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Thio-containing pteridines: Synthesis, modification, and biological activity

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Abstract

The present article is devoted to searching for biologically active agents among novel thio-containing pteridines. Synthetic protocols based on the condensation of 5,6-diamino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-ones with dicarbonyl compounds were elaborated and used for the synthesis of target products. The directions for further modification of the obtained thio-containing pteridines were substantiated and realized. The spectral properties of the obtained compounds were studied and described. The results of the in silico study revealed that the predicted affinity of the obtained compounds to the dihydrofolate reductase (DHFR) active site is comparable with the affinity of methotrexate, despite the differences in the nature of the ligand–enzyme interactions. The in vitro study of DHFR-inhibiting activity revealed that the most active compounds **3.9** and **4.2** have lg IC_{50} values of −5.889 and −5.233, respectively, significantly inferior to methotrexate (lg IC_{50} = −7.605). Additionally, the synthesized compounds were studied for their antiradical activity as a possible mechanism of pharmacological effects. Among the obtained pteridines, compounds **5.1** (lg EC_{50} = −4.82) and **5.3** (lg EC_{50}= −4.92) have antiradical activity higher than the reference compound ascorbic acid (lg EC_{50}= −4.81). The conducted structure–activity relationship analysis provided valuable data for the further search for biologically active agents among thio-containing pteridines and related compounds.

KEYWORDS
DHFR inhibitors, free-radical scavenging activity, modification, synthesis, thio-containing pteridines

1 | INTRODUCTION

Pteridines and related heterocyclic systems have been intensively studied within the development of novel drugs.[1,2] Most pteridines that are used in medical practice are inhibitors of dihydrofolate reductase (DHFR), thymidylate synthase, blockers of α-folate receptors with antitumor, antimicrobial, and other properties. Despite the proven clinical effectiveness and mechanism of activity of pteridines as well as the availability of comprehensive information about the structure of biological targets of classic antifolates (methotrexate), the search for novel biologically active agents among the aforementioned derivatives

is actively carried out. The design and search of biologically active pteridines are conducted in the following directions: modification of *p*-aminobenzoyl-1-glutamate fragment in 6th position of folic acid by alkylation of amino-group or its replacement by methylene fragment, functionalization of pteridine cycle, bioisosteric replacement of pteridine ring by another heterocycle, structural modification of pteridine system, modification of antifolates by introduction of additional pharmacophore fragments (hybrid-pharmacophore approach), search of biologically active pteridines with alternative biological targets and type of biological activity.

At the same time, thio-containing pteridines and their condensed derivatives as promising bioactive compounds are insufficiently studied. 2-Substituted mercaptopteridin-4-ols combined with different heterocycles (pyrrolidine, oxazole, benzimidazole, pyrimidine, quinoline) through the alkyl "linker" with growth inhibition activity against cancer cell lines MCF7, NCI-H460 and SF-268 up to 47%[3] and substituted 8-R^1-4-R^2-2-(propylthio)-5,8-dihydropteridine-6,7-diones with growth inhibiting activity against MGC-803, SGC-7901, A549, and PC-3 cancer cell lines (IC_{50} 4.32–>100 μM)[4,5] should be mentioned among the known bioactive thio-containing pteridines. Likewise, series of substituted thiazolo[4,5-*d*]pteridines and thiazolo[4,5-*d*]pyrimidines were synthesized by Walters et al. via condensation of 2-[(2,3-difluorobenzyl)thio)-6-R-pyrimidine-4,5-diamines with mono- and dinucleophiles.[6] It was shown, that obtained compounds are high active CXCR2-antagonists.

Guiney et al. described approaches to synthesis of polyfunctional 6-substituted 2-methyl-(benzyl-)thiopteridines by modified Wittig reaction using 2-(methyl-(benzyl-)thio)-4-oxo-3,4-dihydropteridine-6-carbaldehydes as initial compounds.[7] A series of promising anti-inflammatory agents were described by Ghoneim et al.[8] The authors described the synthesis of thioxobenzo[*g*]pteridines and studied its interaction with the active center of cyclooxygenase type-2 (COX-2).

Thus, thio-containing pteridines are promising biologically active compounds and can be used for the treatment of widespread and severe diseases including cancer, inflammatory processes, and neurodegenerative pathologies. The present paper is a sequel to our previous investigations aimed at the search for biologically active compounds among substituted pteridines[9–12] and is devoted to the search for DHFR inhibitors among a series of thio-contained pteridines that are structurally similar to the known medicines with this mechanism of action (Figure 1).[13] Evaluation of radical-scavenging activity of obtained compounds was the additional aim of the present study.

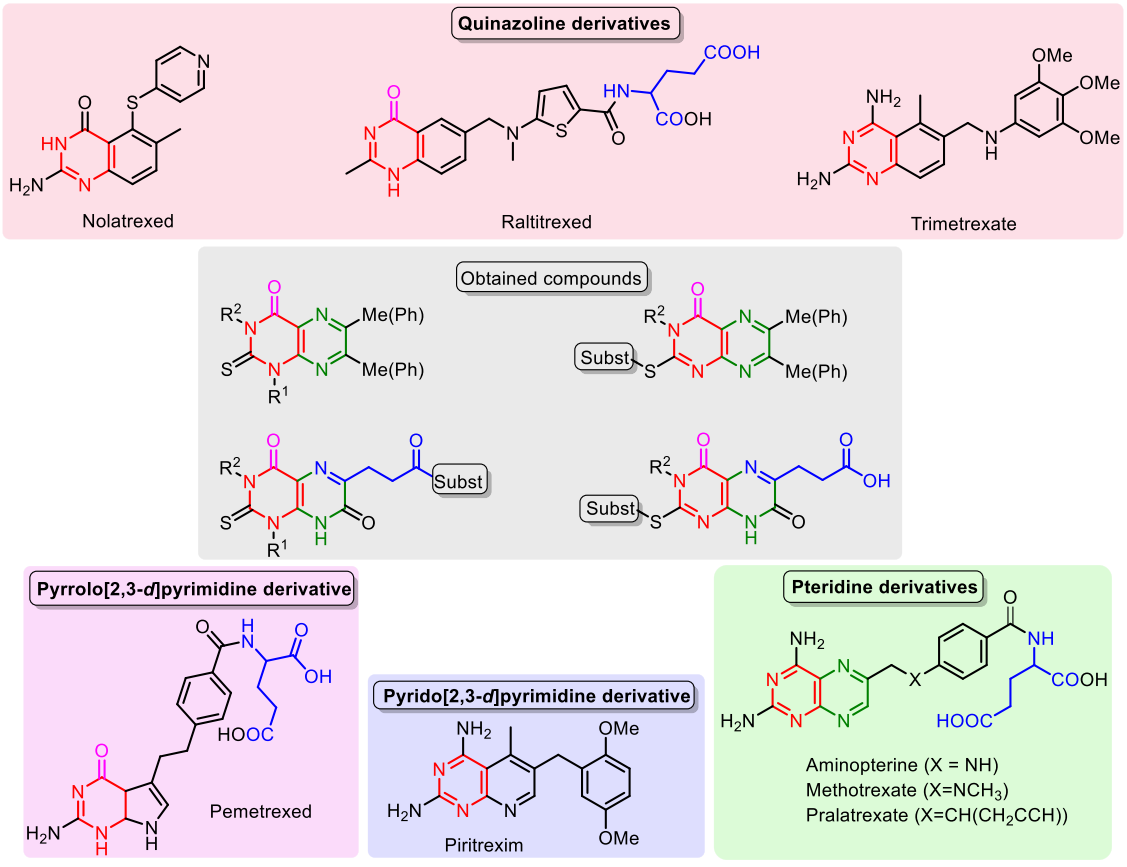


FIGURE 1 Previously reported dihydrofolate reductase-inhibitors and based on the design of novel thio-containing pteridines

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The design of the potential bioactive pteridines includes a modification of initial compound **2** by introduction of substituents to the 7th and 8th position (variation of lipophilicity), modification of thioxo-group by introduction of various pharmacophores, modification of carboxyalkyl fragment by its transformation to amide fragment for enhancement of DHFR-inhibiting activity.

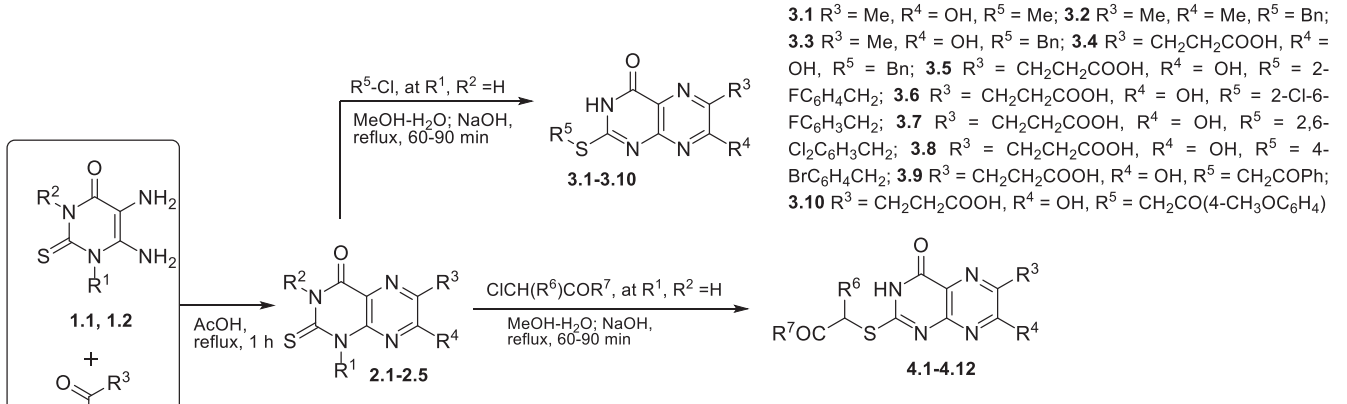
Substituted 5,6-diamino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-ones (**1**) were used as initial compounds for the synthesis of designed potential bioactive agents. The aforementioned starting compounds were obtained by known procedures.[14,15] Synthesis of substituted 2-thioxo-2,3-dihydropteridin-4(1*H*)-ones (**2**) was conducted via condensation of substituted 5,6-diamino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-ones (**1**) with carbonyl compounds or ketocarboxylic acids (Scheme 1).[9–11] The formation of the thio-containing pteridines was proved by the presence of signals of protons at nitrogen in positions N1 and N3 that were observed at the 13.31–12.24 ppm and 12.7–10.87 ppm correspondingly. Additionally, ^{1}H NMR spectra of compounds **2.3–2.5** were characterized by signals of hydroxy-group protons in 7th position at the 13.72–13.33 ppm, and compounds **2.4**, **2.5**, by signals of carboxylic groups protons at the 11.94–11.79 ppm. The AB-system that consists of two triplets at the 3.17–3.05 ppm and 2.78–2.73 ppm was registered in the high field of compounds **2.4**, **2.5** ^{1}H NMR spectra.[16]

The following step of chemical modification consists of alkylation of compound **2** by alkyl halides or halogen-containing carboxylic acid thioxo-group in 2nd position. The above-mentioned reaction was conducted according to the standard procedure[17] and proceeded in agreement with HSAB theory by thioxo-group in 2nd position. The ^{1}H NMR-spectra of compound **3** were characterized by the presence of signals of substituents at the sulfur atom, namely three-proton singlet at the 2.56 ppm of the methyl group (compound **3.1**), two proton singlet at the 4.01–4.87 ppm of methylene fragment and set of the signals that correspond to the benzene or substituted benzene moiety (compounds **3.2**–**3.10**). The complex of signals caused by the presence of carboxyalkyl fragment was observed in ^{1}H NMR spectra of compounds **4.1**–**4.4** which proved their structure. The structures of synthesized compounds were additionally proved by ^{13}C NMR data.

Synthesis of propionamides **5** was conducted by a previously described procedure[11] that was based on the reaction between anilines or amino acids and obtained in situ lactones (A) (Scheme 2).[9–11] The structures of compound **5** were proven by the presence of signals that correspond to the protons at the nitrogen atom, that were registered at the 8.08–7.95 ppm for aliphatic amides (compounds **5.1–5.3**) or at the 9.88 and 10.21 ppm for aromatic amines (compounds **5.4**, **5.5**). The signals of carboxy alkyl moiety (for compounds **5.1–5.3**) and aromatic fragment (for compounds **5.4**, **5.5**) were observed as well.

2.2 | Molecular docking and DHFR inhibition assay

Molecular docking results showed that predicted affinity scores of compounds **2.2**, **3.3**, **3.5**, **3.7–3.10**, **4.2**, **5.2**, **5.4**, and **5.5** are similar to scores of reference compound MTX (Table 1). Obtained data additionally proved the reasonability of the study of DHFR-inhibiting activity of synthesized compounds in vitro. According to the in vitro studies, results obtained compounds in concentration of



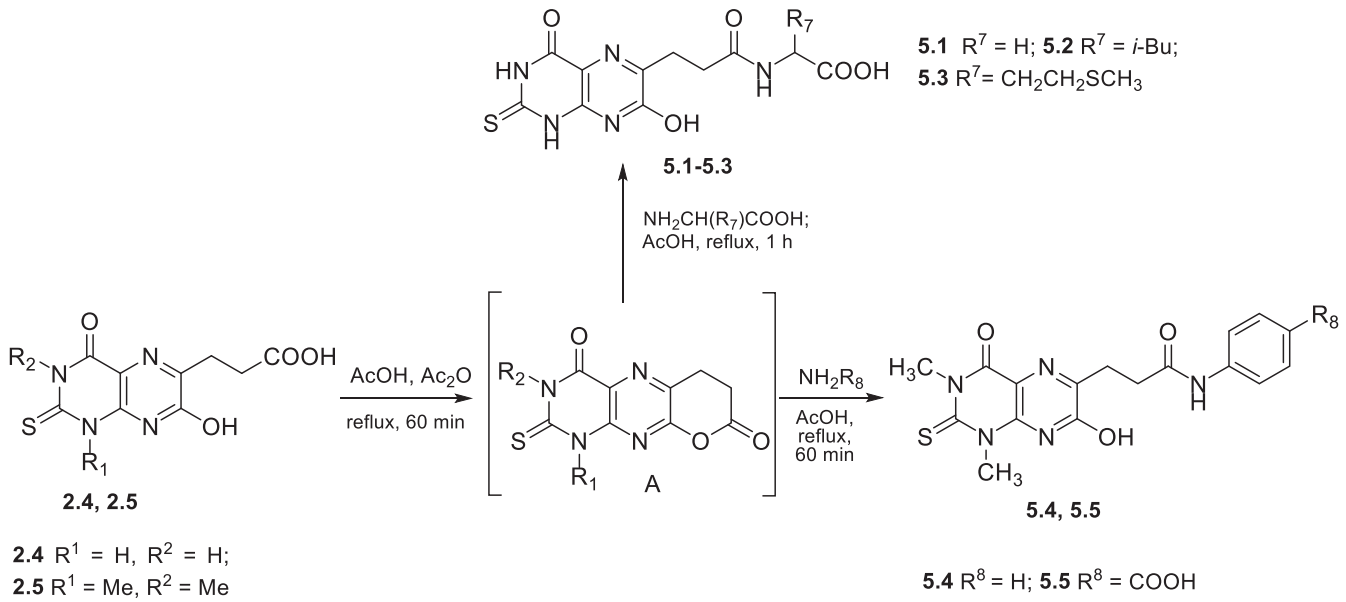
3.1 R^3 = Me, R^4 = OH, R^5 = Me; **3.2** R^3 = Me, R^4 = Me, R^5 = Bn; **3.3** R^3 = Me, R^4 = OH, R^5 = Bn; **3.4** R^3 = CH_2CH_2COOH, R^4 = OH, R^5 = Bn; **3.5** R^3 = CH_2CH_2COOH, R^4 = OH, R^5 = 2-$FC_6H_4CH_2$; **3.6** R^3 = CH_2CH_2COOH, R^4 = OH, R^5 = 2-Cl-6-$FC_6H_3CH_2$; **3.7** R^3 = CH_2CH_2COOH, R^4 = OH, R^5 = 2,6-$Cl_2C_6H_3CH_2$; **3.8** R^3 = CH_2CH_2COOH, R^4 = OH, R^5 = 4-$BrC_6H_4CH_2$; **3.9** R^3 = CH_2CH_2COOH, R^4 = OH, R^5 = CH_2COPh; **3.10** R^3 = CH_2CH_2COOH, R^4 = OH, R^5 = $CH_2CO(4\text{-}CH_3OC_6H_4)$

1.1 R^1 = H, R^2 = H; **1.2** R^1 = Me, R^2 = Me

2.1 R^1 = H, R^2 = H, R^3 = Me, R^4 = Me; **2.2** R^1 = H, R^2 = H, R^3 = Ph, R^4 = Ph; **2.3** R^1 = H, R^2 = H, R^3 = Me, R^4 = OH; **2.4** R^1 = H, R^2 = H, R^3 = CH_2CH_2COOH, R^4 = OH; **2.5** R^1 = Me, R^2 = Me, R^3 = CH_2CH_2COOH, R^4 = OH

4.1 R^3 = Me, R^4 = Me, R^6 = H, R^7 = OH; **4.2** R^3 = Ph, R^4 = Ph, R^6 = H, R^7 = OH; **4.3** R^3 = Me, R^4 = OH, R^6 = H, R^7 = OH; **4.4** R^3 = CH_2CH_2COOH, R^4 = OH, R^6 = H, R^7 = OH; **4.5** R^3 = CH_2CH_2COOH, R^4 7= OH, R^6 = H, R^7 = OH; **4.6** R^3 = Ph, R^4 = Ph, R^6 = H, R^7 = NH_2; **4.7** R^3 = Me, R^4 = Me, R^6 = H, R^7 = 3-FC_6H_4NH; **4.8** R^3 = Me, R^4 = Me, R^6 = H, R^7 = 3-ClC_6H_4NH; **4.9** R^3 = Me, R^4 = Me, R^6 = H, R^7 = 4-ClC_6H_4NH; **4.10** R^3 = Me, R^4 = OH, R^6 = H, R^7 = 3-FC_6H_4NH; **4.11** R^3 = CH_2CH_2COOH, R^4 = OH, R^6 = H, R^7 = 3-FC_6H_4NH; **4.12** R^3 = CH_2CH_2COOH, R^4 = OH, R^6 = H, R^7 = 3-ClC_6H_4NH

SCHEME 1 Synthesis route toward the designed potential bioactive agents



SCHEME 2 Synthesis of propionamides **5**

TABLE 1 The results of synthesized compounds' molecular docking and inhibition of dihydrofolate reductase (DHFR)

Compound	Affinity (kcal/mol) to DHFR (1RG7)	Inhibition of DHFR (%)	Compd.	Affinity (kcal/mol) to DHFR (1RG7)	Inhibition of DHFR (%)	Compd.	Affinity (kcal/mol) to DHFR (1RG7)	Inhibition of DHFR (%)
2.1	−6.5	24.56	**3.7**	−8.1	80.63	**4.7**	−8.4	29.18
2.2	−8.0	82.57	**3.8**	−8.1	22.53	**4.9**	−8.6	36.02
2.3	−6.3	10.91	**3.9**	−8.5	94.19	**5.1**	−7.1	59.33
2.4	−7.1	72.89	**3.10**	−8.5	34.62	**5.2**	−7.5	16.72
3.1	−6.2	28.40	**4.1**	−7.7	67.07	**5.3**	−6.9	10.32
3.2	−8.3	43.83	**4.2**	−8.7	98.06	**5.4**	−9.2	20.59
3.3	−7.9	18.56	**4.3**	−7.3	43.83	**5.5**	−9.0	26.40
3.4	−8.0	12.85	**4.4**	−6.5	31.12	MTX[a]	−8.5	89.57
3.5	−7.9	7.03	**4.5**	−7.1	0.71	-	-	-
3.6	−8.4	2.65	**4.6**	−8.1	37.19	-	-	-

Note: [a], Methotrexate.

100 μM inhibited the activity of DHFR by 7%–98%. (Table 1). The highest DHFR-inhibiting activity was characteristic for compounds **2.2** (82.57%), **3.7** (80.63%), **3.9** (94.19%), and **4.2** (98.06%). The dose-dependent study of DHFR-inhibiting activity was conducted for compounds **3.9** and **4.2**. It was found that lg IC_{50} of compounds **3.9** and **4.2** were −5.889 and −5.233 correspondingly, thus studied compounds were much lower in activity in comparison with the reference compound methotrexate (lg IC_{50} = −7.605).

The visualization of molecular docking study results was carried out for a series of compounds (**3.7**, **3.9**, and **4.2**) and methotrexate to estimate the nature of possible ligand–DHFR interactions.[18]

The visualization of the X-ray diffraction study of the DHFR-MTX complex (Table 1, Figure 2a) reveals the nature of ligand–enzyme interactions. It was shown that the molecule of MTX forms numerous conventional hydrogen bonds that can be considered as electron-donating interactions of amino groups in 2nd and 4th position of the pteridine cycle with amino acids ASP A:27 (2.64 Å) and ILE A:5 (2.77 Å), ILE A:94 (2.90 Å), TYR A:100 (3.23 Å), correspondingly. For pteridine system π-sigma interactions with ALA A:19 (3.65 Å), ILE A:5 (5.23 Å), ASN A:18 (3.60 Å), and π-alkyl interactions with ALA A:19 (4.15 Å) and ALA A:7 (4.49 Å) are characteristic. Attractive interactions, conventional and carbon-hydrogen bonds between MTX and enzyme are formed as a result of bonding of carboxylic and methylamine group of *p*-methylaminobezoylglutamate fragment ARG A:57 (2.96 Å), LYS A:32 (4.40 Å), ARG A:52 (4.20 Å), ARG A:57 (2.56 Å) and ARG A:57

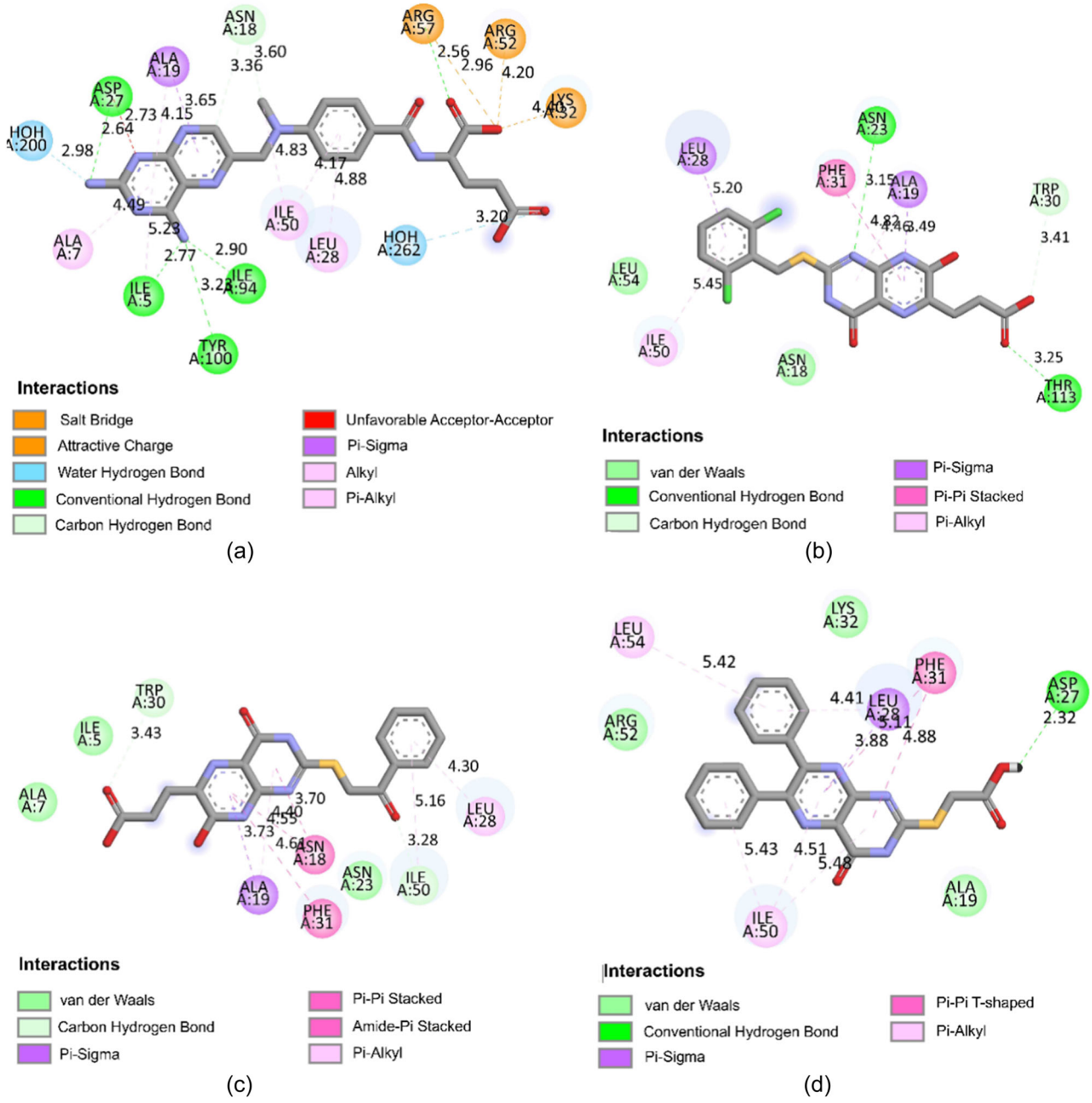


FIGURE 2 Visualization of affinity according to the docking study of methotrexate (a), compounds **3.7** (b), **3.9** (c), and **4.2** (d) with dihydrofolate reductase (1RG7)

(2.56 Å) and ASN A:18 (3.36 Å). Besides, in the active center of the enzyme MTX molecule forms hydrogen bonds between water and amino-group in 2nd position and carboxylic group of glutamine moiety (HOH A: 2.98 Å and HOH200a [3.20 Å], respectively) (Figure 2a).

At the same time between –COOH group and A:ASP27 (2.32 Å) conventional hydrogen bond was observed for the most active compound **4.2** (Table 2, Figure 2d). Between pteridine cycle and A:LEU28 (3.88 Å), A:PHE31 (4.88 Å), A:PHE31 (5.11 Å), A:ILE50 (5.48 Å), A:ILE50 (4.51 Å) hydrophobic π-σ, π-Alk, and π–π T-shaped interactions were observed. Also, with diphenyl moieties, hydrophobic π-Alk interactions with the following amino acid residues were shown: A:ILE50 (5.43 Å), A:LEU28 (4.41 Å), A:LEU54 (5.42 Å). Visualization of the interactions formed by compound **3.9** and the active center of enzyme revealed (Table 2, Figure 2c) two conventional hydrogen bonds between both carbonyl groups of the compound and A:TRP30 (3.43 Å), A:ILE50 (3.28 Å). Other types of interactions were hydrophobic π-σ, π–π stacked, amide-π stacked, and π-Alk with the following amino acids A:ALA19 (3.73 Å), A:PHE31 (4.61 Å), A:ASN18 (4.40 Å), A:ASN18 (3.79 Å), A:ALA19 (4.55 Å), A:LEU28 (4.30 Å), A:ILE50 (5.16 Å). For compound **3.7** visualization showed three hydrogen bonds between –COOH group and nitrogen

TABLE 2 The main types of interactions of synthesized compounds and pharmacological standards with amino acid residues of dihydrofolate reductase

Compound	The main interactions types between compounds, pharmacological standards, and amino acid residues of enzymes*
MTX	ARG57[a] (2.96 Å), LYS32[h] (4.40 Å), ARG52[h] (4.20 Å), HOH200[a] (3.20 Å), HOH262[a] (2.98 Å), ARG57[a] (2.56 Å), ASP27[a] (2.64 Å), ILE5[a] (2.77 Å), ILE94[a] (2.90 Å), TYR100[a] (3.23 Å), ASN18[b] (3.36 Å), ASN18[b] (3.60 Å), ALA19[c] (3.65 Å), ILE5[e] (5.23 Å), ALA7[e] (4.49 Å), ALA19[e] (4.15 Å), LEU28[e] (4.88 Å), ILE50 [e] (4.17 Å)
3.7	A:ASN23[a] (3.15 Å), A:THR113[a] (3.25 Å), A:TRP30[b] (3.41 Å), A:ALA19[c] (3.49 Å), A:LEU28[c] (3.98 Å), A:LEU28[c] (3.84 Å), A:PHE31[i] (4.82 Å), A:ILE50[d] (3.98 Å), A:LEU54[d] (4.79 Å), A:PHE31[e] (4.01 Å), A:ALA19[e] (4.46 Å), A:ILE50[e] (5.45 Å)
3.9	A:TRP30[b] (3.43 Å), A:ILE50[b] (3.28 Å), A:ALA19[c] (3.73 Å), A:PHE31[i] (4.61 Å), A:ASN18[f] (4.40 Å), A:ASN18[f] (3.79 Å), A:ALA19[e] (4.55 Å), A:LEU28[e] (4.30 Å), A:ILE50[e] (5.16 Å)
4.2	A:ASP27[a] (2.32 Å), A:LEU28[c] (3.88 Å), A:PHE31[g] (4.88 Å), A:PHE31[g] (5.11 Å), A:ILE50[e] (5.48 Å), A:ILE50[e] (4.51 Å), A:ILE50[e] (5.43 Å), A:LEU28[e] (4.41 Å), A:LEU54[e] (5.42 Å)

Note: [a] Conventional hydrogen bond. [b] Carbon hydrogen bond, [c] Hydrophobic (π-σ). [d] Hydrophobic (Alk); [e] Hydrophobic (π-Alk); [f] Hydrophobic (amide-π stacked). [g] Hydrophobic (π–π T-shaped). [h] Electrostatic. [i] Hydrophobic (π–π stacked).

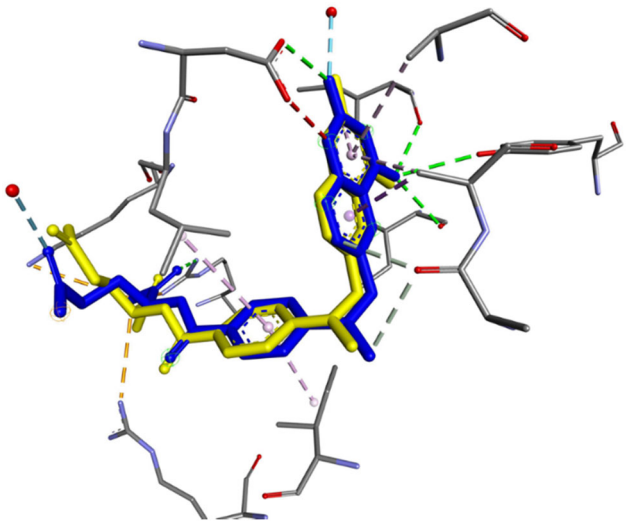


FIGURE 3 3D visualization of the arrangement of the reference native ligand (blue structure) and the reference ligand (yellow structure) in the active site of dihydrofolate reductase

of pteridine cycle with A:ASN23 (3.15 Å), A:THR113 (3.25 Å), and A:TRP30 (3.41 Å) (Table 2, Figure 2b). Other types of interactions were hydrophobic π-σ, π–π stacked, amide-π stacked, and π-Alk with the following amino acids A:ALA19 (3.49 Å), A:LEU28 (3.98 Å), A:LEU28 (3.84 Å), A:PHE31 (4.82 Å), A:ILE50 (3.98 Å), A:LEU54 (4.79 Å), A:PHE31 (4.01 Å), A:ALA19 (4.46 Å), A:ILE50 (5.45 Å).

The validity of the docking study can be estimated by the value of RMSD, which characterizes the degree of reliable docking probability. For our research, the RSMD value between the experimental and the reference conformation of the methotrexate equals 0.912 Å, therefore the study is reliable.[19] A visual comparison between the two poses is shown in Figure 3.

Thus, the visualization of molecular docking results showed that studied compounds have otherwise location and type of interactions with the active center of DHFR. Obtained compounds were found to be low or moderate active inhibitors of DHFR and cannot be considered promising in the scope of the search for biologically active compounds with chemotherapeutic properties.

2.3 | Antiradical activity of the synthesized compounds

The key role of pteridines in redox processes in living organisms motivated us to study the antiradical activity of synthesized thio-containing pteridines. It is well known, that their activity is associated with dihydro- and tetrahydro-levels which are proven by the co-enzyme activity of tetrahydropteridine in biochemical hydroxylation and mitochondrial electron transport processes.[20] Moreover, pteridine derivatives in dependence on the saturation state of the cycle and experiment conditions can be both donors and acceptors of free radicals and reveal anti- and prooxidant activity.[21] It is found that thio-containing pteridines exhibit moderate antiradical activity (Table 3). Certain attention should be paid to compounds **3.9** (lg EC_{50}= −4.78), **5.1** (lg EC_{50}= −4.82), and **5.3** (lg EC_{50}= −4.92).

2.4 | SAR-analysis among obtained thio-containing pteridines

SAR-analysis revealed that level of synthesized thio-containing pteridine's DHFR-inhibiting activity depends on the nature of substituents in 6th and 7th positions. Thus, replacement of the methyl group in 6th and 7th positions (compound **2.1**) by phenyl (**2.2**) or carboxyethyl and hydroxy groups (**2.4**) led to the increase in activity level. The following structural modification of compound **2** via alkylation of the sulfur atom in most cases resulted in the disappearance of enzyme-inhibiting activity (compounds **3.1**–**3.6**, **3.8**). The exceptions were compounds **3.7** and **3.9** which contained 2,6-dichlorbenzyl and phenethyl fragments, respectively. The increasing of DHFR-inhibiting activity was observed as a result of introduction of the acetic acid moiety to the sulfur atom (compounds **4.1**, **4.2**). At the same time, the following transformation of

TABLE 3 The results of synthesized compounds' antiradical activity

Compound	Antiradical activity (%)					lg EC_{50}
	10^{-3} M	10^{-4} M,	5×10^{-5} M	10^{-5} M	10^{-6} M	
2.1	76.54	79.13	48.67	26.11	10.05	−4.13
2.2	89.83	74.31	55.34	24.66	14.14	−4.39
2.3	13.24	9.49	7.42	6.12	5.02	>−3.00
2.4	10.12	10.46	4.29	7.72	5.66	>−3.00
3.1	19.06	0	0	0	0	>−3.00
3.2	26.18	0	0	0	0	>−3.00
3.3	19.09	17.56	13.04	11.03	0	>−3.00
3.4	5.24	0	0	0	0	>−3.00
3.5	49.48	21.89	20.17	12.24	11.03	>−3.00
3.6	32.24	10.86	10.17	9.14	8.79	>−3.00
3.7	28.79	11.03	9.31	7.76	7.24	>−3.00
3.8	11.49	7.38	6.69	7.03	4.63	>−3.00
3.9	72.04	54.55	52.66	31.90	6.86	−4.78
3.10	16.13	10.00	9.52	4.84	4.03	>−3.00
4.1	24.08	0	0	0	0	>−3.00
4.2	13.21	8.75	6.17	9.09	4.80	>−3.00
4.3	21.23	9.44	7.13	6.22	3.00	>−3.00
4.4	46.15	24.05	19.08	18.02	11.04	>−3.00
4.5	49.66	15.86	12.59	9.66	7.76	>−3.00
5.1	99.31	95.71	67.24	9.09	2.92	−4.53
5.2	95.81	93.98	91.79	23.73	0	−4.82
5.3	98.28	94.17	93.31	38.25	5.32	−4.92
5.4	42.75	20.00	16.13	15.65	15.02	>−3.00
5.5	26.13	18.23	16.61	15.97	15.81	>−3.00
Ascorbic acid	95.88	87.65	81.82	30.87	2.06	−4.81

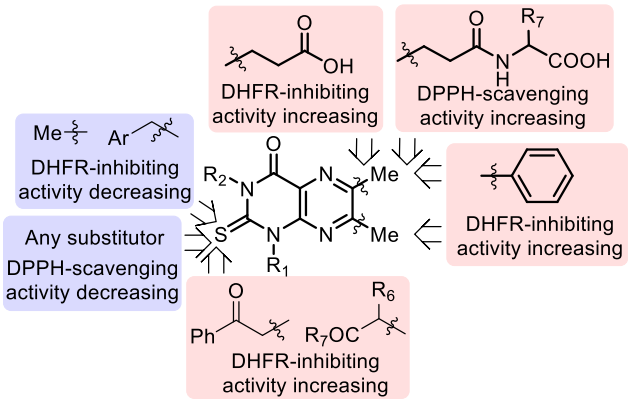


FIGURE 4 SAR-analysis among obtained thio-containing pteridines

compounds **2.4**, **2.5**, and **4.1–4.3** to corresponding amides **4.4–4.9** and **5.1–5.5** independently on the nature of amine led to the significant decrease of DHFR-inhibiting activity (Figure 4).

It was shown that the presence of exchangeable protons is essential for the presence of antiradical activity. The alkylation of the sulfur atom dramatically decreases the level of activity, at the same time introduction of the amino acid moiety to 6th position increases it (Figure 4).

3 | CONCLUSION

It was found that products of condensation of 5,6-diamino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one with dicarbonyl compounds is an effective approach for the synthesis of thio-containing pteridines as initial compounds for further modification aimed to the search of the

promising biologically active compounds. The predicted affinity of some synthesized compounds is comparable with the affinity of the reference compound methotrexate. Despite this, the differences in the ligand are enzyme interactions nature. Obtained compounds revealed low or moderate DHFR-inhibiting activity. Compounds **2.2**, **3.7**, **3.9**, and **4.2** were the most active at a concentration of 100 μM. The enzyme-inhibiting activity of obtained compounds depends on as structure of substituents in 6th and 7th positions so the nature of substituents at the sulfur atom. Compounds **3.9**, **5.1**, and **5.3** revealed high antiradical activity that was apparently associated with the presence of exchangeable protons and the possibility of tautomeric transformations. Obtained pteridines are of no interest in the scope of creation of novel chemotherapeutic agents but can be studied for activities associated with influence on redox processes in organisms.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

Melting points were determined in open capillary tubes in a «Mettler Toledo MP 50» apparatus and were uncorrected. The elemental analyses (C, H, N, S) were performed using the ELEMENTAR vario EL cube analyzer (USA). Analyses were indicated by symbols of the elements or functions within ±0.3% of the theoretical values. ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded on a Varian-Mercury 400 (Varian Inc.) spectrometer with TMS as an internal standard in DMSO-d_6 solution (see the Supporting Information). LC-MS were recorded using a chromatography/mass spectrometric system which consists of high-performance liquid chromatography «Agilent 1100 Series» (Agilent) equipped with diode-matrix and mass-selective detector «Agilent LC/MSD SL» (atmospheric pressure chemical ionization [APCI]). Electron impact mass spectra (EI-MS) were recorded on a Varian 1200L instrument at 70 eV (Varian). The purity of all obtained compounds was checked by ^{1}H-NMR and LC-MS.

Substances **1.1** (CAS: 1004-76-8), **2.1** (CAS:54030-51-2),[14,15] **2.2** (CAS: 14892-97-8) and other starting materials and solvents were obtained from commercially available sources and used without additional purification.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | General procedure for the synthesis of 6-R^3-7-R^4-2-thioxo-2,3-dihydropteridin-4(1*H*)-ones **(2.1–2.5)**

Ten millimoles of the corresponding dicarbonyl compound was added to the suspension of 10 mmol of 5,6-diamino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (**1.1**) in 30 ml of acetic acid. The formed mixture was refluxed for 1 h and cooled. The formed precipitate was filtered off, washed with water, and dried.

*7-Hydroxy-6-methyl-2-thioxo-2,3-dihydropteridin-4(1H)-one (**2.3**)*
Yield: 69.8%; M.p.: >300°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.33–12.46 (m, 2H, 1-NH, 7-OH), 12.34 (s, 1H, 3-NH), 2.38 (s, 3H, 6-CH_3); LC-MS: m/z = 211 [M+1]; Anal. calcd. for $C_9H_8N_4O_4S$: C, 40.00; H, 2.88; N, 26.65; S, 15.25; Found: C, 40.06; H, 2.93; N, 26.68; S, 15.27.

*3-(7-Hydroxy-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl) propanoic acid (**2.4**)*
Yield: 66.7%; M.p.: 293–295°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.72 (s, 1H, 7-OH), 12.24 (s, 1H, 1-NH), 11.79 (s, 1H, COOH), 10.87 (s, 1H, 3-NH), 3.17 (t, J = 7.3 Hz, 2H, $\underline{CH_2}CH_2COOH$), 2.78 (t, J = 7.3 Hz, 2H, $CH_2\underline{CH_2}COOH$); LC-MS: m/z = 269 [M+1]; Anal. calcd. for: $C_9H_8N_4O_4S$: C, 40.30; H, 3.01; N, 20.89; S, 11.95; Found: C, 40.37; H, 3.08; N, 20.92; S, 12.02.

*3-(7-Hydroxy-1,3-dimethyl-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl)propanoic acid (**2.5**)*
Yield: 79.1%; M.p.: 279–281°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.51 (s, 1H, 7-OH), 11.94 (s, 1H, COOH), 4.00 (s, 3H, 1N-CH_3), 3.76 (s, 3H, 3N-CH_3), 3.05 (t, J = 7.3 Hz, 2H, $\underline{CH_2}CH_2COOH$), 2.73 (t, J = 7.3 Hz, 2H, $CH_2\underline{CH_2}COOH$); ^{13}C NMR (126 MHz, DMSO-d_6) δ 177.16 (C-2), 174.02 (COOH), 160.06 (C-4), 157.84 (C-7), 146.73 (C-6), 144.12 (C-8a), 119.71 (C-4a), 36.85 (1-N-CH_3), 35.89 (3-N-CH_3), 30.49 (−$CH_2\underline{CH_2}COOH$), 27.12 (−$\underline{CH_2}CH_2COOH$); LC-MS: m/z = 297 [M+1]; Anal. calcd. for: $C_{11}H_{12}N_4O_4S$: C, 44.59; H, 4.08; N, 18.91; S, 10.82; Found: C, 44.63; H, 4.12; N, 18.96; S, 10.87.

4.1.3 | General procedure for the synthesis of 2-methyl-(benzyl-, phenacyl-)thio-6-R^3-7-R^4-pteridin-4(3*H*)-ones **(3.1–3.10)**

The appropriate quantity of sodium hydroxide (10 mmol, 0.4 g) for compounds **2.1**, **2.2**, 20 mmol (0.8 g.) for compound **2.3**, 30 mmol (1.2 g.) for compound **2.4**) was added to the suspension of 10 mmol of 6-R^3-7-R^4-2-thioxo-2,3-dihydropteridin-4(1*H*)-ones (**2.1–2.4**) in 20 ml of methanol/water (1:1) mixture. The formed mixture was heated until the precipitate was dissolved and 10 mmol of the proper alkylating reagent was added. The mixture was refluxed until the neutral pH value (around 1 h) and cooled. The mixture was filtered and the filtrate was acidified up to pH 3–4, the formed precipitate was filtered off, washed with water, and dried.

*7-Hydroxy-6-methyl-2-(methylthio)pteridin-4(3H)-one (**3.1**)*
Yield: 89.3%; M.p.: 214–216°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.00–12.10 (m, 2H, 3-NH, 7-OH), 2.56 (s, 3H, –SCH_3), 2.34 (s, 3H, 6-CH_3), LC-MS: m/z = 225 [M+1]; Anal. calcd. for: $C_8H_8N_4O_2S$: C, 42.85; H, 3.60; N, 24.99; S, 14.30; Found: C, 42.88; H, 3.66; N, 25.03; S, 14.35.

*2-(Benzylthio)-6,7-dimethylpteridin-4(3H)-one (**3.2**)*

Yield: 83.7%; M.p.: 226–228°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.84 (s, 1H, 3-NH), 7.41 (d, J = 7.3 Hz, 2H, Ar H-2,6), 7.25 (t, J = 7.3 Hz, 2H, Ar H-3,5), 7.21–6.95 (m, 1H, Ar H-4), 4.48 (s, 2H, –SCH_2–), 2.61 (s, 3H, CH_3), 2.59 (s, 3H, CH_3). LC-MS: m/z = 299 [M+1]; Anal. calcd. for: $C_{15}H_{14}N_4OS$: C, 60.38; H, 4.73; N, 18.78; S, 10.75; Found: C, 60.41; H, 4.77; N, 18.81; S, 10.78.

*2-(Benzylthio)-7-hydroxy-6-methylpteridin-4(3H)-one (**3.3**)*

Yield: 86.3%; M.p.: >300°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.42 (d, J = 7.5 Hz, 2H, Ar H-2,6), 7.38–7.12 (m, 3H, Ar H-3,4,5), 4.01 (s, 2H, –SCH_2–), 2.20 (s, 3H, CH_3); LC-MS: m/z = 301 [M+1]; Anal. calcd. for: $C_{14}H_{12}N_4O_2S$: C, 55.99; H, 4.03; N, 18.66; S, 10.67; Found: C, 56.03; H, 4.09; N, 18.71; S, 10.72.

*3-[2-(Benzylthio)-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl] propanoic acid (**3.4**)*

Yield: 74.5%; M.p.: 283–285°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.34–12.56 (m, 2H, 3-NH, 7-OH), 12.05 (s, 1H, COOH), 7.45 (d, J = 7.2 Hz, 2H, Ar H-2,6), 7.31–7.08 (m, 3H, Ar H-3,4,5), 4.36 (s, 2H, –SCH_2–), 2.89 (t, J = 7.2 Hz, 2H, $\underline{CH_2}CH_2COOH$), 2.62 (t, J = 7.2 Hz, 2H, $CH_2\underline{CH_2}COOH$); ^{13}C NMR (126 MHz, TFA) δ 179.86 (COOH), 165.05 (C-4), 162.24 (C-7), 158.24 (C-2), 157.20 (C-8a), 147.86 (C-6), 134.02 (Ph C-4), 128.72 (Ph C-2, 6), 128.38 (Ph C-3, 5), 127.87 (Ph C-1), 112.85 (C-4a), 35.58 (–SCH_2–), 28.79 (–$CH_2\underline{C}H_2COOH$), 25.85 (–$\underline{C}H_2CH_2COOH$); LC-MS: m/z = 358 [M+1]; Anal. calcd. for: $C_{16}H_{14}N_4O_4S$: C, 53.62; H, 3.94; N, 15.63; S, 8.95; Found: C, 53.66; H, 3.98; N, 15.69; S, 9.01.

*3-{2-[(2-Fluorobenzyl)thio]-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl} propanoic acid (**3.5**)*

Yield: 73.5%; M.p.: 286–288°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.78 (s, 2H, 3-NH, 7-OH), 11.86 (s, 1H, COOH), 7.76 (t, J = 7.0 Hz, 1H, Ar H-6), 7.24 (q, J = 6.1 Hz, 1H, Ar H-4), 7.13–6.93 (m, 2H, Ar H-3,5), 4.38 (s, 2H, –SCH_2–), 2.90 (t, J = 7.2 Hz, 2H, –$C\underline{H_2}CH_2COOH$), 2.63 (t, J = 7.2 Hz, 2H, –$CH_2C\underline{H_2}COOH$); ^{13}C NMR (126 MHz, TFA/DMSO) δ 179.90 (COOH), 164.96 (C-4), 162.14 (C-7), 161.72 (C-2), 159.23 (d, J = 237.9 Hz, Ar C-2), 157.32 (C-8a), 147.88 (C-6), 130.94 (d, J = 2.8 Hz, Ar C-6), 130.08 (d, J = 8.4 Hz, Ar C-4), 123.97 (d, J = 3.3 Hz, Ar C-5), 121.44 (d, J = 14.3 Hz, Ar C-1), 115.11 (d, J = 21.4 Hz, Ar C-3), 112.94 (C-4a), 29.18 (d, J = 2.5 Hz, –SCH_2–), 28.83 (–$CH_2\underline{C}H_2COOH$), 26.10 (–$\underline{C}H_2CH_2COOH$); LC-MS: m/z = 377 [M+1]; Anal. calcd. for: $C_{16}H_{13}FN_4O_4S$: C, 51.06; H, 3.48; N, 14.89; S, 8.52; Found: C, 51.11; H, 3.52; N, 14.90; S, 8.56.

*3-{2-[(2-Chloro-6-fluorobenzyl)thio]-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl}propanoic acid (**3.6**)*

Yield: 81.7%; M.p.: 297–299°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.70 (s, 2H, 3-NH, 7-OH), 11.91 (s, 1H, COOH), 7.33 (q, J = 8.0 Hz, 1H, Ar H-4), 7.25 (d, J = 8.0 Hz, 1H, Ar H-3), 7.12 (t, J = 8.5 Hz, 1H, Ar H-5), 4.58 (s, 2H, –SCH_2–), 2.91 (t, J = 7.1 Hz, 2H, –$C\underline{H_2}CH_2COOH$), 2.64 (t, J = 7.1 Hz, 2H, –$CH_2C\underline{H_2}COOH$); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.72 (COOH), 160.84 (d, J = 249.6 Hz, Ar C-6), 160.09 (C-4), 158.55 (C-7), 156.38 (C-2), 156.17 (C-8a), 147.46 (C-6), 134.7 (d, J = 4.6 Hz, Ar C-2), 130.92 (d, J = 9.7 Hz, Ar C-4), 125.81 (d, J = 2.9 Hz, Ar C-3), 121.32 (d, J = 18.0 Hz, Ar C-1), 114.85 (d, J = 21.8 Hz, Ar C-5), 113.25 (Ar C-4a), 29.68 (–$CH_2\underline{C}H_2COOH$), 27.37 (–$\underline{C}H_2CH_2COOH$), 25.74 (d, J = 2.1 Hz, –SCH_2–); LC-MS: m/z = 411 [M+1]; Anal. calcd. for: $C_{16}H_{12}ClFN_4O_4S$: C, 46.78; H, 2.94; N, 13.64; S, 7.80; Found: C, 46.82; H, 2.98; N, 13.69; S, 7.87.

*3-{2-[(2,6-Dichlorobenzyl)thio]-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl}propanoic acid (**3.7**)*

Yield: 87.8%; M.p.: 289–291°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.14–12.47 (m, 2H, 3-NH, 7-OH), 11.93 (s, 1H, COOH), 7.39 (d, J = 8.2 Hz, 2H, Ar H-3,5), 7.32–7.25 (m, 1H, Ar H-4), 4.71 (s, 2H, –SCH_2–), 2.91 (t, J = 7.1 Hz, 2H, –$C\underline{H_2}CH_2COOH$), 2.64 (t, J = 7.2 Hz, 2H, –$CH_2C\underline{H_2}COOH$); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.17 (COOH), 160.75 (C-4), 158.98 (C-7), 156.83 (C-2), 156.60 (C-8a), 147.93 (C-6), 135.65 (Ar C-2,6), 131.50 (Ar C-1), 131.26 (Ar C-4), 129.29 (Ar C-2,5), 113.69 (C-4a), 31.10 (–SCH_2–), 30.11 (–$CH_2\underline{C}H_2COOH$), 27.81 (–$\underline{C}H_2CH_2COOH$); LC-MS: m/z = 427 [M+1]; Anal. calcd. for: $C_{16}H_{12}Cl_2N_4O_4S$: C, 44.98; H, 2.83; N, 13.11; S, 7.50; Found: C, 45.02; H, 2.86; N, 13.18; S, 7.56.

*3-{2-[(4-Bromobenzyl)thio]-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl} propanoic acid (**3.8**)*

Yield: 92.5%; M.p.: >300°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.07–12.36 (m, 2H, 3-NH, 7-OH), 11.91 (s, 1H, COOH), 7.46 (d, J = 8.4 Hz, 2H, Ar H-3,5), 7.36 (d, J = 8.4 Hz, 2H, Ar H-2,6), 4.30 (s, 2H, –SCH_2–), 2.90 (t, J = 5.8 Hz, 2H, $\underline{CH_2}CH_2COOH$), 2.62 (t, J = 7.2 Hz, 2H, $CH_2\underline{CH_2}COOH$). LC-MS: m/z = 436 [M+1]; Anal. calcd. for: $C_{16}H_{13}BrN_4O_4S$: C, 43.95; H, 3.00; N, 12.81; S, 7.33; Found: C, 43.99; H, 3.06; N, 12.88; S, 7.38.

*3-{7-Hydroxy-4-oxo-2-[(2-oxo-2-phenylethyl)thio]-3,4-dihydropteridin-6-yl}propanoic acid (**3.9**)*

Yield: 83.4%; M.p.: 257–259°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.46–12.32 (m, 2H, 3-NH, 7-OH), 11.91 (s, 1H, COOH), 8.02 (d, J = 7.5 Hz, 2H, Ph H-2, 6), 7.61 (t, J = 7.3 Hz, 1H, Ph H-4), 7.51 (t, J = 7.5 Hz, 2H, Ph H-3, 5), 4.87 (s, 2H, –SCH_2–), 2.87 (t, J = 7.2 Hz, 2H, –$C\underline{H_2}CH_2COOH$), 2.61 (t, J = 7.2 Hz, 2H, –$CH_2C\underline{H_2}COOH$); ^{13}C NMR (151 MHz, DMSO-d_6) δ 192.82 (CO), 174.17 (COOH), 161.16 (C-4), 159.00 (C-7), 156.80 (C-2), 156.31 (C-8a), 147.81 (C-6), 135.85 (Ph C-1), 134.20 (Ph C-4), 129.27 (Ph C-2, 6), 128.83 (Ph C-3, 5), 113.57 (C-4a), 39.30 (–SCH_2–), 30.13 (–$CH_2\underline{C}H_2COOH$), 27.77 (–$\underline{C}H_2CH_2COOH$); LC-MS: m/z = 387 [M+1]; Anal. calcd. for: $C_{17}H_{14}N_4O_5S$: C, 52.85; H, 3.65; N, 14.50; S, 8.30; Found: 52.87; H, 3.68; N, 14.54; S, 8.37.

*3-(7-Hydroxy-2-{[2-(4-methoxyphenyl)-2-oxoethyl]thio}-4-oxo-3,4-dihydropteridin-6-yl)propanoic acid (**3.10**)*

Yield: 82.4%; M.p.: 274–276°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.24–12.17 (m, 2H, 3-NH, 7-OH), 11.94 (s, 1H, COOH), 7.99 (d, J = 8.5 Hz, 2H, Ar H-2,6), 6.98 (d, J = 8.5 Hz, 2H, Ar H-3,5), 4.78 (s, 2H, –SCH_2–), 3.85 (s, 3H, OCH_3), 2.87 (t, J = 7.0 Hz, 2H,

$\underline{CH_2}CH_2COOH$), 2.61 (t, J = 7.2 Hz, 2H, $CH_2\underline{CH_2}COOH$); LC-MS: m/z = 417 [M+1]; Anal. calcd. for: $C_{18}H_{16}N_4O_6S$: C, 51.92; H, 3.87; N, 13.46, S, 7.70; Found: C, 51.98; H, 3.88; N, 13.51, S, 7.76.

4.1.4 | General procedure for the synthesis of 2-[(6-R^3-7-R^4-4-oxo-3,4-dihydropteridin-2-yl)thio]-alkylcarboxylic acids **(4.1–4.12)**

The appropriate quantity of sodium hydroxide (20 mmol (0.8 g) for compounds **2.1**, **2.2**, 30 mmol (0.12 g) for compound **2.3**, 40 mmol (1.6 g.) for compound **2.4**) was added to the suspension of 10 mmol of 6-R^3-7-R^4-2-thioxo-2,3-dihydropteridin-4(1*H*)-ones (**2.1–2.4**) in 20 ml of methanol/water (1:1) mixture. The formed mixture was heated until the precipitate was dissolved and 10 mmol of the 2-chloroacetic or 2-chloropropanoic acids and their amides were added. The mixture was refluxed until the neutral pH value (around 1 h) and cooled. The mixture was filtered and the filtrate was acidified up to pH 3-4, the formed precipitate was filtered off, washed with water, and dried.

*2-[(6,7-Dimethyl-4-oxo-3,4-dihydropteridin-2-yl)thio]acetic acid (**4.1**)*

Yield: 67.3%; M.p.: 240–242°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.27–12.61 (m, 2H, 3-NH, 7-OH), 4.06 (s, 2H, –SCH_2–), 2.62–2.55 (m, 6H, 6-CH_3, 7-CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.65 (COOH), 160.71 (C-4), 159.67 (C-7), 158.81 (C-2), 152.86 (C-8a), 152.12 (C-6), 128.89 (C-4a), 33.19 (SCH_2), 23.01 (6-CH_3), 22.24 (7-CH_3); LC-MS: m/z = 267 [M+1]; Anal. calcd. for: $C_{10}H_{10}N_4O_3S$: C, 45.11; H, 3.79; N, 21.04; S, 12.04; Found: C, 45.16; H, 3.83; N, 21.11; S, 12.09.

*2-[(4-Oxo-6,7-diphenyl-3,4-dihydropteridin-2-yl)thio]acetic acid (**4.2**)*

Yield: 68.8%; M.p.: 274–276°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.94 (s, 1H, 3-NH), 11.64 (s, 1H, COOH), 7.54–7.40 (m, 4H, 6-Ar H-2,6, 7-Ar H-2,6), 7.40–7.22 (m, 6H, 6-Ar H-3, 4, 5, 7-Ar H-3,4,5), 3.99 (s, 2H, –SCH_2–); ^{13}C NMR (151 MHz, DMSO-d_6) δ 170.15 (COOH), 162.60 (C-4), 161.74 (C-8a), 157.07 (C-2), 152.97 (C-7), 150.62 (C-6), 138.50 (6-Ph C-1), 138.31 (7-Ph C-1), 130.09 (6-Ph C-2, 6), 130.01 (7-Ph C-2,6), 129.77 (7-Ph C-4), 129.09 (6-Ph C-4), 128.59 (6-Ph C-3, 5), 128.53 (7-Ph C-3, 5), 34.02 (–SCH_2); LC-MS: m/z = 391 [M+1]; Anal. calcd. for: $C_{20}H_{14}N_4O_3S$: C, 61.53; H, 3.61; N, 14.35; S, 8.21; Found: C, 61.57; H, 3.65; N, 14.42; S, 8.27.

*2-[(7-Hydroxy-6-methyl-4-oxo-3,4-dihydropteridin-2-yl)thio]acetic acid (**4.3**)*

Yield: 57.8%; M.p.: 289–291°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.92–12.03 (m, 3H, COOH, 3-NH, 7-OH), 4.01 (s, 2H, –SCH_2–), 2.33 (s, 3H, 6-CH_3); ^{13}C NMR (126 MHz, DMSO-d6) δ 169.58 (COOH), 160.92 (C-4), 158.95 (C-7), 157.13 (C-2), 155.13 (C-8a), 147.98 (C-6), 113.60 (C-4a), 33.10 (–SCH_2), 20.34 (6-CH_3); LC-MS: m/z = 269 [M+1]; Anal. calcd. for: $C_9H_8N_4O_4S$: C, 40.30; H, 3.01; N, 20.89; S, 11.95; Found: C, 40.35; H, 3.08; N, 20.91; S, 11.98.

*3-{2-[(Carboxymethyl)thio]-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl}propanoic acid (**4.4**)*

Yield: 59.2%; M.p.: 266–268°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.18–11.77 (m, 4H, 2-COOH, 3-NH, 7-NH), 4.01 (s, 2H, –SCH_2–), 2.95–2.88 (m, 2H, $C\underline{H_2}CH_2COOH$), 2.66 (t, J = 7.0 Hz, 2H, $CH_2C\underline{H_2}COOH$); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.18 (7-COOH), 169.58 (3-COOH), 161.15 (C-4), 158.98 (C-7), 156.79 (C-2), 156.40 (C-8a), 147.88 (C-6), 33.15 (SCH_2), 30.12 (–$CH_2\underline{C}H_2COOH$), 27.78 (–$\underline{C}H_2CH_2COOH$); LC-MS: m/z = 327 [M+1]; Anal. calcd. for: $C_{11}H_{10}N_4O_6S$: C, 40.49; H, 3.09; N, 17.17; S, 9.83; Found: C, 40.52; H, 3.11; N, 17.22; S, 9.86.

*2-{[6-(2-Carboxyethyl)-7-hydroxy-4-oxo-3,4-dihydropteridin-2-yl]thio}propanoic acid (**4.5**)*

Yield: 61.4%; M.p.: 247–249°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.18–12.05 (m, 4H, 2-COOH, 6-COOH, 3-NH, 7-OH), 4.54 (q, J = 7.1 Hz, 1H, –$SC\underline{H}(CH_3)$), 2.90 (t, J = 7.1 Hz, 2H, $\underline{CH_2}CH_2COOH$), 2.63 (t, J = 7.1 Hz, 2H, $CH_2\underline{CH_2}COOH$), 1.58 (d, J = 7.2 Hz, 3H, CH_3). LC-MS: m/z = 343 [M+1]; Anal. calcd. for: $C_{12}H_{14}N_4O_6S$: C, 42.10; H, 4.12; N, 16.37; S, 9.37; Found: C, 42.16; H, 4.19; N, 16.42; S, 9.41.

*2-[(6,7-Diphenyl-4-oxo-3,4-dihydropteridin-2-yl)thio]-acetamide (**4.6**)*

Yield: 92.5%; M.p.: >300°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.18 (s, 1H, 3-NH), 7.58 (s, 1H, NH_2), 7.49–7.40 (m, 4H, 6-Ar H -2,6, 7-Ar H-2,6), 7.39–7.21 (m, 6H, 6-Ar H-3,4,5, 7-Ar H-3,4,5), 7.10 (s, 1H, NH_2), 4.02 (s, 2H, SCH_2), LC-MS: m/z = 390 [M+1]; Anal. calcd. for: $C_{18}H_{12}N_4OS$: C, 61.68; H, 3.88; N, 17.98; S, 8.23; Found: C, 61.71; H, 3.93; N, 18.02; S, 8.26.

*2-[(6,7-Dimethyl-4-oxo-3,4-dihydropteridin-2-yl)thio]-N-(3-fluorophenyl)acetamide (**4.7**)*

Yield: 87.3%; M.p.: 246–248°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.06 (s, 1H, 3-NH), 10.56 (s, 1H, Ar-NH), 7.54 (d, J = 11.2 Hz, 1H, Ar H-2), 7.41–6.93 (m, 2H, Ar H-4,5), 6.98–6.11 (m, 1H, Ar H-6), 4.19 (s, 2H, –SCH_2–), 2.72–2.55 (m, 6H, 6-CH_3, 7-CH_3); LC-MS: m/z = 360 [M+1]; Anal. calcd. for: $C_{16}H_{14}FN_5O_2S$: C, 53.47; H, 3.93; N, 19.49; S, 8.92; Found: C, 53.51; H, 3.97; N, 19.52; S, 8.98.

*N-(3-Chlorophenyl)-2-[(6,7-dimethyl-4-oxo-3,4-dihydropteridin-2-yl)thio]acetamide (**4.8**)*

Yield: 86.1%; M.p.: 267–269°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.06 (s, 1H, 3-NH), 10.54 (s, 1H, NH), 7.75 (s, 1H, Ar H-2), 7.47 (d, J = 8.8 Hz, 1H, Ar H-4), 7.24 (t, J = 8.0 Hz, 1H, Ar H-5), 7.00 (d, J = 7.9 Hz, 1H, Ar H-6), 4.19 (s, 2H, –SCH_2–), 2.70–2.61 (m, 6H, 6-CH_3, 7-CH_3); LC-MS: m/z = 376 [M+1]; Anal. calcd. for: $C_{16}H_{14}ClN_5O_2S$: C, 51.13; H, 3.75; N, 18.63; S, 8.53; Found: C, 51.20; H, 3.79; N, 18.68; S, 8.59.

*N-(4-Chlorophenyl)-2-[(6,7-dimethyl-4-oxo-3,4-dihydropteridin-2-yl) thio]acetamide (**4.9**)*

Yield: 79.7%; M.p.: 281–283°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.04 (s, 1H, 3-NH), 10.46 (s, 1H, Ar-NH), 7.60 (d, *J* = 8.8 Hz, 2H, Ar H-3,5), 7.22 (d, *J* = 8.8 Hz, 2H, Ar H-2,6), 4.19 (s, 2H, –SCH_2–), 2.63–2.58 (m, 6H, 6-CH_3, 7-CH_3); LC-MS: *m/z* = 376 [M+1]; Anal. calcd. for: $C_{16}H_{14}ClN_5O_2S$: C, 51.13; H, 3.75; N, 18.63; S, 8.53; Found: C, 51.17; H, 3.81; N, 18.67; S, 8.58.

*N-(3-Fluorophenyl)-2-[(7-hydroxy-6-methyl-4-oxo-3,4-dihydropteridin-2-yl)thio]acetamide (**4.10**)*

Yield: 91.0%; M.p.: 258–260°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.93 (s, 1H, 3-NH), 12.66 (s, 1H, 7-OH), 10.01 (s, 1H, Ar-NH), 7.54 (d, *J* = 11.4 Hz, 1H, Ar H-2), 7.37–7.17 (m, 2H, Ar H-4, 5), 6.76 (t, *J* = 7.8 Hz, 1H, Ar H-6), 4.02 (s, 2H, –SCH_2–), 2.34 (s, 3H, CH_3); LC-MS: *m/z* = 362 [M+1]; Anal. calcd. for: $C_{15}H_{12}FN_5O_3S$: C, 49.86; H, 3.35; N, 19.38; S, 8.87; Found: C, 49.92; H, 3.40; N, 19.43; S, 8.90.

*3-[2-({2-[(3-Fluorophenyl)amino]-2-oxoethyl}thio)-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl]propanoic acid (**4.11**)*

Yield: 79.7%; M.p. 277–259°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.70 (s, 2H, 3-NH, 7-OH), 12.05 (s, 1H, COOH), 10.01 (s, 1H, Ar-NH), 7.54 (d, *J* = 11.1 Hz, 1H, Ar H-2), 7.36–7.10 (m, 2H, Ar H-4, 5), 6.75 (t, *J* = 7.7 Hz, 1H, Ar H-6), 4.02 (s, 2H, –SCH_2–), 2.92 (t, *J* = 6.8 Hz, 2H, $\underline{CH_2}CH_2COOH$), 2.66 (t, *J* = 7.1 Hz, 2H, $CH_2\underline{CH_2}COOH$), LC-MS: *m/z* = 420 [M+1]; Anal. calcd. for: $C_{17}H_{14}FN_5O_5S$: C, 48.69; H, 3.36; N, 16.70; S, 7.64; Found: C, 48.76; H, 3.42; N, 16.78; S, 7.67.

*3-[2-({2-[(3-Chlorophenyl)amino]-2-oxoethyl}thio)-7-hydroxy-4-oxo-3,4-dihydropteridin-6-yl]propanoic acid (**4.12**)*

Yield: 94.3%; M.p.: 287–289°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (s, 2H, 3-NH, 7-OH), 12.01 (s, 1H, COOH), 9.97 (s, 1H, Ar-NH), 7.75 (s, 1H, Ar H-2), 7.46 (d, *J* = 8.1 Hz, 1H, Ar H-4), 7.25 (t, *J* = 8.1 Hz, 1H, Ar H-5), 7.01 (d, *J* = 7.5 Hz, 1H, Ar H-6), 4.01 (s, 2H, –SCH_2–), 2.93 (t, *J* = 7.1 Hz, 2H, $\underline{CH_2}CH_2COOH$), 2.66 (t, *J* = 7.0 Hz, 2H, $CH_2\underline{CH_2}COOH$). LC-MS: *m/z* = 436 [M+1]; Anal. calcd. for: $C_{17}H_{14}ClN_5O_5S$: C, 46.85; H, 3.24; N, 16.07; S, 7.36; Found: C, 46.91; H, 3.29; N, 16.11; S, 7.41.

4.1.5 | General procedure for the synthesis of 3-(7-hydroxy-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl) propanoic acids amides **(5.1–5.5)**

10 mmol of 3-(7-hydroxy-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl)propanoic acids (**2.4**, **2.5**) was refluxed in 20 ml of acetic acid/acid anhydride (1:1) mixture for 1 h. The reactional mixture was cooled, and the formed precipitate was filtered off and washed with diethyl ether. The precipitate was dissolved in 20 ml of acetic acid and 10 mmol of substituted aniline or amino acid was added to the formed mixture. The reaction mixture was refluxed for 1 h. After finishing the reaction the mixture was cooled, and the formed mixture was filtered off, washed with water, and dried.

*[3-(7-Hydroxy-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl) propanoyl]glycine (**5.1**)*

Yield: 71.3%; M.p.: 273–275°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.17–11.57 (m, 4H, 1-NH, 3-NH, COOH, 7-OH), 8.08 (t, *J* = 5.5 Hz, 1H, –N*H*–), 3.72 (d, *J* = 5.6 Hz, 2H, $NH\underline{CH_2}$), 3.07–2.81 (m, 2H, $\underline{CH_2}CH_2COOH$), 2.70–2.54 (m, 2H, $CH_2\underline{CH_2}COOH$). LC-MS: *m/z* = 326 [M+1]; Anal. calcd. for: $C_{11}H_{11}N_5O_5S$: C, 40.62; H, 3.41; N, 21.53; S, 9.86 Found: C, 40.62; H, 3.41; N, 21.53; S, 9.86.

*[3-(7-Hydroxy-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl) propanoyl]leucine (**5.2**)*

Yield: 73.9%; M.p.: 261–263°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.11–12.44 (m, 3H, 1-NH, COOH, 7-OH), 12.36 (s, 1H, 3-N*H*), 7.95 (d, *J* = 7.9 Hz, 1H, –N*H*–), 4.20 (q, *J* = 8.4 Hz, 1H, $\underline{CH}COOH$), 3.16–2.72 (m, 2H, $\underline{CH_2}CH_2COOH$), 2.74–2.54 (m, 2H, $CH_2\underline{CH_2}COOH$), 1.72–1.60 (m, 1H, $\underline{CH}(CH_3)_2$), 1.57–1.41 (m, 2H, $CHCH_2CH$), 0.91 (d, *J* = 6.5 Hz, 3H, –$CH(\underline{CH_3})_2$)), 0.87 (d, *J* = 6.4 Hz, 3H, $CH(\underline{CH_3})_2$). LC-MS: *m/z* = 382 [M+1]; Anal. calcd. for: $C_{15}H_{19}N_5O_5S$: C, 47.24; H, 5.02; N, 18.36; S, 8.41; Found: C, 47.29; H, 5.11; N, 18.41; S, 8.47.

*[3-(7-Hydroxy-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl) propanoyl]methionine (**5.3**)*

Yield: 68.2%; M.p.: 265–267°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.05–12.51 (m, 3H, 1-NH, 3-NH, 7-OH), 12.37 (s, 1H, COOH), 8.06 (d, *J* = 8.0 Hz, 1H, –N*H*–), 4.39–4.19 (m, 1H, $NH\underline{CH}COOH$), 3.05–2.86 (m, 2H, $\underline{CH_2}CH_2COOH$), 2.61 (t, *J* = 7.4 Hz, 2H, $CH_2\underline{CH_2}COOH$), 2.49–2.34 (m, 2H, CH_2S), 2.05 (s, 3H, SCH_3), 2.01–1.56 (m, 2H, $\underline{CH_2}CH_2S$). LC-MS: *m/z* = 400 [M+1]; Anal. calcd. for: $C_{14}H_{17}N_5O_5S_2$: C, 42.10; H, 4.29; N, 17.53; S, 16.05; Found: C, 42.19; H, 4.36; N, 17.58; S, 16.11.

*3-(7-Hydroxy-1,3-dimethyl-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl)-N-phenylpropanamide (**5.4**)*

Yield: 80.3%; M.p.: 290–292°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.46 (s, 1H, 7-OH), 9.88 (s, 1H, N*H*), 7.57 (d, *J* = 7.8 Hz, 2H, Ar H-2,6), 7.20 (t, *J* = 7.8 Hz, 2H, Ar H-3,5), 6.95 (t, *J* = 7.3 Hz, 1H, Ar H-4), 4.00 (s, 3H, 1N-CH_3), 3.76 (s, 3H, 3N-CH_3), 3.12 (t, *J* = 7.5 Hz, 2H, $\underline{CH_2}CH_2CO$), 2.85 (t, *J* = 7.5 Hz, 2H, $CH_2\underline{CH_2}CO$); LC-MS: *m/z* = 372 [M+1]; Anal. calcd. for: $C_{17}H_{17}N_5O_3S$: C, 54.98; H, 4.61; N, 18.86; S, 8.63; Found: C, 55.04; H, 4.68; N, 18.96; S, 8.68.

*4-[3-(7-Hydroxy-1,3-dimethyl-4-oxo-2-thioxo-1,2,3,4-tetrahydropteridin-6-yl)propanamido]benzoic acid (**5.5**)*

Yield: 89.4%; M.p.: >300°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.97 (s, 1H, COOH), 10.21 (s, 1H, N*H*), 7.84 (d, *J* = 8.6 Hz, 2H, Ar H-2,6), 7.67 (d, *J* = 8.6 Hz, 2H, Ar H-3,5), 4.01 (s, 3H, 1N-CH_3), 3.76 (s, 3H, 3N-CH_3), 3.30–3.10 (m, 2H, $\underline{CH_2}CH_2CO$), 2.95–2.80 (m, 2H, $CH_2\underline{CH_2}CO$); ^{13}C NMR (126 MHz, DMSO-d_6) δ 177.13 (C-2), 171.25 (CONH), 167.33 ($\underline{C}OOH$), 160.11 (C-4), 157.86 (C-7),

146.72 (C-6), 144.40 (Ar C-1), 143.75 (C-8a), 130.74 (Ar C-2, 6), 125.21 (Ar C-4), 119.75 (C-4a), 118.62 (Ar C-3, 5), 36.84 (1-N-CH_3), 35.88 (3-N-CH_3), 33.25 ($CH_2\underline{C}H_2CONH$), 27.26 ($\underline{C}H_2CH_2CONH$); LC-MS: *m/z* = 416 [M+1]; Anal. Calcd. for: $C_{18}H_{17}N_5O_5S$: C, 52.04; H, 4.12; N, 19.26; S, 7.72; Found: C, 52.11; H, 4.19; N, 16.91; S, 7.79.

4.2 | Molecular docking

Research was conducted by flexible molecular docking, as an approach for finding molecules with affinity to a specific biological target. Macromolecules from Protein Data Bank (PDB) were used as biological targets, namely DHFR (PDB ID 1RG7).[22] The choice of biological targets was due to the literature about the mechanism of antitumor drug activity.[13]

4.2.1 | Ligand preparation

Substances were drawn using MarvinSketch 20.20.0 and saved in mol format.[23] After that they were optimized by program Chem3D, using molecular mechanical MM2 algorithm and saved as pdb-files. Molecular mechanics was used to produce more realistic geometry values for most organic molecules, owing to the fact of being highly parameterized. Using AutoDockTools-1.5.6 pdb-files were converted into PDBQT, the number of active torsions was set as default.[24]

4.2.2 | Protein preparation

PDB files were downloaded from the Protein Data Bank. Discovery Studio v 19.1.0.18287 was used to delete water molecules and ligands. Structures of proteins were saved as pdb-files.[25] In AutoDockTools-1.5.6 polar hydrogens were added and saved as PDBQT. Grid box was set as follows: center_x = −1.657, center_y = 22.030, center_z = 23.080, size_x = 14, size_y = 12, size_z = 12. Vina was used to carry out docking.[23] For visualization Discovery Studio v 19.1.0.18287 was used. To validate the docking method the reference ligand, namely methotrexate was extracted and then reused for the redocking process. The calculation of the RMSD value was performed with DockRMSD available online.[26]

4.3 | DHFR inhibition assay

Reagents: Dihydrofolate reductase Assay Kit (Sigma-Aldrich, Catalog Number CS0340, Batch Number 067M4065V) was used for evaluation of the DHFR-inhibitory activity of synthesized compounds. The protein content in supplied dihydrofolate reductase was 0.032 mg/ml and the activity of the enzyme was 3.75 U/mgP.

4.3.1 | The procedure of estimation of studied compounds DHFR-inhibitory activity

To the microcentrifuge tube (volume 2 ml) 966 µl of diluted 1:10 assay buffer was added. Then sequentially 13 µl of DHFR and 10 µl of 100 µM solution of the studied compound in DMSO were added. Compounds **3.9** and **4.2** were selected and tested for dose–response at five concentrations according to standard procedures (100–0.01 µM). The tube was sealed and intensively shacked and the formed mixture was transferred to the 1.4 ml quartz cuvette. To the formed mixture, 6 µl of 10 mM solution of the NADPH was added, and the cuvette was sealed with parafilm and shaken. To the formed mixture, 5 µl of 10 mM solution of dihydrofolic acid was added, the cuvette was sealed by parafilm, shake, and immediately transferred to spectrophotometer ULab 131 UV. The absorption of the sample at 340 nm was measured every 15 s for 150 s.

The activity of the enzyme was calculated according to the following formula:

$$\text{Activity(Units/mgP)} = \frac{\Delta\text{OD/min}_{\text{sample}} - \Delta\text{OD/min}_{\text{blank}}}{12.3 * 0.013 * 0.032},$$

where:

- $\Delta\text{OD/min}_{\text{blank}} = \Delta\text{OD/min}$ for the blank, from the spectrophotometer readings;
- $\Delta\text{OD/min}_{\text{sample}} = \Delta\text{OD/min}$ for the reaction, from the spectrophotometer readings;
- 12.3 = extinction coefficient (ε, $mM^{-1}*cm^{-1}$) for the DHFR reaction at 340 nm;
- 0.013 = Enzyme volume in ml (the volume of enzyme used in the assay);
- 0.032 = Enzyme concentration of the original sample.

The value of DHFR-inhibitory activity in % was calculated according to the formula:

$$\text{DHFR} - \text{inhibitory activity}(\%) = \frac{3.75 - \text{Activity(Units/mg}P)}{3.75} * 100\%$$

Methotrexate was used as a reference compound.

4.4 | Antiradical activity

The in vitro research of antiradical activity was based on the interaction of synthesized compounds with 2,2-diphenyl-1-picrylhydrazyl (DPPH).[27] DPPH is a stable free radical and its alcohol solutions are colored in intense purple color (λ_{max} = 517 nm). DPPH interacted with compounds that are able to bind with free radicals yielding products, which are yellow-colored and does not absorb the light at the specified above wavelengths.

4.4.1 | Research methodology

Compounds were dissolved in DMSO to obtain 1 mM solution. Then, 2 ml of this solution was mixed with 2 ml of 0.1 mM DPPH methanol solution and it was incubated for 30 min at the temperature of 25°C for 30 min. Then optical density (Ad) was measured.[28] The optical density of 2 ml of 0.1 mM DPPH solution in 2 ml of methanol (ADPPH) was determined simultaneously. Antiradical activity (ARA) was calculated by the following formula: ARA % = (ADPPH − Ad)/ADPPH × 100%. In the case of a negative meaning, ARA in % was estimated as 0. Weighing reagents and synthesized compounds were conducted on electronic scales «ANG200C» (Axis, Gdansk, Poland) and the optical density was measured by a spectrophotometer «ULAB 108UV» (Ulab).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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