87% yields (Scheme 1). However, 2,5-dichloroaniline was not reactive enough to form an intermediate imine and thus, finally to undergo reduction under these conditions. The desirable **3e** was obtained by preparation of imine using
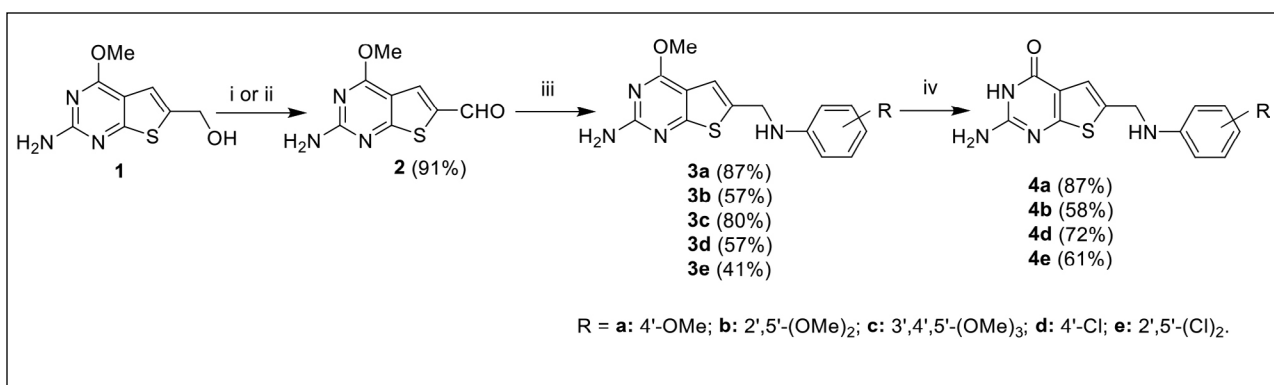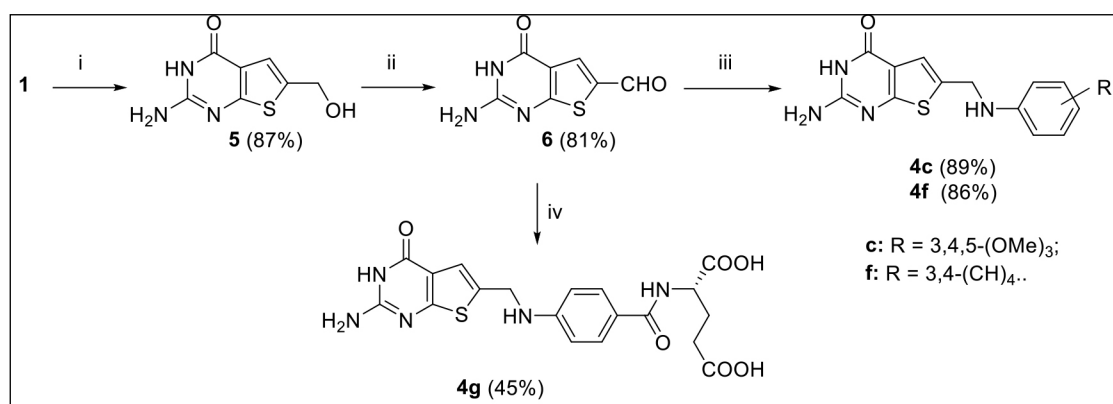
Scheme 1. Reagents and conditions: (i) CAN, ACN, 1 drop H_2O, r.t., 1.5 h; (ii) DMP, 1 drop H_2O, DMF, r.t. 1.5 h; (iii) ArNH$_2$, NaCNBH$_3$, MeOH, pH = 6, r.t. 1 h, 2 days, argon; for **3d**: 1. 2,5-(Cl)$_2$C$_6$H$_3$NH$_2$, TiCl$_4$, C$_6$H$_6$, r.t. overnight, argon; 2. NaCNBH$_3$, MeOH, pH = 8, r.t., overnight, argon; (iv) conc. HCl, reflux, 1.5–4 h

titanium tetrachloride as Lewis acid and following its reduction with sodium cyanoborohydride in methanol [27]. To convert 4-methoxy derivatives **3** into 4-oxothienopyrimidines **4**, the demethylation reaction with concentrated hydrochloric acid was applied. Although compounds **4a, b, d, e** were obtained in 58–87% yields, 6-(3,4,5-trimethoxyphenyl)aminomethyl derivative **3c** under these conditions decomposed and we were not able to isolate pure compound **4c**. Conditions of the demethylation reaction appeared to be too vigorous for the preparation of classical antifolate **4g**, as well. For the synthesis of compounds **4c, f, g**, we developed another way consisting of the demethylation of a 4-methoxy group prior to the reductive amination step (Scheme 2).

Compound **1** was treated with concentrated hydrochloric acid to give 4-oxo derivative **5** in 87% yield [20]. Oxidation of the latter compound with DMP resulted in the formation of carbaldehyde **6**

in the high 81% yield. Reductive amination of 2-amino-6-formylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**6**) with appropriate anilines in the presence of sodium cyanoborohydride gave amines **4c, f**. For the synthesis of **4g**, the alkaline hydrolysis of ester groups of glutamic acid moiety was applied after the successive reductive amination reaction.

Thieno[2,3-*d*]pyrimidine analogues of folic acid **4a–g** were evaluated as inhibitors of DHFR isolated from *Pneumocystis carinii* (pcDHFR), *Toxoplasma gondii* (tgDHFR), *Mycobacterium avium* (maDHFR) and rat liver (rlDHFR). Selectivity ratios (IC$_{50}$ rlDHFR/IC$_{50}$ pc or tg, or ma DHFR, respectively) were determined using DHFR isolated from rat liver (rlDHFR) as the mammalian standard. The results (IC$_{50}$) are presented in the Table. For comparison, analogous DHFR inhibitory data of the known inhibitor trimethoprim are also included.

Scheme 2. Reagents and conditions: (i) conc. HCl, reflux, 70 min; (ii) DMP, DMF, 1 drop H_2O, r.t., 3 h; (iii) ArNH$_2$, NaCNBH$_3$, MeOH, pH = 6, r.t. 2 h; (iv) 1. 4-H$_2$NC$_6$H$_4$CO-L-Glu-Et$_2$, NaCNBH$_3$, MeOH, pH = 5, r.t., 18 h; 2. 1M NaOH, r.t., 15 min; 3. aq. HCl

Table. Inhibitory concentrations (IC$_{50}$, µM) against isolated DHFR

Entry	Compound	DHFR						
		rl	pc	rl/pc	tg	rl/tg	ma	rl/ma
1	**4a**	262	56.5	4.6	6.0	43.7	>60	NA[a]
2	**4b**	>256	129	NA	>256	NA	>256	NA
3	**4c**	>228	214	NA	302	NA	>228	NA
4	**4d**	9.3	49.9	0.2	2.8	3.3	>61	NA
5	**4e**	2.7	23	0.1	2.3	1.2	>249	NA
6	**4f**	37.4	22.5	1.7	11.9	3.1	>25	NA
7	**4g**	1	6.6	0.15	1.06	1	16.9	0.06
8	Trimethoprim[b]	133	12	11.1	2.7	49.0	0.3	600

[a] Not applicable.

[b] Inhibitory activity data of trimethoprim were taken from Refs. [28, 29].

The activity of 4-methoxy derivative **4a** against tgDHFR (IC$_{50}$ = 6.0 µM) and selectivity (4.6 for pcDHFR and 43.7 for tgDHFR) was comparable to that of trimethoprim (11.1 and 49.0, respectively) (entry 1). 2',5'-Dimethoxy **4b** and 3',4',5'-trimethoxy **4c** derivatives were less active than trimethoprim (entries 2, 3). Thus, in a series of methoxyphenyl derivatives **4a–c** the activity against pcDHFR and tgDHFR decreased as the number of methoxy groups increased. 4-Chloro **4d** and 2,5-dichloro **4e** compounds showed a higher activity against rlDHFR (IC$_{50}$ = 9.3 µM and 2.7 µM, respectively) than trimethoprim **6** (IC$_{50}$ = 133 µM), a lower activity against pcDHFR (IC$_{50}$ = 49.9 µM and 23 µM, respectively) than trimethoprim (IC$_{50}$ = 12 µM) and the comparable activity against tgDHFR (IC$_{50}$ = 2.8 µM and 2.3 µM, respectively) with trimethoprim (IC$_{50}$ = 2.7 µM). However, an inversed or a low selectivity for pcDHFR and tgDHFR was observed (entries 4, 5). In a series of chlorophenyl derivatives **4d**, **e** the activity against rl, pc and tgDHFR increased and, interestingly, the selectivity decreased as the number of chloro groups in the aromatic part of molecules increased. β-Naphthyl derivative **4f** showed a moderate activity and a modest selectivity (entry 6). It should be noted that none of the studied lipophilic inhibitors inhibited maDHFR. Classical antifolate **4g** appeared to be the most potent inhibitor and showed a slightly better activity against pcDHFR and tgDHFR compared to that of trimethoprim and was the only one from the studied series, which inhibited maDHFR (entry 7). However, no or

reversed selectivity was observed. In summary, while several synthesised compounds showed a promising activity against microbial DHFR, many of these compounds were also equipotent inhibitors of mammalian DHFR what reduces possibilities of their further applicability. Nevertheless, the obtained data should be useful for the search of novel DHFR inhibitors in the thieno[2,3-*d*]pyrimidine series.

CONCLUSIONS

In this research, a novel series of thieno[2,3-*d*]pyrimidin-4(3*H*)-ones were successfully synthesised as potential antifolate agents. For the synthesis of inhibitors the synthetic pathway included sequential reactions of oxidation of (2-amino-4-methoxy(or 4-oxo)thieno[2,3-d]pyrimidin-6-yl)methanols to the corresponding aldehydes and their reductive amination reactions with the selected anilines and sodium cyanoborohydride. An efficient high-yielding method for the synthesis of thieno[2,3-d]pyrimidine-6-carbaldehydes by using Dess-Martin periodinane in the presence of water has been elaborated. Investigation of the DHFR inhibitory activity of the synthesised compounds revealed that 2-amino-6-[(4-methoxyphenylamino)-, 6--[(4-chlorophenylamino)- and 6-[(2,5-dichlorophenylamino)methyl]thieno[2,3-*d*]pyrimidin-4(3H)-ones had a moderate activity against pcDHFR with IC$_{50}$ 56.5, 49.9 and 23 µM, respectively. Their activity against tgDHFR (IC$_{50}$ 6.0, 2.8 and 2.3 µM, respectively) was comparable with that of trimethoprim (IC$_{50}$ 2.7 µM). The classical inhibitor 2-{4-[(2-amino-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-6-yl)

methylamino]benzamido}-L-glutamic acid showed a better activity against pcDHFR (IC$_{50}$ 6.6 μM) and tgDHFR (IC$_{50}$ 1.06 μM) than trimethoprim and was the only one from the studied series, which inhibited maDHFR (IC$_{50}$ 16.9 mM). However, its selectivity was inferior to that of trimethoprim. Interestingly, none of the lipophilic inhibitors studied was active against maDHFR.

The proposed general synthesis pathway for the preparation of lipophilic and classical antifolates and the obtained DHFR inhibitory data can be useful for the development of novel antifolate libraries.

ACKNOWLEDGEMENTS

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2-AMINO-6-(ARILAMINOMETIL)TIENO[2,3-*d*] PIRIMIDIN-4(3*H*)-ONŲ SINTEZĖ IR DIHIDROFOLATO REDUKTAZES SLOPINANTIS AKTYVUMAS

S a n t r a u k a

Šiame darbe susintetinti nauji tieno[2,3-*d*]pirimidino dariniai ir ištirtas jų slopinantis aktyvumas dihidrofolato reduktazėms (DHFR), išskirtoms iš mikroorganizmų *Pneumocystis carinii* (pc), *Toxoplasma gondii* (tg) ir *Mycobacterium avium* (ma). Slopinančio aktyvumo selektyvumo įvertinimui remiantis žinduolių DHFR standartu buvo naudojama žiurkių kepenų DHFR (rlDHFR). Susintetinti lipofiliniai ir klasikinis antifolatai, turintys būdingus DHFR slopikliams farmakoforus ir metilenamino tiltelį, jungiantį aromatinę molekulių dalį su 2-amino-4-okso-tieno[2,3-*d*]pirimidino fragmentu. Ištyrus DHFR slopinantį aktyvumą, nustatyta, kad 2-amino-6-[(4-metoksifenilamino)-, 6--[(4-chlorofenilamino)-, 6-[(2,5-dichlorofenilamino)metil]tieno[2,3-*d*]pirimidin-4(3*H*)-onai pasižymi vidutiniu pcDHFR slopinančiu aktyvumu (IC$_{50}$ 56,5 µM, 49,9 µM ir 23 µM, atitinkamai), o jų aktyvumas tgDHFR atžvilgiu (IC$_{50}$ 6,0 µM, 2,8 µM ir 2,3 µM, atitinkamai) yra panašus į trimetoprimo aktyvumą. Pažymėtina, kad nė vienas iš tirtų lipofilinių antifolatų nepasižymėjo maDHFR slopinančiu aktyvumu. Klasikinis antifolatas – 2-{4-[(2-amino-4-okso-3,4-dihidrotieno[2,3-*d*]pirimidin-6-il)metilamino]benzamido}-L-glutamo rūgštis slopino visų mikroorganizmų DHFR (IC$_{50}$ 6,6 µM pcDHFR, 1,06 µM tgDHFR ir 16,9 µM maDHFR). Jo slopinantis aktyvumas buvo geresnis nei trimetoprimo, tačiau DHFR selektyvumu, palyginti su rlDHFR slopinimu, nusileido trimetoprimo selektyvumui.

Pasiūlytas bendras tieno[2,3-*d*]pirimidino lipofilinių ir klasikinių antifolatų sintezės būdas ir gauti oportunistinių mikroorganizmų DHFR slopinančio aktyvumo rezultatai turėtų būti naudingi, konstruojant naujus efektyvius DHFR slopiklius.