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Original article

Mechanism-based design, synthesis and biological studies of N^5-substituted tetrahydrofolate analogs as inhibitors of cobalamin-dependent methionine synthase and potential anticancer agents

Zhili Zhang [b], Chao Tian [b], Shouxin Zhou [b], Wei Wang [b], Ying Guo [b], Jie Xia [a], Zhenming Liu [a], Biao Wang [b], Xiaowei Wang [b], Bernard T. Golding [c], Roger J. Griff [c], Yansheng Du [d], Junyi Liu [a,b,*]

[a] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, China
[b] Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China
[c] Department of Chemistry, University of Newcastle, England
[d] Department of Neurology, Indiana University School of Medicine, USA

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ABSTRACT

A number of 8-deazatetrahydrofolates bearing electrophilic groups on N^5 were designed and synthesized based on the action mechanism of methionine synthase, and their biological activities were investigated as well. Compounds (**11b**, **12b** and **16**) showed the most active against methionine synthase (IC_{50}: 8.11 μM, 1.73 μM, 1.43 μM). In addition, the cytotoxicity to human tumor cell lines and dihydrofolate reductase (DHFR) inhibition by target compounds were evaluated.

1. Introduction

Methionine synthase (EC.2.1.1.13) plays a crucial role in folate metabolism due to its catalytic activity of the conversion of homocysteine to methionine. The conversion of homocysteine to methionine can be catalyzed by two distinct enzymes, which are the cobalamin-independent and cobalamin-dependent methionine synthase. The cobalamin-independent enzyme catalyzes the direct transfer of a methyl group from 5-methyltetrahydrofolate (CH_3–H_4folate) to homocysteine, whereas the cobalamin-dependent methionine synthase (MetS), as an intermediary methyl carrier, promotes the transfer of the methyl group in two steps. Firstly, the methyl group is transferred from CH_3–H_4folate to enzyme-bound cob(I)alamin (Cbl(I)) to generate methyl-cob(III)alamin (CH_3–Cbl) and H_4folate, secondly it is transferred from the generated CH_3–Cbl to homocysteine to produce methionine and regenerate the Cbl(I) (Fig. 1) [1]. The cobalamin–independent methionine synthase was found in plants and the cobalamin-dependent methionine synthase was found in mammalian tissues, and either one or both could be found in microorganisms [2].

Since free cob(I)alamin is a strong nucleophile [3], and can easily react with alkyl halides following S_N2 mechanism [4], the postulated double-transfer mechanism has stereochemical and kinetic constraints on the reaction. Zydowsky [2] observed that, using isotope labeled N^5 (HDT) CH_3–H_4folate, steric effect supported the proposed two-step reaction mechanism in which the methyl group was transferred sequentially from CH_3–H_4folate to Cb1(I) and then from the generated enzyme-bound CH_3–Cbl to homocysteine.

The methionine and H_4folate will be further metabolized through the one-carbon methionine transmethylation and folate cycles. Therefore, MetS is involved in many important biochemical pathways. According to the proposed mechanism, inhibition of MetS will result in the decline of the intracellular methionine and S-adenosylmethionine (AdoMet), and accumulation of CH_3–H_4folate in the folate circulation. Besides the redistribution of cellular folate derivatives, inhibition of methionine synthase also leads to a decline in the total intracellular folate level. The major circulation form of folate is CH_3–H_4folate, and MetS activity is required to convert this derivative to forms that can be used for

* Corresponding author. Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China.
E-mail address: lilybmu@bjmu.edu.cn (J. Liu).

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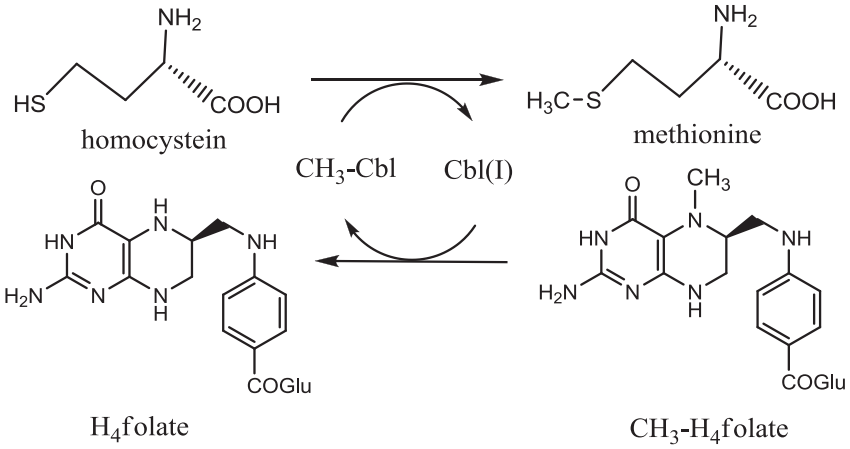


Fig. 1. The cobalamin-dependent methionine synthase catalyzed methyl group transfer. Cbl(I): Cob(I)alamin, CH_3–Cbl: methylcob(III)alamin, Glu: glutamate.

nucleotide biosynthesis [5]. Thus, the deleterious effect of MetS inhibition on cellular nucleotide biosynthesis and overall folate distribution renders this enzyme a particularly interesting target for chemotherapeutic intervention.

2. Design of inhibitors

Although the biochemical role of MetS is well understood and it can be an excellent target for rational drug design, no anticancer agents directed toward this enzyme is available to date. The enzyme is efficiently and specifically inhibited by the cell-signaling molecule nitrous oxide (N_2O) [6,7] through the oxidation of the cobalamin cofactor [8]. There are also other inhibitors such as methylmercury through binding to homocysteine [9], ethanol and acetaldehyde through acetaldehyde-induced inhibition (IC_{50}: 2 mM) [10], S-AdoMet derivatives [11] and various cobalamin analogs through decrease of the coenzyme cobalamin (activity decreased to 20%) [12] reported in literature. However, it appears that the design of folate analogs as inhibitors of MetS has been overlooked. Consequently, no prototype folate analog inhibitor is available for synthetic studies.

Recently, benzimidazole and benzothiadiazole derivatives have been designed and evaluated in a cell free system as specific MetS inhibitors by Banks et al. (Fig. 2) [13]. The activity of the inhibitors and the action mechanism have been investigated by using purified rat liver MetS. It was found that some benzimidazoles and nitrobenzothiadiazoles gave IC_{50} values close to or below 100 μM. According to CH_3–H_4folate-binding model with MetS, some structural requirements for the design of inhibitors should be met. For example, N^1 and 2-amino group of CH_3–H_4folate forming hydrogen bonds with amino acid residues of MetS should be kept. A planar region like pyrimidine ring that can be positioned in the center of the active site, with two or three possible hydrogen-bonding groups, is required.

X = H, OCH_3; Y = H, NO_2, NH_2, OCH_3; R = H, CH_3; Z = H, NO_2, NH_2

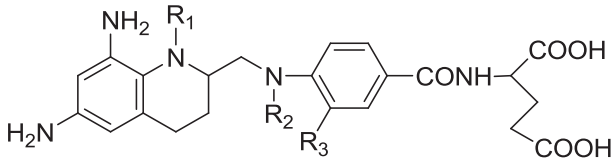
Fig. 2. Structures of benzimidazole and benzothiadiazole derivatives synthesized by Banks et al.

Based on the action mechanism and the model with an active site for docking the substrate CH_3–H_4folate, we designed and synthesized a series of N^8-deazaH_4folates bearing electrophilic substituents at N^5-position in which carbons have partial positive charge due to the polarity of the carbon–oxygen π bond or carbon–halogen bond (Fig. 3) as novel inhibitors of MetS. These compounds should bind to MetS competitively to replace CH_3–H_4folate, and inhibit MetS activity by formation of covalent bonds with the nucleophilic cob(I)alamin cofactor of the enzyme, as shown in Fig. 4.

Literature survey revealed that numerous derivatives of folate have been synthesized to date as potential antagonists. There were structural modifications in either the pteridine ring, the p-aminobenzoyl region, or the glutamate side-chain [14]. So far, very few studies on the effect of N^5-substituent of H_4folate have been reported [15]. One obstacle in the synthesis of such compounds is the autoxidation tendency of H_4folates under relatively mild conditions [15]. In contrast, their 5-or 8-deaza analogs are significantly more stable. Therefore, we designed 8-deaza and N^5-substituted H_4folate analogs, which should have higher reactivity and stability than CH_3–H_4folate. The N^5-electron-deficient substituent of N^8-deazatetrahydrofolates could easily form covalent bonds with the nucleophilic cob(I)alamin of MetS, thus inhibiting the activity of MetS.

3. Chemistry

The synthesis of N^5-substituted 8-deazatetrahydrofolate derivatives **8b**–**11b** was accomplished via the reactions outlined in Scheme 1. Intermediate 6-bromomethyl-2,4-diaminopyrido[3,2-d] pyrimidine (**1**) was prepared by a procedure initially reported by Srinivasan and Broom [16] and then modified by DeGraw et al. [17] Chlorination of 6-acetoxymethyl-2,4-dioxopyrido[3,2-*d*]pyrimidine with $POCl_3$ in the presence of a catalytic amount of triethylamine under reflux to afford 6-acetoxymethyl-2,4-dichloro



R_1 = -CHO, -TS, $-CH_2CHBrCH_2Br$; R_2=H, $-COCH_3$, $-CH_3$; R_3=H, Br

Fig. 3. N^8-deazatetrahydrofolates bearing N^5 electrophilic substituents.

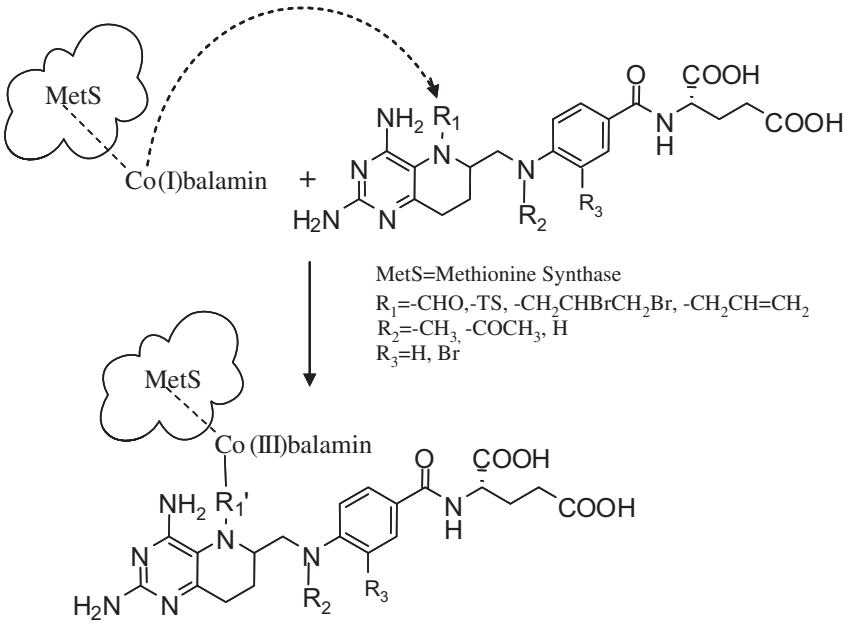
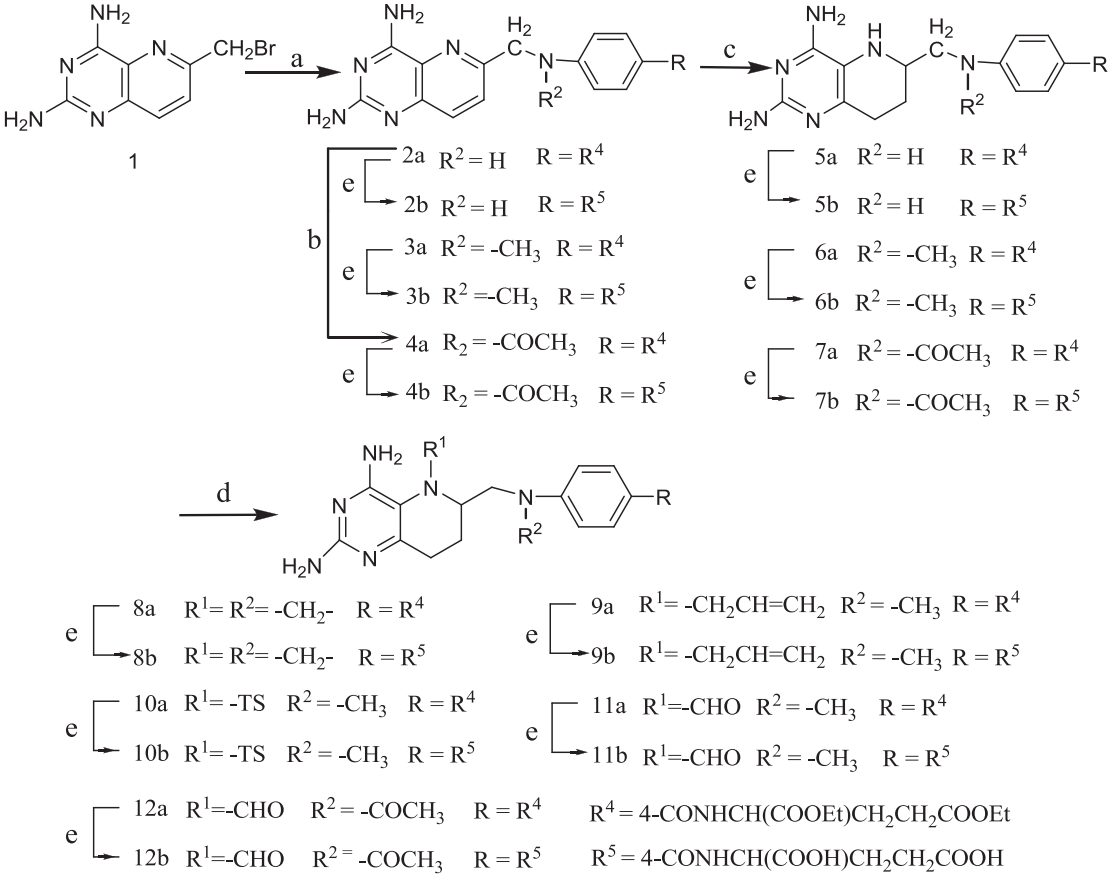


Fig. 4. Reactions of designed inhibitors with MetS.

pyrido[3,2-d]pyrimidine. Subsequent hydrolytic amination with ammonia–methanol in a sealed vessel at 150 °C furnished 2,4-diamino-6-hydroxymethylpyrido[3,2-*d*]pyrimidine, which was further transformed to crude product **1** by bromination with PBr_3.

Intermediate **1** [16] was condensed with diethyl *N*-(*p*-aminobenzoyl)-L-glutamate and diethyl *N*-[*p*-(methylamino)benzoyl]-L-glutamate in dry dimethylacetamide (DMAc) to afford the compounds **2a** and **3a**, respectively. The reaction partner *N*-[*p*-



Scheme 1. Reagents and conditions: (a) DMAc, diethyl *N*-(*p*-aminobenzoyl) -L-glutamate or diethyl *N*-[*p*-(methylamino)benzoyl]-L-glutamate, rt, 48 h; (b) acetic anhydride, acetic acid, rt, 2 h; (c) PtO_2, 0.3–0.4 MPa, AcOH or trifluoroactyl acid, ethanol, rt, 2–3 d; (d) 8a: paraformaldehyde, THF, H_2O, sonication, rt, 4 h; 9a: allyl bromide, Cs_2CO_3, DMF, rt, 15 h; 10a: Tos-Cl, $(CH_3CH_2)_3N$, CH_3CN, rt, 10 h; 11a: formic acid, ethyl formate, rt, 1 h; 12a: formic acid, 60 °C, 2 h; (e) 1 mol/L NaOH solution, THF, rt.

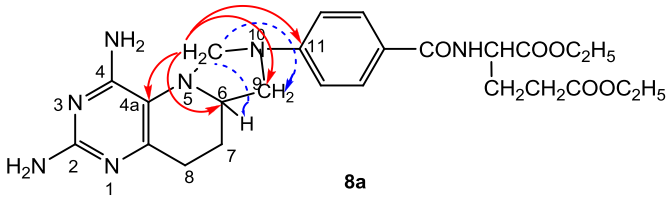


Fig. 5. Long-range coupled correlation of the methylene groups of **8a**. $\rightarrow$ ^{1}H of methylene group and ^{13}C in three-bond distance, $\dashrightarrow$ ^{13}C of methylene group and ^{1}H in three-bond distance.

(methylamino)benzoyl]-L-glutamate was synthesized from commercially available diethyl *N*-(*p*-aminobenzoyl)-L-glutamate by using a modified method reported Yang et al. [18] Reductive benzylation and methylation of *N*-(*p*-aminobenzoyl)-L-glutamate by benzaldehyde, sodium cyanoborohydride, acetic acid, and formaldehyde in one pot afforded diethyl *N*-[*p*-(*N*-benzoyl-*N*-methylamino) benzoyl]-L-glutamate [19]. Then hydrogenolytic debenz ylation with Pd/C in 1 N HCl at 0.3 MP furnished diethyl *N*-[(*p*-methylamino)benzoyl]-L-glutamate.

With the condensed products in hand, treatment of diethyl 8-deaza aminopterin **2a** with a mixture of acetic anhydride and acetic acid gave the N^{10}-acetyl derivative **4a** directly. Reduction of **2a** and **3a** with PtO_2 and HOAc in ethanol at 40 °C gave compounds **5a** and **6a** in 45% yield along with over-reduced by-products [14]. By treatment with trifluoroacetic acid, **4a** could be transformed to **7a** in a high yield (74%), possibly because of the electron-withdrawing acetyl group. Compounds **5a**, **6a** and **7a** were diastereomeric mixtures due to the appearance of another chiral center C6, and the ratio determined by chiral high performance liquid chromatography is 1:1.

The N^{5},N^{10}-methylenated compound **8a** was synthesized from **5a** and paraformaldehyde at room temperature by nucleophilic addition and dehydration reactions. It was found that the reaction rate could be increased under sonication. And no products of hydroxymethylation on N^{5} or N^{10} of **5a** was observed in the reaction. The structure assignment of **8a** was achieved based on detailed MS and NMR spectroscopic studies. The ESI-TOF-MS showed the molecular ion peak $[M + H]^{+}$ was at *m*/*z* 512. Signals at δ 3.78, 4.92 (d, each, J = 4.8 Hz) in the ^{1}H NMR (DMSO-d_6) spectrum and signals at δ 65.8 in the ^{13}C NMR (DMSO-d_6) spectrum were assigned to N–CH_2–N. In addition, correlation signals between the long-range J-coupled H of methylene groups and C-6 (δ 56.3), C-4a (δ 113.7), C-9 (δ 51.2), C-11 (δ 148.2) could be observed at cross points (Fig. 5). Also the coupling signals between the carbon of methylene and H-6 (δ 3.65), H-9 (δ 3.33) could be found. Additional gHMBC results confirmed the assigned structure of **8a**.

The N^{5}-allyl N^{10}-methyl derivative **9a** was synthesized in 40% yield from **6a** and allyl bromide (1.1 eq) in DMF with K_2CO_3 as the base, and the yield was improved to 76% when using Cs_2CO_3 instead of K_2CO_3. Treatment of **6a** with TsCl and $(C_2H_5)_3N$ gave the N^{5}-tosyl N^{10}-methyl derivative **10a**. Treatment of **6a** and **7a** with a mixture of ethyl formate and formic acid gave the N^{5}-formyl derivatives **11a** and **12a** in 75 and 86%, respectively.

Finally saponification of the diesters of **2a**–**12a** with 0.3 mol/L NaOH in ethanol or THF gave the target compounds **2b**–**11b**.

4. Results and discussion

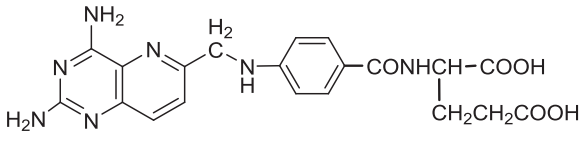
4.1. Methionine synthase and dihydrofolate reductase (DHFR) inhibition

The target compounds and intermediates were evaluated against MetS of HL-60 and recombinant human DHFR, respectively, and the half-inhibitory concentrations (IC_{50}) are presented in Table 1. Compounds **13**, **14**, **15** and **16** previously reported [14] as inhibitors of DHFR have the same substitution on N^{5}-position [Fig. 6]. So they were evaluated at the same time to get more information of structure activity relationship.

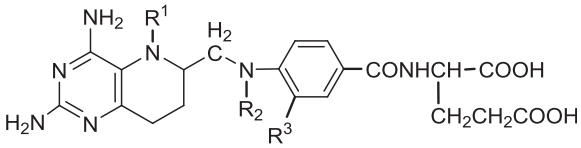
IC_{50} values of N^{5}-substituted compounds **8b**–**11b** and **13**–**16** on MetS were below 66.38 μM except that of **9b**. Comparing the activity results, it was apparent that the alkyl and acyl groups at N^{5}-position were associated with stronger inhibition (**5b** compared with **13**, **6b** compared with **11b**, **7b** compared with **12b**) whereas the N^{5}-unsubstituted analogs (**2b** and **5**–**7b**) showed no inhibition of MetS.

5-Dibromosubstituted compound **16**, the most potent inhibitor of MetS in this series, showed a greater activity, about 15-fold with IC_{50} of 1.43 μM, than the corresponding 5-alky compound **15** (IC_{50} = 21.00 μM). Similarly, compounds **11b** and **12b** with formyl groups on N^{5} were stronger MetS inhibitors than **6b** and **7b**. The

Table 1
In vitro inhibitory effects against MetS and DHFR.



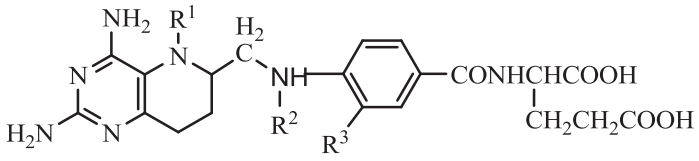
2a



5b-12b, 13-16

	R^1	R^2	R^3	MetS (IC_{50}, μM)	DHFR (IC_{50}, μM)
2b	–	–	–	>100	0.018 ± 0.00098
5b	H	H	H	87.91 ± 5.81	0.56 ± 0.060[a]
6b	H	$-CH_3$	H	>100	0.033 ± 0.0027
7b	H	$-COCH_3$	H	>100	0.033 ± 0.0051
13	$-CH_2CH{=}CH_2$	H	H	27.83 ± 5.19	1.41 ± 0.12[a]
14	$-CH_2CH{=}CH_2$	$-CH_2CH{=}CH_2$	H	15.87 ± 1.43	2.15 ± 0.16[a]
8b	$-CH_2-$	$-CH_2-$	H	66.38 ± 10.99	0.80 ± 0.00005
9b	$-CH_2CH{=}CH_2$	$-CH_3$	H	>100	0.42 ± 0.063
10b	–TS	$-CH_3$	H	55.00 ± 3.74	1.07 ± 0.32
11b	–CHO	$-CH_3$	H	8.11 ± 1.43	0.032 ± 0.0017
12b	–CHO	$-COCH_3$	H	1.73 ± 0.67	>100
15	$-CH_2CH{=}CH_2$	H	Br	21.00 ± 1.31	2.37 ± 0.11[a]
16	$-CH_2CHBrCH_2Br$	H	Br	1.43 ± 0.40	9.34 ± 0.56[a]

[a] Date derived from ref. [14].



13 R^1= -$CH_2CH{=}CH_2$ R^2= H R^3 = H **15** R^1= -$CH_2CH{=}CH_2$ R^2= H R^3 = Br

14 R^1= R^2= -$CH_2CH{=}CH_2$ R^3 = H **16** R^1= -$CH_2CHBrCH_2Br$ R^2= H R^3 = Br

Fig. 6. Structures of compounds **13**, **14**, **15** and **16**.

results indicated that the partial positive charge on the N^5-substituents was necessary as inhibitors of nucleophilic MetS. Moreover, the results that **11b**, **12b** and **16** had higher inhibitory activity were consistent with our prediction. However, as shown in Table 1, weaker inhibition was found for **11b** compared to **8b**. The later docking poses of them revealed that the specific tricyclic geometry of **8b** didn't fit well into the enzyme pocket.

Compounds **2b**, **6**–**8b**, **11**–**12b** were evaluated against recombinant human DHFR also because the bicyclic pyrimidine analogs of folic acid containing 2,4-diamino substitution pattern are usually inhibitors of dihydrofolate reductase [20]. As shown in Table 2, compound **2b**, a 8-deaza derivative of aminopterin which is a classical antifolate and antitumor agent, was the strongest inhibitor of DHFR (IC_{50} = 0.018 μM). Compounds **6b**, **7b** and **11b** were almost equipotent against the DHFR (IC_{50} = 0.033 μM, 0.033 μM, and 0.032 μM) to the standard MTX (IC_{50} = 0.0312 μM). Compared to **6b**, compounds **9b** and **10** bearing bulkier substituents on N^5 position showed weaker inhibition.

4.2. Molecular modeling

To understand the possible binding mode of 8-deazatetrahydrofolate derivatives with electrophilic groups on MetS, compound **16** was docked into the CH_3–H_4folate-binding pocket of cobalamin-dependent methionine synthase (1–566) from thermotoga maritima (cd^{2+}, hcy, methyltetrahydrofolate complex) (PDB ID: 1Q8J) by using the ligand minimization of Discovery Studio (DS) 2.5. Default parameters were used as described in the manual unless otherwise specified. It was found that compound **16** bound to MetS in the pocket of CH_3–H_4folate in an extended mode, and formed almost the same key hydrogen bonds with the protein [Fig. 7].

The pterin ring, similar to that of CH_3–H_4folate, was positioned by hydrogen bonds with the same three residues Asn-411, Asp-473, and Asn-508 except Asp-390 which initially formed a hydrogen bond with N^8 of CH_3–H_4folate. There was an interaction between p-aminobromobenzoic acid side chain and Asn-323 as well as Arg-516, similar to the interaction with CH_3–H_4folate. No matter whether the N^5–Br was in S or R configuration, the skeleton was in the same position and formed the same hydrogen bonds. The dibromopropyl group on N^5 in both configurations would be in suitable cave which was near the "opening" site of the protein [Fig. 8].

4.3. Evaluation of human tumor cell lines

Compounds **7b**–**12b** and **13**–**16** were evaluated for their cytotoxicity toward tumor cell lines. For comparison, the intermediates **2b** and **5b** were evaluated as well. The potency of inhibiting the growth of tumor cells was measured as IC_{50} compared to MTX, and the results are listed in Table 2. To discuss adequately, the previously reported values of compounds **5b**, **13**–**16** against BGC-823, Hela, Bel-7402 were indicated [14]

Compound **2b**, an 8-deaza counterpart of aminopterin, displayed a much stronger cytotoxicity against all five cell lines than MTX. Besides **2b**, only **10b** among the compounds showed good activity against KB and Bei-7402 cell lines. IC_{50} values of other compounds were more than 1 M and even more than 50 M. That means the 8-deazafolate derivatives might be insensitive against these two cell lines and might be tissue-specific chemotherapeutic agents for cancers.

It's interesting to note that the inhibition values of N^5,N^{10}-methylenated tricyclic compound **8b** against BGC-823 and Hela tumor cells was better than those of **9b** and **11b**, almost close to that of **MTX**. These data suggest that **8b** maybe inhibit other enzyme of folic acid metabolism except MetH.

Analog **16**, with the dibromopropyl group on N^5, inhibited the growth of SKOV3 cells by 50% at concentrations of 0.042 μM, which clearly indicated that **16** was a considerably stronger anti-tumor agent than MTX by the fact that it was 1.4 times more effective for SKOV3. Another two compounds **11b** and **12b,** which inhibited MetS better too, have poor activity. Maybe we need more evidence

Table 2
Inhibition concentrations (IC_{50}, in μM) against selected tumor cell lines.

	BGC-823	Hela	SKOV3	KB	Bel-7402
2b	0.010 ± 0.002	0.003 ± 0.0007	0.010 ± 0.0025	2.70 ± 0.64	18.30 ± 3.12
5b	0.44 ± 0.10[a]	0.96 ± 0.10[a]	0.24 ± 0.063	>100	87.9 ± 1.97[a]
7b	>100	>100	>100	31.05 ± 2.327	>100
13	5.20 ± 0.92[a]	7.85 ± 1.73[a]	0.87 ± 0.21	>100	42.2 ± 8.6[a]
8b	0.18 ± 0.04	0.13 ± 0.023	37.41 ± 7.77	>100	64.7 ± 13.8
9b	2.09 ± 0.41	0.2273 ± 0.0382	0.573 ± 0.0565	>100	61.5 ± 14.9
10b	0.289 ± 0.0538	0.434 ± 0.0873	0.450 ± 0.0711	0.336 ± 0.0623	0.325 ± 0.0640
11b	6.90 ± 1.43	6.0 ± 1.30	>100	>130	65.2 ± 12.5
12b	27.01 ± 0.619	41.59 ± 0.992	34.62 ± 0.531	20.03 ± 0.556	12.35 ± 0.762
14	2.43 ± 0.41[a]	27.1 ± 5.60[a]	50.24 ± 1.195	6.722 ± 0.449	7.1 ± 1.8[a]
15	6.59 ± 1.5[a]	0.90 ± 0.21[a]	>100	>100	1.2 ± 0.23[a]
16	22.56 ± 5.05[a]	19.4 ± 6.12[a]	0.042 ± 0.01	>100	>100[a]
MTX	0.11 ± 0.01[a]	0.10 ± 0.023[a]	0.06 ± 0.012	20.9 ± 3.70	82.3 ± 10.2[a]

[a] Date derived from ref [14].

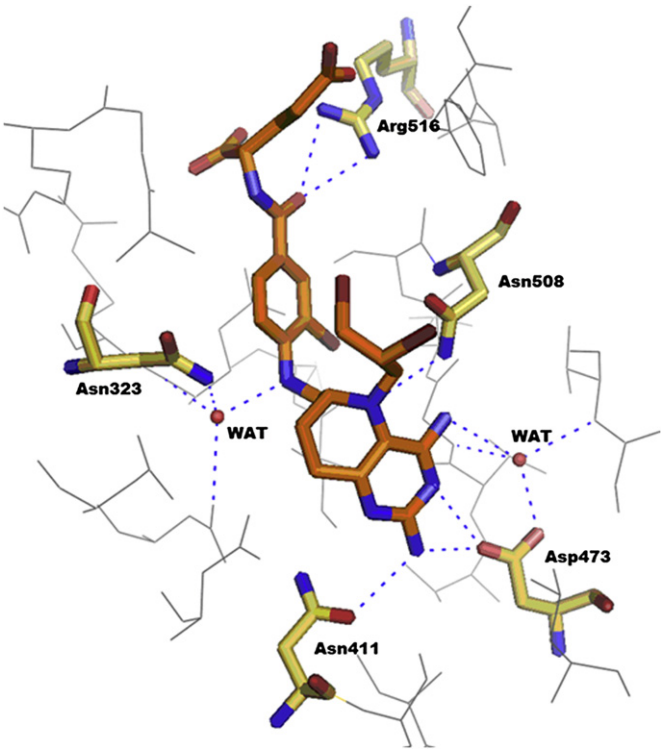


Fig. 7. Superimposition of the **16**'s best docking pose into 1Q8J considering the hydrogen bonds with water and amino acid residues. The viewpoint is approximately along the expected approach of the corrin ring. On the piperidine ring of the pterin, the C6 side chain substituent is axial and the N^5-dibromo propyl group is equatorial, pointing toward the reader.

to support the association of MetH inhibition with cancer cell death. Further studies against more cancer cell lines and on the transport mechanism and accumulation will be required.

5. Conclusion

Based on the action mechanism of methionine synthase, 8-deaza-5,6,7,8-tetrahydrofolate bearing electrophilic substituents on N^5 were designed and synthesized to explore their MetS inhibition activity as well as antitumor activity. Compounds **11b**, **12b** and **16** showed high activities against MetS. And the function was confirmed by docking study into the CH_3–H_4folate-binding pocket of MetS. Compounds **11b**, **12b** didn't show good activity against cancer cell lines. Further studies will be done on this class of compounds.

6. Experimental section

6.1. Biological evaluation

Human dihydrofolate reductase and MTX were purchased from Sigma Chemical Co. RPMI-1640 medium was produced from GIBCO.; Sulforhodamine B (SRB), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), tetrazolium salt and propidium iodide were purchased from Sigma Chemical co.; Microplate reader (FLUOsta OPTIMA, Germany); BECScan flow cytometer (Becton Sickinson FACScan, American).

6.1.1. Cell lines and culture conditions

Human promyelocytic leukemic cell line (HL-60), human hepatocellular carcinoma cell line (Bel-7402), stomach adenocarcinoma (BCG-823), SKOV3, KB and cervical cancer (Hela) were grown and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units mL^{-1}), streptomycin concentration at 37 °C in a humidified incubators in an atmosphere of 5% CO_2. All of the experiments were performed on exponentially growing cancer cells. Inhibition of cell growth was analyzed by using MTT and SRB assays [21,22] after 48 h treatment of different dosages of compounds. The data was presented in Table 1 as the means of three independent experiments.

6.1.2. Methionine synthase activity assay

Cells of HL60 at the density of 1 × 106 cells/well in 24-well plates were serum-starved before treating with various concentrations of test compounds for 3 h. Then the cells were harvested and lysed with lysis buffer (20 mmol/L HEPES pH 7.5, 3 mmol/L $MgCl_2$, 14 mmol/L NaCl, 5% glycerol, 0.5% Igepal CA-630, 1 mmol/L DTT, 1 mg/mL aprotonin, 1 mg/mL eupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride) [23]. After centrifugation (10,000 rpm, 10 min, at 4 °C), the supernatants were assayed in 96-well microplates for MetS activity, following the method of Drummond et al. [24] and Jarret et al. [25] with modification. The assay solution contained 1 mol/L potassium phosphate buffer (pH 7.2) 20 μL, 1 mol/L DTT 5 μL, 4.2 mmol/L 5-methyltetrahydrofolate 12 μL, 0.76 mmol/L SAM 5 mL, 97 μL double distilled water, and 40 μL cell lysate. After adding 0.5 mmol/L hydroxocobalamin 20 μL into the mixture, it was immediately preincubated at 37 °C for 5 min immediately. Then the reaction was initiated by mixing with 100 mmol/L L-homocysteine 1 μL and incubated for 10 min at 37 °C. The reaction was terminated by the addition of 5 mmol/L HCl/60% formic acid 50 μL and incubated at 80 °C for 10 min. The total volume in the well of a 96-well microplate was 250 μL. The plate was read at 350 nm by using FLUOstar OPTIMA microplate multidetection reader (BMG Offenburg, Germany).

6.1.3. Dihydrofolate reductase (DHFR) assay

All recombinant human (rh) DHFR enzymes were assayed spectrophotometrically in a solution containing 0.1 mM dihydrofolate, 0.3 mM NADPH, and 100 mM KCl at pH 7.5 and 32 °C. The

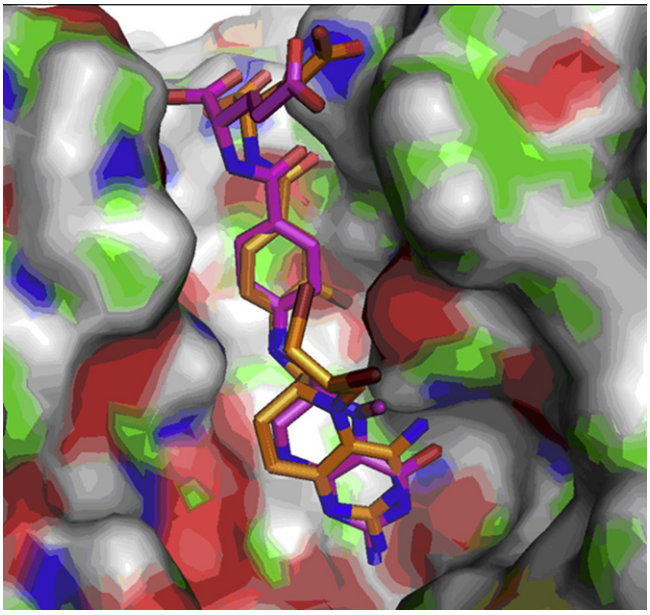


Fig. 8. Superposition of MetS structure in complex with **16** (orange) and CH_3–H_4folate (magenta). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

change in absorbance was measured at 340 nm by microplate spectrophotometer [26,27]

6.2. Chemistry

Melting points (uncorrected) were determined with an X_4-type apparatus. All evaporations were carried out *in vacuo* with a rotary evaporator.^{1}H-and ^{13}C NMR spectra were recorded with Jeol-AL-300 and Varian INOVA-500 with DMSO-d_6 as solvent and Me_4Si as an internal standard. J-values are expressed in Hz. The ESI-TOF spectra were taken on a QSTAR (ABI. SUA) mass spectrometer by using methanol as the solvent. HPLC purity determinations were carried out using a DIKMA AS-H 4.6 × 250 nm column. Mobile phase was isopropanol/n-hexane (1:1–1:3.5). All test compounds were confirmed to be ≥95% by HPLC. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV254 silica gel plates with fluorescent indicator, and the spots were visualized under 254 nm illumination.

6.2.1. Diethyl N-[4-[(2,4-diaminopyrido[3,2-d]pyrimidin-6-ylmethyl)methylamino]benzoyl]–L-glutamate **3a**

A solution of dried **1** (2.15 g, 7.96 mmol) in dimethylacetamide (180 mL) was added to diethyl *N*-(*p*-methylaminobenzoyl)-L-glutamate (2.85 g, 8.43 mmol) and the solution was stirred for 2 days at room temperature. The solvent was removed under reduced pressure and water (100 mL) was added to the gummy residue. The pH of the suspension was adjusted to 8 by adding $NaHCO_3$ (5%) and the resulting solution was extracted with chloroform (3 × 100 mL). The organic layer was concentrated to a small volume and was purified by column chromatography (silica gel). Elution with CH_2Cl_2–CH_3OH (15:1) gave pure **3a** (2.9 g, 72%) as a yellow solid. mp 78–80 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18–1.31 (m, 6H), 2.09–2.15 (m, 1H), 2.26–2.29 (m, 1H), 2.41–2.90 (m, 2H), 3.18 (s, 3H), 4.05–4.25 (m, 4H), 4.72 (s, 2H), 4.75–4.80 (m, 1H), 5.17 (bs, 2H), 6.71–6.74 (d, 2H, J = 9.0 Hz), 6.84–6.87 (d, 1H, J = 9.0 Hz), 7.32–7.35 (d, 1H, J = 9.0 Hz), 7.62–7.65 (d, 1H, J = 9.0 Hz), 7.69–7.72 (d, 2H, J = 9.0 Hz). MS (ESI) m/z 510.3 $[M + H]^+$.

6.2.2. N-[4-[(2,4-Diaminopyrido[3,2-d]pyrimidin-6-ylmethyl) methylamino]benzoyl]-L-glutamic acid **3b**

A solution of **3a** (110 mg, 0.216 mmol) in THF (2 mL) was added to 1 N NaOH (1 mL), and the mixture was stirred at room temperature about 2 h indicted by the TLC. The organic solvent was removed by evaporation under reduced pressure, the remaining aqueous solution was acidified carefully with 0.5 N HCL, and the solid was collected by filtration, washed with water, and dried *in vacuo* to give **3b** (70 mg, 71%) as a white solid. mp 200–202 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.93–2.06 (m, 2H), 2.29–2.34 (t, 2H, J = 7.2 Hz), 3.18 (s, 3H), 4.29–4.37 (m, 1H), 4.77 (s, 2H), 6.77–6.80 (d, 2H, J = 8.1 Hz), 7.35–7.38 (d, 1H, J = 8.7 Hz), 7.54–7.57 (d, 1H, J = 8.4 Hz), 7.71–7.74 (d, 2H, J = 8.4 Hz), 8.10–8.12 (d, 1H, J = 7.5 Hz). MS (ESI) m/z 454.0 $[M + H]^+$.

6.2.3. Diethyl N-[4-[(2,4-diaminopyrido[3,2-d]pyrimidin-6-ylmethyl)acetylamino]benzoyl]-L-glutamate **4a**

A solution of **2a** (330 mg, 0.67 mmol) in CH_3COOH (20 mL) was added to acetic anhydride (1 mL), and the solution was stirring at room temperature for 2 h. Then the mixture was poured into 15 mL of water, extracted with $CHCl_3$ (3 × 10 mL) and dried over Na_2SO_4. After evaporation of solvent, the residue was purified by column chromatography (silica gel). Elution with CH_2Cl_2–CH_3OH (13:1) gave pure **4a** (350 mg, 88%) as a white solid. mp 99–100 °C ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.19–1.32 (m, 6H), 1.93 (s, 3H), 2.12–2.36 (m, 2H), 2.39–2.55 (m, 2H), 4.07–4.26 (m, 4H), 4.73–4.80 (m, 1H), 5.01 (s, 2H), 6.30 (br, 2H), 6.86 (br, 2H), 7.16–7.18 (d, 2H, J = 7.8 Hz), 7.53–7.66 (m, 3H), 7.81–7.84 (d, 2H, J = 8.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.1, 22.6, 26.8, 29.6, 52.5, 54.2, 60.9, 61.8, 126.7, 127.8, 128.7, 131.2, 133.2, 145.9, 151.6, 159.1, 162.2, 166.2, 170.4, 172.0, 173.3, 177.5. MS(ESI) m/z 538.2 $[M + H]^+$.

6.2.4. N-[4-[(2,4-Diaminopyrido[3,2-d]pyrimidin-6-ylmethyl) acetylamino]benzoyl]-L-glutamic acid **4b**

A procedure similar to the preparation of **3b** was followed to prepare **4b** from **4a**. The yield of **4b** was 56% as a white solid. mp 200 °C (dec). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.93 (s, 3H), 2.04–2.37 (m, 4H), 3.96–4.02 (m, 1H), 5.02 (s, 2H), 6.18 (s, 2H), 6.95 (br, 2H), 7.42–7.43 (d, 2H, J = 8.0 Hz), 7.49–7.54 (m, 2H), 7.78–7.80 (d, 2H, J = 8.0 Hz), 7.91–7.92 (d 1H, J = 6.5 Hz). MS(ESI) m/z 482.2 $[M + H]^+$.

6.2.5. Diethyl N-[4-[2-(2,4-diamino-5,6,7,8-tetrahydropyrido[3,2-d] pyrimidin-6-ylmethyl) methylamino]benzoyl]-L-glutamate **6a**

A solution of **3a** (397 mg, 0.78 mmol) in 80 mL of ethanol and acetic acid (v/v = 1/70) was added to platinum oxide catalyst (40 mg), and the suspension was hydrogenated (0.4 MPa) for 48 h. The reaction mixture was filtered through celite and the filtrate was adjusted to pH7–8 with $NaHCO_3$ (5%), and evaporated to dryness under reduced pressure. The residue was added to $CHCl_3$ (100 mL × 2) and filtered. The filtrate was concentrated to a small volume, and was purified by column chromatography (silica gel). Elution with CH_2Cl_2–CH_3OH (12:1) gave pure **6a** (173 mg, 43%) as a light-yellow solid; mp 119–121 °C ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.15–1.20 (m, 6H), 1.65–1.71 (m, 1H), 1.91–1.95 (m, 1H), 1.97–2.12 (m, 2H), 2.40–2.43 (t, 2H, J = 7.5 Hz), 2.56–2.58 (m, 2H), 3.04 (s, 3H), 3.37–3.44 (m, 2H), 3.57–3.61 (m, 1H), 4.03–4.12 (m, 4H), 4.37–4.41 (m, 1H), 4.79 (s, 1H), 6.73–6.75 (m, 4H), 7.58 (bs, 2H), 7.75–7.76 (d, 2H, J = 8.5 Hz), 8.32–8.33 (d, 1H, J = 7.5 Hz). ^{13}C NMR (500 MHz, DMSO-d_6) δ 14.1, 21.0, 22.6, 23.8, 25.8, 30.2, 48.8, 51.8, 55.5, 59.9, 60.4, 110.4, 115.8, 120.1, 128.9, 132, 151.2, 151.4, 157.3, 166.4, 172.2, 172.2. MS (ESI) m/z 514.1 $[M + H]^+$.

6.2.6. N-[4-[2-(2,4-Diamino-5,6,7,8-tetrahydropyrido[3,2-d] pyrimidin-6-ylmethyl)methylamino] benzoyl]-L-glutamic acid **6b**

A procedure similar to the preparation of **3b** was followed to prepare **6b** from **6a**. The yield of **6b** was 46% as a light-yellow solid. mp 204–206 °C ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.92–1.93 (m, 1H), 2.03–2.05 (m, 1H), 2.31–2.32 (m, 4H), 2.40–2.45 (m, 2H), 2.88–3.01 (m, 2H), 3.07 and 3.18 (s, 3H), 3.44–3.45 (m, 1H), 4.33 (m, 1H), 4.73–4.76 (s, 1H), 6.54 (bs, 2H), 6.76–6.79 (m, 2H), 7.71–7.74 (m, 2H), 8.11–8.14 (m, 1H). MS (ESI) m/z 458.2 $[M + H]^+$. HRMS calcd for $C_{21}H_{28}N_7O_5$ ($M + H^+$), 458.21464; found, 458.21509.

6.2.7. Diethyl N-[4-[2-(2,4-diamino-5,6,7,8-tetrahydropyrido[3,2-d] pyrimidin-6-yl methyl)acetylamino]benzoyl]-L-glutamate **7a**

A solution of **4a** (520 mg, 0.97 mmol) in ethanol (100 mL) and trifluoroactyl acid (0.2 mL) was added to platinum oxide catalyst (55 mg), and the suspension was hydrogenated (0.3 MPa) for 72 h. The reaction mixture was filtered through celite and the filtrate was adjusted to pH7–8 with $NaHCO_3$ (5%), and evaporated to dryness under reduced pressure. The residue was added to $CHCl_3$ (60 mL × 2) and filtered. The filtrate was concentrated to a small volume, and was purified by column chromatography (silica gel). Elution with CH_2Cl_2–CH_3OH (18:1) gave pure **7a** (390 mg, 74%) as a white solid; mp: 104–106 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.14–1.22 (m, 6H), 1.56–1.91 (m, 2H), 1.83 (s, 3H), 1.99–2.14 (m, 2H), 2.43–2.48 (t, 2H, J = 7.5 Hz), 3.23–3.36 (m, 3H), 3.68–3.81 (m, 2H), 4.01–4.15 (m, 4H), 4.41–4.49 (m, 1H), 4.54 (s, 1H), 7.02 (s, 2H), 7.57–7.57 (d, 2H, J = 8.4 Hz), 7.73 (br, 2H), 7.94–7.97 (d, 2H, J = 8.4 Hz), 8.82–8.85 (d, 1H, J = 7.2 Hz). MS (ESI) m/z 542.3 $[M + H]^+$.

6.2.8. N-[4-[2-(2,4-Diamino-5,6,7,8-tetrahydropyrido[3,2-d] pyrimidin-6-ylmethyl)acetylamino] benzoyl]-L-glutamic acid ***7b***

A procedure similar to the preparation of **3b** was followed to prepare **7b** from **7a**. The yield of **7b** was 76% as a light-yellow solid. mp 173 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.56–1.91 (m, 2H), 1.83 (s, 3H), 1.99–2.14 (m, 2H), 2.43–2.48 (t, 2H, J = 7.5 Hz), 3.23–3.68 (m, 3H), 3.73–3.81 (m, 2H), 4.41–4.49 (m, 1H), 4.54 (s, 1H), 7.02 (s, 2H), 7.47 (br, 2H), 7.94–7.97 (d, 2H, J = 7.5 Hz), 7.54–7.57 (d, 2H, J = 7.5 Hz), 8.82–8.85 (d, 1H, J = 7.2 Hz). MS (ESI) m/z 546.5 $[M + H]^+$.

6.2.9. Diethyl N-[4-[(2-2,4-diamino-5,10-methylene-5,6,7,8-tetrahydropyrido[3,2-d]pyrimidin-6-yl methyl)amino]benzoyl]-L-glutamate ***8a***

A solution of **5a** (400 mg, 0.8 mmol) and paraformaldehyde (120 mg) in THF (1 mL) and water (1.2 mL) was reacted under sonication for 4 h at room temperature. TLC in $CHCl_3$–CH_3OH (8:2) indicated complete disappearance of compound 8. The organic solvent was removed by evaporation under reduced pressure and the remaining aqueous solution was added to 10 mL of water, and extracted with $CHCl_3$ (5 mL × 3). The organic layer was dried over anhydrous sodium sulfate, concentrated to a small volume, and poured onto a column (silica gel). Elution with $CHCl_3$–CH_3OH (16:1) gave 317 mg (77.4%) of pure **8a** as a light yellow solid. mp 95–97 °C ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.15–1.24 (m, 6H), 1.72–1.87 (m, 2H), 1.97–2.11 (m, 2H), 2.41–2.46 (m, 2H), 2.50–2.58 (m, 2H), 3.33–3.34 (m, 1H), 3.63–3.69 (m, 2H), 3.78–3.79 (d, 1H, J = 4.5 Hz), 4.03–4.13 (m, 4H), 4.38–4.43 (m, 1H), 4.91–4.92 (d, 1H, J = 4.0 Hz), 5.64 (s, 2H), 6.24 (s, 2H), 6.53–6.55 (d, 2H, J = 9.0 Hz), 7.80–7.78 (d, 2H, J = 9.0 Hz), 8.33–8.35 (d, 1H, J = 7.0 Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ 14.1, 21.9, 25.8, 28.2, 30.2, 51.2, 51.9, 56.3, 59.9, 60.4, 65.8, 110.5, 113.7, 120.6, 129.0, 148.2, 153.7, 158.8, 159.5, 166.5, 172.2, 172.2. MS (ESI) m/z 512.3 $[M + H]^+$, m/z 534.3 $[M + Na]^+$.

6.2.10. N-[4-[[2-(2,4-Dimino-5,10-methylene-5,6,7,8-tetrahydropyrido[3,2-d]pyrimidin-6-yl)methyl] amino]benzoyl]-L-glutamic acid ***8b***

A procedure similar to the preparation of **3b** was followed to prepare **8b** from **8a**. The yield of **8b** was 68% as a white solid. mp 95 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.76–2.11 (m, 4H), 2.29–2.34 (m, 2H), 2.49–2.73 (m, 2H), 3.33–3.90 (m, 1H), 3.66–3.69 (m, 2H), 3.79–3.80 (d, 1H, J = 3.9 Hz), 4.29–4.36 (m, 1H), 4.92–4.93 (d, 1H, J = 3.9 Hz), 6.48 (bs, 2H), 6.53–6.56 (d, 2H, J = 8.7 Hz), 6.77 (bs, 2H), 7.79–7.76 (d, 2H, J = 8.7 Hz), 8.13–8.16 (d, 1H, J = 7.5 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ 21.5, 26.6, 30.9, 51.2, 52.2, 56.7, 65.8, 110.6, 113.9, 121.2, 128.9, 148.1, 150.1, 157.1, 160.1, 166.1, 174.4. IR (KBr) υ 3330.64, 2928.96, 1607.55, 1505.89, 1392.01, 1275.19, 1201.58. MS (ESI) m/z 456 $[M + H]^+$. HRMS calcd for $C_{22}H_{26}N_6O_5$ ($M + H^+$), 456.19899; found, 456.19933.

6.2.11. Diethyl N-[4-[[2-(2,4-diamino-5-allyl-5,6,7,8-tetrahydropyrido[3,2-d]pyrimidin-6-yl)methyl]methylamino] benzoyl]-L-glutamate ***9a***

A solution of **6a** (80 mg, 0.16 mmol) in anhydrous DMF (1 mL) was added to allyl bromide (15 μL, 0.18 mmol) and Cs_2CO_3 (60 mg, 0.18 mmol) and the reaction mixture was stirred at room temperature for about 15 h. Solvent was removed under reduced pressure. The residue was stirred with water (20 mL), and the mixture was extracted with CH_2Cl_2 (2 × 20 mL). The CH_2Cl_2 layers were combined and dried over anhydrous Na_2SO_4. After evaporation of solvent to a small volume the residue was purified by column chromatography (silica gel). Elution with CH_2Cl_2–CH_3OH (15:1) gave pure **9a** (66 mg, 77%) as a light yellow solid. mp 88–90 °C ^{1}H NMR (500 MHz, $CDCl_3$) δ:1.21–1.31 (m, 6H), 1.70–1.74 (m, 1H), 2.11–2.19 (m, 2H), 2.28–2.34 (m, 1H), 2.39–2.55 (m, 2H), 2.63–2.77 (m, 2H), 2.95–2.96 (d, 3H, J = 3.5 Hz), 3.12–3.12 (d, 1H, J = 4.0 Hz), 3.15 (d, 1H, J = 4.0 Hz), 3.34–3.37 (d, 1H, J = 16.0 Hz), 3,42 (bs, 1H), 3.46–3.49 (d, 1H, J = 13.5 Hz), 4.09–4.25 (m, 4H), 4.76–4.81 (m, 1H), 5.10–5.25 (m, 2H), 5.66–5.73 (m, 1H), 6.11 (bs, 2H), 6.51–6.52 (d, 2H, J = 8.5 Hz), 7.64–7.66 (d, 2H, J = 7.5 Hz). ^{13}C NMR (500 MHz, $CDCl_3$) δ 14.1, 17.6, 21.9, 27.3, 29.6, 30.6, 51.9, 52.3, 52.8, 55.8, 60.8, 60.6, 110.7, 110.8, 116.8, 117.5, 120.5, 128.8, 134.8, 151.5, 151.6, 155.2, 161.1, 167.2, 172.5, 173.4. MS (ESI) m/z 554.2 $[M + H]^+$.

6.2.12. N-[4-[[2-(2,4-Diamino-5-allyl-5,6,7,8-tetrahydropyrido [3,2-d]pyrimidin-6-yl)methyl] methylamino]benzoyl]-L-glutamic acid ***9b***

A procedure similar to the preparation of **3b** was followed to prepare **9b** from **9a**. The yield of **9b** was 69.4% as a light-yellow solid. m.p. 208–210 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.63–1.67 (m, 1H), 1.90–1.97 (m, 1H), 1.99–2.10 (m, 2H), 2.30–2.35 (t, 2H, J = 7.2 Hz), 2.44–2.51 (m, 2H), 2.95 (s, 3H), 3.05–3.13 (m, 1H), 3.30 (bs, 4H), 4.30–4.36 (m, 1H), 4.96–5.18 (m, 2H), 5.82–5.84 (m, 1H), 6.58–6.61 (d, 2H, J = 9.0 Hz), 6.79 (bs, 2H), 7.70–7.72 (d, 2H, J = 7.8 Hz), 8.09–8.11 (d, 1H, J = 6.0 Hz). MS (ESI) m/z 498.3 $[M + H]^+$. HRMS calcd for $C_{24}H_{32}N_7O_6$ ($M + H^+$), 498.24594; found, 498.24675.

6.2.13. Diethyl N-[4-[2-(2,4-diamino-5-tosyl-5,6,7,8-tetrahydropyrido[3,2-d]pyrimidin-6-ylmethyl) methylamino] benzoyl]-L-glutamate ***10a***

A solution of **6a** (47 mg, 0.092 mmol) in CH_3CN (2 mL) was added to Tos-Cl (22.6 mg, 0.021 mmol) and $(CH_3CH_2)_3N$ (50 μL) and the mixture was stirred at room temperature for 10 h. Solvent was removed under reduced pressure. The residue was stirred with water (30 mL), and the mixture was extracted with CH_2Cl_2 (2 × 20 mL). The CH_2Cl_2 layers were combined and dried over anhydrous Na_2SO_4. After evaporation of solvent to a small volume, the residue was purified by preparative thin layer chromatography (silica gel). Elution with $CHCl_3$–CH_3OH (9:1) gave pure **10a** (11 mg, 18%) as a light yellow solid. mp 88–90 °C ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21–1.34 (m, 6H), 1.94–1.99 (m, 2H), 2.09–2.20 (m, 2H), 2.29–2.33 (m, 2H), 2.40 (s, 3H), 2.44–2.56 (m, 2H), 3.08–3.09 (d, 3H, J = 1.2 Hz), 3.18–3.24 (m, 1H), 3.76–3.85 (m, 1H), 4.09–4.16 (m, 2H), 4.21–4.28 (m, 2H), 4.35–4.40 (m, 1H), 4.81–4.82 (m, 1H), 4.93 (bs, 2H), 5.54 (bs, 2H), 6.76–6.79 (d, 2H, J = 8.7 Hz), 7.24–7.27 (d, 2H, J = 9.0 Hz), 7.50–7.53 (d, 2H, J = 7.8 Hz), 7.75–7.78 (d, 2H, J = 9.0 Hz). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.1, 21.6, 27.3, 27.4, 28.1, 29.6, 30.5, 52.1, 54.9, 57.4, 60.7, 61.6, 106.0, 110.9, 120.8, 127.5, 128.9, 129.9, 133.4, 144.7, 150.9, 159.9, 162.0, 167.0, 172.4, 173.3. MS (ESI) m/z 668.5 $[M + H]^+$.

6.2.14. N-[4-[2-(2,4-Diamino-5-tosyl-5,6,7,8-tetrahydropyrido[3,2-d] pyrimidin-6-ylmethyl) methylamino]benzoyl]-L-glutamatic acid ***10b***

A procedure similar to the preparation of **3b** was followed to prepare **10b** from **10a**. The yield of **10b** was 59.5% as a light-yellow solid. m.p. 191–192 °C ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.48 (m, 1H), 1.76 (m, 1H), 1.89–1.97 (m, 2H), 2.08–2.18 (m, 2H), 2.29–2.37 (m, 5H), 2.99 (s, 3H), 3.17–3.21 (m, 1H), 3.74–3.76 (m, 1H), 4.21 (m, 1H), 4.39 (m, 1H), 6.74 (d, 2H, J = 7.0 Hz), 7.39–7.40 (d, 2H, J = 12.0 Hz), 7.70-7.71 (d, 2H, J = 7.0 Hz), 7.89–7.91 (d, 2H, J = 9.0 Hz), 8.25–8.26 (d, 1H, J = 5.5 Hz). MS (ESI) m/z 612.3 $[M + H]^+$. HRMS calcd for $C_{28}H_{34}N_7O_7S_1$ ($M + H^+$), 612.22349; found, 612.22393.

6.2.15. Diethyl N-[4-[2-(2,4-diamino-5-formyl-5,6,7,8-tetrahydropyrido[3,2-d]pyrimidin-6-yl methyl)methylamino] benzoyl]-L-glutamate ***11a***

A solution of **6a** (48 mg, 0.094 mmol) in ethyl formate (10 mL) was added formic acid (0.4 mL, 0.106 mmol) and the mixture was

stirred at room temperature for 1 h. Then acetyl anhydride (0.2 mL) was added and the resulting solution was stirred for 0.5 h again. $NaHCO_3$ (5%, 10 mL) was added to the reaction mixture and extracted with CH_2Cl_2 (10 mL × 2). The organic layer was dried over anhydrous sodium sulfate, concentrated to a small volume, and poured onto a column (silica gel). Elution with $CHCl_3$–CH_3OH (18:1) gave 38 mg (75%) of pure **11a** as a white solid. mp 87–89 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21–1.33 (m, 6H), 1.84 (m, 1H), 2.12–2.17 (m, 1H), 2.28–2.40 (m, 2H), 2.44–2.55 (m, 2H), 2.65–2.73 (m, 2H), 2.99 (s, 3H), 3.12–3.14 (m, 1H), 3.26 (m, 1H), 3.50–3.55 (m, 1H), 4.08–4.27 (m, 4H, J = 7.2 Hz), 4.76–4.83 (m, 1H), 5.06 (bs, 2H), 5.29 (s, 2H), 6.60–6.63 (d, 2H, J = 8.7 Hz), 6.97 (bs, 1H), 7.73–7.75 (d, 2H, J = 8.7 Hz), 7.87–7.90 (d, 1H, J = 7.2 Hz). ^{13}C NMR (300 MHz, $CDCl_3$) δ 14.1, 24.0, 26.1, 26.9, 27.3, 30.5, 45.9, 52.2, 54.2, 60.7, 61.9, 110.9, 121.4, 121.4, 129.0, 150.4, 150.4, 158.6, 160.4, 161.1, 166.8, 173.3, 173.3. MS (ESI) m/z 542.3 $[M + H]^+$.

6.2.16. *N-[4-[2-(2,4-Diamino-5-formyl-5,6,7,8-tetrahydropyrido [3,2-d]pyrimidin-6-ylmethyl) methylamino]benzoyl]-L-glutamic acid* ***11b***

A procedure similar to the preparation of **3b** was followed to prepare **11b** from **11a**. The yield of **11b** was 84% as a white solid. mp 209–210 °C ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.626–1.652 (m, 1H), 1.89–1.95 (m, 3H), 2.01–2.05 (m, 2H), 2.28 (m, 2H), 3.03 (s, 3H), 3.36–3.40 (m, 2H), 3.53–3.56 (m, 1H), 4, 28–4.31 (m, 1H), 6.38 (bs, 2H), 6.89 (bs, 2H), 6.71–6.72 (d, 2H, J = 5.4 Hz), 7.18 (bs, 2H), 7.69–7.71 (d, 2H, J = 6.3 Hz), 7.99–8.03 (m, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ 23.2, 24.1, 26.7, 29.3, 31.1, 48.9, 52.3, 55.6, 110.4, 115.4, 120.6, 128.7–128.8, 151.1, 152.5, 157.0, 160.2, 161.3, 165.9, 174.3, 174.5. HRMS calcd for $C_{22}H_{28}N_7O_6$ ($M + H^+$), 486.20956; found, 486.20976.

6.2.17. *Diethyl N-[4-[2-(2,4-diamino-5-formyl-5,6,7,8-tetrahydropyrido[3,2-d]pyrimidin-6-yl methyl)acetylamino] benzoyl]-L-glutamate* ***12a***

A solution of **7a** (50 mg, 0.092 mmol) in formic acid (2 mL) was stirred at 60 °C for 2 h. After the solution was cooled to room temperature, the mixture was adjusted to pH 7 with $NaHCO_3$ (5%) and extracted with $CHCl_3$ (3 mL × 3). The organic layer was dried over anhydrous sodium sulfate, concentrated to a small volume, purified by preparative thin layer chromatography (silica gel). Elution with $CHCl_3$–CH_3OH (9:1) gave pure **12a** (45 mg, 86%) as a light yellow solid. mp 118–120 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15–1.23 (m, 6H), 1.49–1.60 (m, 1H), 1.80 (s, 3H), 1.91–2.14 (m, 3H), 2.32–2.36 (m, 2H), 2.50–2.51 (m, 2H), 3.35–4.01 (m, 3H), 4.02–4.16 (m, 4H), 4.46 (m, 1H), 6.18 (bs, 2H), 7.53–7.98 (m, 4H), 8.15 (s, 1H), 8.82–8.87 (t, 1H, J = 7.2 Hz). MS(ES^+) m/z 570.0.

6.2.18. *N-[4-[2-(2,4-Diamino-5-formyl-5,6,7,8-tetrahydropyrido[3,2-d] pyrimidin-6-ylmethyl)acetylamino]benzoyl]-L-glutamic acid* ***12b***

A procedure similar to the preparation of **3b** was followed to prepare **12b** from **12a**. The yield of **12b** was 79% as a light-yellow solid. mp 145–147 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ1.80 (s, 3H), 1.91–2.14 (m, 2H), 2.32–2.51 (m, 4H), 3.35–4.01 (m, 3H), 4.45–4.52 (m, 2H), 6.18 (bs, 2H), 7.53–7.98 (m, 4H), 8.15 (s, 1H), 8.82–8.87 (d, 1H, J = 6.9 Hz). MS (ESI) m/z 514.2 $[M + H]^+$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.09.027.

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