Synthesis and Antitumor Activities of Novel 6-5 Fused Ring Heterocycle Antifolates: *N*-[4-[ω-(2-Amino-4-substituted-6,7-dihydrocyclopenta[*d*]pyrimidin-5-yl)alkyl]benzoyl]-L-glutamic Acids

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Novel antifolates with a 6-5 fused ring system, 6,7-dihydrocyclopenta[*d*]pyrimidine, (**3a,b** and **4a,b**) were synthesized on the basis of combined modification of the heterocycle and bridge regions of the folate molecule. The synthetic method involves (1) synthesis of key intermediates of *tert*-butyl 4-[ω-(2-substituted-3-oxocyclopentanyl)alkyl]benzoates (**8a,b** and **9a,b**) by a carbon–carbon radical coupling of *tert*-butyl 4-(ω-iodoalkyl)benzoates (**7a,b**) with 2-substituted-2-cyclopenten-1-ones (**5** and **6**) utilizing tributyltin hydride, (2) cyclization of either the methyl enol–ethers derived from the 2-cyanocyclopentanones (**8a,b**) or the 2-(methoxycarbonyl)cyclopentanones (**9a,b**) themselves by treatment with guanidine which leads to 6,7-dihydrocyclopenta[*d*]pyrimidines with a 4-(*tert*-butoxycarbonyl)phenylalkyl group (**11a,b** and **14a,b**), (3) deprotection to the corresponding carboxylic acids (**12a,b** and **15a,b**), and (4) amidation with diethyl glutamate and deesterification. Potent dihydrofolate reductase inhibition and highly potent cell growth inhibition were found with 2,4-diaminopyrimidine-fused cyclopentene compounds containing the trimethylene (**3a**) or ethylene bridge (**3b**) but not with the corresponding 2-amino-4-hydroxy analogs (**4a,b**). Compounds **3a** and **3b** were more growth inhibitory to several tumor cell lines (P388, colon 26, colon 38, and KB) than was methotrexate, with **3a** being the most potent. Both **3a** and **3b** gave increases in the lifespan of P388 leukemic mice comparable to that observed with MTX. Both compounds were therapeutic against colon 26 colorectal carcinoma in mice. Compound **3a** was highly effective against LC-6 non-small cell lung carcinoma in nude mice.

In this paper we report on the synthesis and antitumor activities of a new series of antifolates containing 6,7-dihydrocyclopenta[*d*]pyrimidine, a novel 6-5 fused ring system in the antifolate area, and methylene bridges of various lengths.

Hitherto, extensive structural modifications in the folate molecule have been made. One recent marked progress has been the advent of various deaza analogs of folic acid (FA) and the related compounds which have proven to have extraordinarily high antitumor efficacies[1] as compared to those of the first generation of antifolate drugs. 5,8-Dideazafolates (e.g., D1694[2]), 5,10-dideazafolates and their tetrahydro derivatives (e.g., DDATHF[3]), 10-deazaaminopterins and their 10-ethyl derivatives (10-EDAM[4]) are the best known examples of recent promising antifolates. These findings have led to the concept that isosteric modifications of the folate skeleton by replacing nitrogen atoms in the heterocycle region and/or in the bridge region[1] by carbon atoms should provide antifolates with better therapeutic spectrum and potency. In this case, it should be noted that most antifolates with antitumor activity belong to a class of analogs possessing a 6-6 fused ring in the heterocycle region.

Several antifolates with a 6-5 fused ring system have recently been reported to be antifolate active and promising as antitumor agents. Compounds with 2,4-diaminopyrrolo[2,3-*d*]pyrimidine (**1**, TNP351[5]), and those with 2-amino-4-hydroxypyrrolo[2,3-*d*]pyrimidine (**2**, LY231514[6]) are typical examples. Although these antifolates are structurally unique in the heterocycle region, their 6-5 fused rings are limited to the pyrrolo[2,3-*d*]pyrimidine ring system, and therefore it remains unknown whether other 6-5 fused ring systems meet the structural requirements for antitumor activity.

To answer this question, we searched for 6-5 fused ring heterocycle antifolates. This paper deals with the synthesis and antitumor activity of 6,7-dihydrocyclopenta[*d*]pyrimidine antifolates (**3a,b** and **4a,b**), the first examples of a novel type of folate analog.

Chemistry

The structural characteristics of this series of antifolates are the presence of the cyclopentene-fused pyrimidine nucleus in place of pteridine in the FA structure and methylene groups of various lengths in place of the $-CH_2-NH-$ bridge. The present compounds are classified in two groups, 2,4-diamino-6,7-dihydro-5*H*-cyclopenta[*d*]-pyrimidine antifolates (**3a,b**) and 2-amino-4-hydroxy-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidine antifolates[7] (**4a,b**).

To obtain the key intermediates, oligomethylene-containing cyclopentanones (**8a,b**), we required a reliable synthetic method; a one-step synthesis would be ideal. The strategy we selected for approaching **8a** and **8b** involved introduction of the alkyl group already suitably functionalized to 2-substituted-2-cyclopenten-1-ones (**5** and **6**). Hitherto, the addition of alkyl radicals to alkenes has been extensively studied,[8] but no previous report is available relating to the reaction using cyclic alkenones. Thus, *tert*-butyl 4-(3-iodopropyl)benzoate **7a** was successfully subjected to a carbon–carbon radical coupling reaction with freshly prepared 2-cyano-2-cyclopentenone (**5**)[9] in the presence of tributyltin hydride and a catalytic amount of a radical initiator (AIBN) in boiling benzene, and the target trimethylene-containing cyclopentanone **8a** was obtained. Similarly, a carbon–carbon radical

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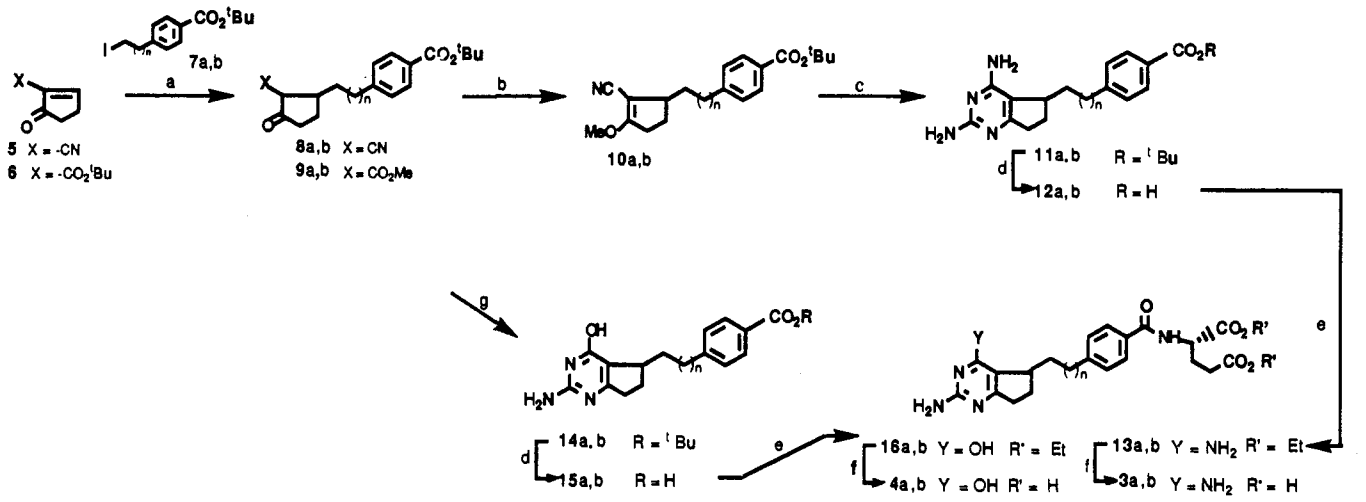
Scheme 1[a]



[a] Reagents: (a) benzene, Bu_3SnH/benzene, AIBN; (b) $TMSCHN_2$/hexane, diisopropylaminé, MeOH–CH_3CN; (c) guanidine H_2CO_3/tBuOH; (d) 1 N HCl–AcOH; (e) diethyl L-glutamate hydrochloride, DMF, DPPA, Et_3N; (f) (i) 1 N NaOH–EtOH, (ii) 1 N HCl–EtOH; (g) guanidine hydrochloride, tBuOK/tBuOH.

coupling of the 2-iodoethyl compound **7b** with the cyclic enone **5** progressed successfully to give an ethylene-bridged compound containing 2,3-disubstituted cyclopentanone **8b** (Scheme 1).

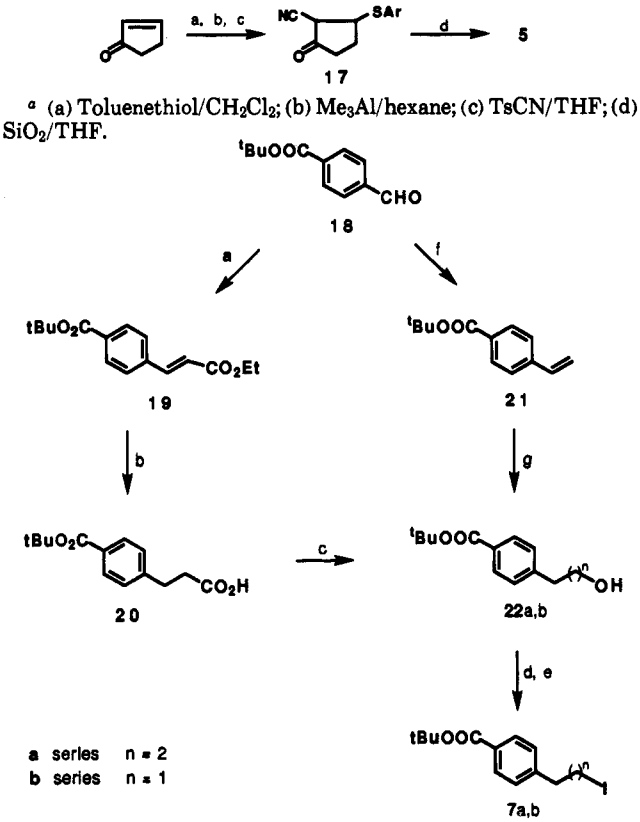
Synthesis of the key intermediates (**8a,b**) opened an entry to the new series of 6-5 fused ring antifolates (**3a,b** and **4a,b**) as shown below. Attempts to form the pyrimidine ring by heating **8a** with guanidine were unsuccessful, presumably due to insufficient stability of the cyano ketone (**8a**) under the reaction conditions. To prevent decomposition during the cyclization, **8a** was converted to its enol methyl ether (**10a**) by treatment with (trimethylsilyl)diazomethane. Cyclization by treating compound **10a** with a 5-fold molar excess of guanidine carbonate in *tert*-butyl alcohol at 160 °C progressed smoothly to give the desired 6-5 fused heterocyclic compound, *tert*-butyl 4-[3-(2,4-diamino-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-5-yl)propyl]benzoate (**11a**) in a good yield. Deprotection of the ester group of **11a** using mild acid to the corresponding carboxylic acid (**12a**) and the subsequent coupling reaction with diethyl L-glutamate in the presence of diphenyl phosphorazidate (DPPA) gave the antifolate diester **13a** smoothly. Final deprotection with dilute aqueous alkali in EtOH at room temperature gave the desired diacid **3a**.

Because the bridge-region structure is one of major determinants of antifolate activity, modification in this region of antifolate structures has been another site of interest.[1] Presumably the structure of the bridge region controls a conformational flexibility of the molecule, thereby determining whether it can interact with the enzyme active site. To obtain a shorter chain antifolate, synthesis according to an identical reaction sequence was undertaken starting from 4-(2-iodoethyl)benzoate (**7b**), and compound **3b** was obtained.

In light of what is now known about 2-amino-4-hydroxypyrimidine-fused heterocycle antifolates (quinazoline antifolates,[2] DDATHF,[3] and pyrrolo[2,3-*d*]pyrimidine antifolates[6]) which possess excellent antitumor properties, it is also of interest to examine whether the 4-amino group of compounds in this series can be replaced by a 4-hydroxy group without loss of biological activity. Therefore, we added 4-hydroxycyclopenta[*d*]pyrimidine antifolates to our list of synthetic targets. In the first study, hydrolysis of the 4-amino group of esters (**11a,b**) or carboxylic acids (**12a,b**) under alkaline conditions[10] was attempted but without success. Therefore, we started the synthesis of the 4-hydroxy analogs (**4a,b**) with a reaction sequence similar to the one mentioned above. The synthesis of the key intermediates **9a** and **9b** requires the use of 2-carbomethoxy-2-cyclopenten-1-one (**6**)[11] in place of compound **5** as the radical coupling partner. The reaction of **6** with either **7a** or **7b** in the presence of tri-*n*-butyltin hydride progressed similarly and gave 3-alkyl-2-carbomethoxycyclopentan-1-ones (**9a,b**) in a moderate yield. Because the ketones (**9a,b**) were stable relative to **8a,b**, direct use of **9a,b** in the subsequent cyclization was found to be useful. Thus, the cyclization reaction of **9a,b** with an equimolar amount of guanidine progressed successfully to yield the 2-amino-4-hydroxypyrimidine-fused cyclopentanes (**14a,b**). Subsequent deprotection of the ester group to the acids (**15a,b**) using mild acid, followed by amidation with diethyl L-glutamate and deesterification, gave the target antifolates (**4a,b**, Scheme 1), respectively. Obviously, this series of antifolates (**3a,b** and **4a,b**) have two chiral centers at position 5 and at the α-carbon of the L-glutamate moiety, and therefore synthesis according to this route (Scheme 1) gave compounds which are racemic about position 5 and hence consist of two diastereomers in equal amounts. Synthesis and biological properties of each of these diastereoisomers (the *d*-L- and *l*-L-diastereomers) will be reported elsewhere.

Synthesis of *tert*-butyl (ω-iodoalkyl)benzoates (**7a,b**), which were previously undescribed, have been achieved by the route shown in Scheme 2. The aldehyde **18** was employed as a starting compound. Condensation of **18** with ethyl (diethylphosphono)acetate by a Horner–Emmons reaction[12] gave the phenylacrylic acid ester **19**.

Scheme 2[a,b]



[a] (a) Toluenethiol/CH_2Cl_2; (b) Me_3Al/hexane; (c) TsCN/THF; (d) SiO_2/THF.

[b] (a) (i) NaH, THF; (ii) $(EtO)_2POCH_2CO_2Et$, THF; (b) (i) Pd/C, H_2, EtOH, (ii) aqueous NaOH, EtOH, (iii) aqueous HCl; (c) (i) BH_3/THF, (ii) MeOH; (d) MsCl/Et_3N, CH_2Cl_2; (e) NaI/acetone; (f) THF, *n*-BuLi/hexane, $MePh_3P^+Br^-$/THF; (g) (i) 9-BBN, THF, (ii) aqueous NaOH, aqueous H_2O_2.

Catalytic hydrogenation of the double bond, followed by alkaline hydrolysis and then acidification, gave the phenylpropionic acid **20**, which on reduction with excess borane under cooling in a dry ice bath gave the functionalized propanol **22a**. Preparation of the corresponding ethanol **22b** began with the one carbon extension[13] of the aldehyde **18** with methylenetriphenylphosphorane to give the olefine **21**. Hydroboration with 9-BBN of this Wittig reaction product gave the appropriately substituted ethanol **22b**. Both of the alcohols (**22a,b**) were converted to the corresponding iodides (**7a,b**) via mesylation followed by the treatment with alkali iodide. A sequence of these reactions proceeded with good yields.

Preparation of 2-cyano-2-cyclopenten-1-one (**5**)[9] was prerequisite for the synthesis of key intermediates (**8a,b**), but to our knowledge no synthetic method has ever been reported. We synthesized this compound the first time according to the method which involved (1) treatment of 2-cyclopentenone with trimethylaluminum[14] and aryl thiol, which yielded the dimethylaluminum salt of 3-(arylthio)-2-cyclopenten-1-one, (2) subsequent treatment with tosyl cyanide which gave 3-(arylthio)-2-cyanocyclopentan-1-one (**17**), and (3) elimination of the arylthio group from **17** which progressed very rapidly upon contact with silica gel to yield the cyclic enone **5** in a quantitative yield. Synthesis of the present new class of antifolates mentioned above made it possible to evaluate the effect on antitumor activity of combined modification of the heterocycle and bridge regions of the antifolate molecule.

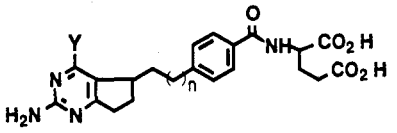
Biological Evaluation

The antifolate diacids (**3a,b** and **4a,b**) were assayed[15] as inhibitors of DHFR partially purified[16] from P388 mouse leukemia cells and purified from bovine liver cells (Sigma, D-6385). The results are expressed as IC_{50} values. The DHFR inhibitors, methotrexate (MTX) and 10-EDAM,[17] were included as positive controls. The present antifolate diacids were tested for their growth inhibition[18] of P388 leukemia cells (MTX-sensitive and MTX-resistant strains), colon 26 colorectal carcinoma cells, colon 38 colorectal carcinoma cells, and KB epidermoid carcinoma cells in culture. The results were expressed as IC_{50} values. Compounds **3a** and **3b** were further tested for their *in vivo* efficacy against P388 leukemia,[19] colon 26 colorectal carcinoma, and LC-6 human non-small cell lung carcinoma in mice.

Results and Discussion

The dihydrocyclopenta[*d*]pyrimidine antifolates (**3a,b** and **4a,b**) were evaluated in comparison with MTX for their abilities to inhibit DHFR[15] and to inhibit the growth of five tumor cell lines in culture.[18] The 2,4-diamino compounds (**3a,b**) showed potent inhibitory activity against DHFR from the two sources, P388 cells and bovine liver cells. The inhibitory effect of **3a** on the activity of DHFR was almost as potent as that of MTX, and that of **3b** was approximately 10–17-fold lower than that of MTX (Table 1). IC_{50} values obtained with the two enzymes did not differ by more than 3.5-fold. *In vitro* cell growth inhibition of these compounds was examined in MTX-sensitive and MTX-resistant P388 mouse leukemia cell lines, two mouse colorectal carcinoma cell lines, colon 26 and colon 38, and a KB human epidermoid carcinoma cell line. On prolonged drug exposure (72 h), **3a** and **3b** were excitingly inhibitory to the growth of these tumor cell lines, and their potencies were higher than that of MTX (Table 1). Against P388 MTX-sensitive and MTX-resistant cells, IC_{50} values were found to be 2.5 and 19 nM for **3a** and 7.1 and 64 nM for **3b**, while those of MTX were 23 and 223 nM, respectively. Against colon 26 mouse colorectal carcinoma cells, colon 38 colorectal carcinoma cells and KB human epidermal carcinoma cells, **3a** and **3b** inhibited the cell growth with IC_{50} values in the range of 3–8, 5–10, and 1.7–4.1 nM. The sensitivity of these solid tumor cell lines to each compound was similar to that of P388 MTX-sensitive cells. The results indicate that cell growth inhibitory activity of the 2,4-diamino compounds (**3a,b**) were enhanced considerably more than would be expected from the measured DHFR inhibition activity, that is, **3a** was 9–12-fold and **3b** was 3–7-fold more potent than MTX on direct comparison. The IC_{50} values of **3a** and **3b** were comparable to the 3–11 nM range of 10-EDAM. The elevated potency in cell growth inhibition probably results from a marked increase in efficient uptake and polyglutamation relative to those of MTX, based on the results obtained by McGuire et al.[20] The growth inhibitory effect has been shown to be primarily mediated by DHFR inhibition and not by TS inhibition.[20] This indicates that the target enzyme of **3a** is different from those of pyrrolo[2,3-*d*]pyrimidine antifolates (**1** and **2**),[5,6] another series of 6-5 fused heterocycle antifolates. Changing the length of the methylene bridge from trimethylene (**3a**) to ethylene (**3b**) resulted in a 10-fold decrease in the inhibition of DHFR activity but did not greatly affect the growth inhibitory activity against tumor cells (approximately only

Table 1. Dihydrofolate Reductase Inhibition and Tumor Cell Growth Inhibition by Antifolates



			IC_{50}, nM[a]						
			DHFR						
compd	Y	n	P388	bovine liver	P388	P388:MTXr (E-2)	colon 26	colon 38	KB
MTX			6.6 ± 0.8[b]	1.3 ± 0.1[b]	23 ± 3[c]	223 ± 22[c]	31 ± 2[d]	66 ± 7[d]	19 ± 2[c]
10-EDAM			11 ± 2.0[c]	3.8 ± 0.3[c]	4.3 ± 0.2[c]	44 ± 3[c]	4.8 ± 0.3[e]	11 ± 1[c]	3.0 ± 0.2[e]
3a	NH_2	2	7.1 ± 1.5[b]	2.5 ± 0.4[f]	2.5 ± 0.4[e]	19 ± 2[e]	3.0 ± 0.9[f]	5.1 ± 0.5[b]	1.7 ± 0.2[e]
3b	NH_2	1	72	22	7.1 ± 0.8[b]	64 ± 2[b]	8	10[g]	4.1 ± 0.9[b]
4a	OH	2	ND[h]	60×10^3	$>1.0 \times 10^3$	$>1.0 \times 10^3$	$>1.0 \times 10^3$	ND	$>1.0 \times 10^3$
4b	OH	1	ND	2.1×10^3	$>1.0 \times 10^3$	$>1.0 \times 10^3$	$>1.0 \times 10^3$	$>1.0 \times 10^3$	$>1.0 \times 10^3$

[a] Methods described in the Experimental Section. Data are presented as mean ± SE. Data presented without annotation are from a single determination ($N = 1$). [b] $N = 3$–4. [c] $N \geq 19$. [d] $N = 12$–13. [e] $N = 9$. [f] $N = 5$–6. [g] $N = 2$. [h] ND = not determined.

a 2-fold decrease) on prolonged drug exposure. The elevated inhibitory effect of **3a** and **3b** on DHFR and tumor cell growth appears to be due primarily to the presence of the 2,4-diaminopyrimidine-fused cyclopentene ring and secondarily to the presence of the oligomethylene bridge.[21] An increase in lipophilic character of the molecules (**3a,b**) due to replacement of the nitrogen containing ring B[1] (e.g., pyrazine) by cyclopentene and the bridge nitrogen by carbon probably contributes to an increase in inhibitory activity both *in vitro* and *in vivo*.

Earlier workers had shown that 2-amino-4-hydroxypyrrolo[2,3-*d*]pyrimidine antifolates (**2**, LY231514) and their 7,8-dihydro derivatives (LY288601) had good antitumor activities and that the mechanism of action of **2** was likely to be both DHFR (K_i = 0.56 μM) and TS inhibition (K_i = 0.55 μM).[6] On the basis of structural similarity to LY231514, one might have expected that the introduction of the 4-hydroxy group on a cyclopenta[*d*]-pyrimidine ring in place of the 4-amino group might not impair the cytotoxic activity of the molecule due to inhibition of certain folate-relating enzymes. Therefore, we synthesized the 2-amino-4-hydroxy compounds (**4a,b**) and tested inhibitory activity to assess whether the nitrogen atom at position 7 in the 4-hydroxypyrrolo[2,3-*d*]pyrimidine series could be replaced by carbon without loss of antifolate activity. However, this did not prove to be correct. In fact, the 4-hydroxy congeners (**4a,b**) were less effective as inhibitors of DHFR and cell growth, irrespective of changes in the bridge methylene length. Compounds **4a** and **4b** were respectively approximately 10^4- and 10^2-fold weaker than the corresponding 4-amino compounds (**3a,b**) as inhibitors of DHFR and failed to inhibit tumor cell growth at concentrations of up to 1 μM even under conditions of prolonged exposure (Table 1). Replacing the 4-amino group on the dihydrocyclopenta-[*d*]pyrimidine ring by hydroxyl led to a marked decrease in growth inhibitory activity. Apparently, the existence of the 4-amino group in the present series of compounds is essential for cell growth inhibitory activity. In contrast, earlier reports showed that in the series of the ethylene bridge containing antifolates including the pyrrolo[2,3-*d*]pyrimidine,[6] its dihydro derivative,[6a] and 5-deaza-5,6,7,8-tetrahydropteridine,[3] the presence of the 4-amino group did not appear to be an absolute structural requirement for potent inhibition of enzyme activity and cell growth and could be replaced by the 4-hydroxy group without a marked decrease of activity. A possible explanation of this discrepancy between these classes of deaza heterocycle antifolates and the present series of cyclopenta[*d*]pyrimidine antifolates is that the existence of the NH group of ring B in LY231514 and DDATHF should play a critical hydrogen-bonding role in the interaction with a folate-relating enzyme cavity to hold the heterocycles in a favorable conformation which is required for binding to recognition sites on the folate-relating enzyme.[22] This explanation is supported by the findings reported earlier that 5,10-dideazafolic acid,[3a] 5,8,10-trideazafolic acid,[23] and its 5,6,7,8-tetrahydro derivative[24] in which no such NH group exist on heterocycle ring B possess only minimal inhibitory activity against tumor cells. These deaza compounds are structurally similar to **4a** and **4b** with respect to the lack of both the ring B NH group and the bridge nitrogen of the folate skeleton. As a reflection of this structural similarity,[25] both groups of 4-hydroxy compounds exhibit similarly poor antifolate activities.

Table 2. Growth Inhibition of Colon 26 Mouse Colorectal Carcinoma on 4-h Drug Exposure

compd	IC_{50}, μM[a]
MTX	>40
10-EDAM	>40
3a	1.7
3b	0.19

[a] The method is described in the Experimental Section.

Next, we determined growth inhibitory activity of 2,4-diamino compounds (**3a,b**) on short term exposure[26] in colon 26 cells. As expected, when the exposure time was decreased to 4 h, the IC_{50} values increased to 1.7 μM for **3a**, 0.19 μM for **3b**, and >40 μM for MTX and 10-EDAM. The inhibitory activity of **3a** and **3b** relative to that of MTX was found to be 24-fold and over 200-fold more potent, respectively (Table 2). Hence, on short-term exposure the differences in activity among these compounds were markedly greater as compared to the corresponding differences on prolonged exposure. This is likely to be due to a combination of factors such as the efficient uptake[20] and polyglutamation[20] of these compounds in addition to their elevated potency against DHFR. The sensitivity of the cells to short-term drug exposure might provide more information about the efficacy of antifolates *in vivo*.[26] The finding of the higher sensitivity of the cells to **3a** and **3b** on short term exposure suggested their *in vivo* efficacies which are greater than that of MTX at least in the case of colon 26 carcinoma.

Table 3. Activity against P388 Leukemia in Mice[a]

compd	T/C, %, at dose, mg/kg										
	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50
MTX					122	137	149	168	172	(–)[b]	
10-EDAM			114	135	136	155	162	165	(157)[c]		
3a	120	130	147	158	170	180	(138)[d]				
3b					129	ND[f]	135	ND	147	ND	(148)[e]

[a] Life span assay; see the Experimental Section. Controls died in an average of 9.8 days. [b] All mice died of toxic effects on day of evaluation (before day 10). [c] One toxic death. [d] Three toxic deaths. [e] Two toxic deaths. [f] ND = not determined.

Table 4. Activity against Colon 26 Mouse Colorectal Carcinoma in Mice[a]

compd	T/C, %, at dose, mg/kg							
	0.3	0.6	1.25	2.5	5	10	20	40
MTX						71	65	31
10-EDAM						70	(64)[b]	
3a	71	49	38	31				
3b				95	62	41		

[a] Tumor weight assay; see the Experimental Section. [b] One toxic death assessed before day 14.

Table 5. Activity against LC-6 Non-small Cell Lung Carcinoma in Mice[a]

compd	T/C, %, at dose, mg/kg						
	0.65	1.3	2.5	5	10	20	40
MTX					100	53[b]	(35)[c]
10-EDAM					63	(38)[c]	
3a		ND	6	(23)[d]			
10-EDAM					22	1	(1)[c]
3a	ND	4	9	1			

[a] Tumor weight assay; see the Experimental Section. [b] One toxic death assessed on day of evaluation (before day 14). [c] Four toxic deaths. [d] Two toxic deaths. [e] ND = not determined.

As mentioned below, this prediction proved to be essentially true.

Compounds **3a** and **3b** were tested in mice with transplantable tumors: P388 leukemia cells, colon 26 mouse colorectal carcinoma cells, and LC-6 human non-small cell lung carcinoma cells. As a primary screen, the tests were carried out with the P388 leukemic mice. The compounds were administered intraperitoneally (ip) daily from day 1 to day 4 (q1d × 4) at dose levels of 0.05–3.12 mg/kg for **3a** and 0.78–50 mg/kg for **3b**. MTX and 10-EDAM were included as positive controls. As shown in Table 3, all of the compounds tested gave an increase in life span compared to untreated controls. The activity of compound **3a** was comparable to that of MTX on direct comparison. However, it should be noted that effective doses and the dose range (0.05–1.56 mg/kg) for **3a** were found to be much lower and broader than those for MTX. Compound **3b** was active at a dose of 0.78–12.5 mg/kg under the same experimental conditions, but the therapeutic effect appeared to be slightly less than that of MTX. The results were sufficient to warrant further *in vivo* testing of **3a** and **3b** against a variety of solid tumors.

Treatment of the mice subcutaneously (sc) inoculated with colon 26 cells with **3a** and **3b** on a q2d × 5 schedule showed a definite therapeutic activity, with **3a** being more effective (Table 4). MTX showed activity only at a dose of 40 mg/kg, which appeared to be nearly the maximum tolerated dose under the given conditions and was accompanied by toxic weight loss, while 10-EDAM was much less effective even at the maximum tolerated dose in the same test. The findings that the colon 26 tumors in mice were sensitive to both **3a** and **3b** at doses much lower than those of the positive controls, sensitive to MTX only at its maximally tolerated dose, and resistant to 10-EDAM were in good agreement with the prediction based on the results obtained in short-term drug exposure tests. Next, each of these folate inhibitors except **3b** was subjected to evaluation for activity against the LC-6 human non-small cell lung carcinoma in mice, and the results are summarized in Table 5. Compounds **3a** and 10-EDAM gave excellent antitumor activity against human xenografts of LC-6. Intraperitoneal injection of **3a** on a q2d × 5 schedule at a dose of 1.3–5 mg/kg produced a 91–99% inhibition relative to untreated controls without any death due to toxicity. Treatment with 10–20 mg/kg of 10-EDAM afforded a 78–99% inhibition. Lack of material prevented us from clarifying the efficacy of **3b** in this tumor system. MTX did not show any significant inhibition of tumor growth. Although the number of these *in vivo* test systems was limited, **3a** showed therapeutic activity superior to that of the other two reference drugs. It is interesting to note that, against these three tumor cells, **3a** showed *in vivo* activity at much lower doses (nearly 10-fold lower) and wider effective dose ranges than those of MTX and 10-EDAM. The lower dosage requirement is likely to be due to a combination of factors such as increased enzyme binding, increased cellular uptake, and possibly increased intracellular polyglutamylation.[20]

Compounds **3a** and **3b** are the first examples of a novel type of antifolate containing 6,7-dihydrocyclopenta[*d*]-pyrimidine, different from those heterocyclic nuclei of previously published antifolates. The *in vivo* effectiveness of **3a** against experimental solid tumors which respond poorly to MTX could afford a marked therapeutic advantage. Efforts along these lines are currently in progress and will be the subject of forthcoming papers.

Experimental Section

Column chromatography was performed on silica gel (Merck, particule size 0.063–0.200 mm for normal chromatography and 6.3–40 μm for flash chromatography). TLC analyses were done on silica gel plates (Merck, Art 5715). All meting points were determined on a Yanagimoto micromelting point apparatus and uncorrected. IR spectra were obtained on a Nicolet 205 FT-IR spectrometer. ^{1}H-NMR spectra were measured on a Varian Unity 400 (400-MHz) spectrometer, and chemical shifts are expressed in δ units from tetramethylsilane (TMS) as an internal standard; coupling constant constants (*J*) are reported in herz; abbreviations are as follows; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad peak; Hz, herz. Mass spectra (FAB-MS and DI-EI-MS) were obtained on a JEOL SX102 mass spectrometer. Analytical results indicated by element symbols were within ±0.4% of the theoretical values.

***tert*-Butyl 4-[3-(2-Cyano-3-oxocyclopentanyl)propyl]benzoate (8a).** A solution of the propyl iodide **7a** (7.6 g, 23 mmol) in benzene (230 mL) was kept at refluxing temperature with constant stirring under N_2. To this solution were added dropwise simultaneously over 1 h two solutions: a solution of 2-cyano-2-cyclopenten-1-one **5** (4.9 g, 46 mmol) in benzene (50 mL) and a solution of tributyltin hydride (10 g, 35 mmol) in benzene (50 mL) containing a catalytic amount of AIBN. After the addition

was complete, the mixture was refluxed for 20 min and evaporated to dryness. The residue was dissolved in Et_2O (300 mL) and washed with saturated KF.[13] The resulting precipitate was removed by filtration, and the filtrate was allowed to separate into two phases. The organic phase was collected, dried over $MgSO_4$, and evaporated. The residue was purified by chromatography on a silica gel column using hexane–AcOEt (3:1) as the eluent to give **8a** as a colorless oil: 1.94 g (19%); IR (neat) ν 2958, 2932, 2248, 1760, 1710, 1311, 1294, 1167, 1117, 912, 734 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.40–1.66 (2 H, m), 1.59 (9 H, s), 1.70–1.92 (3 H, m), 2.20–2.56 (4 H, m), 2.60–2.80 (2 H, m), 2.81 (1 H, d, J = 11.6 Hz), 7.22 (2 H, d, J = 8.0 Hz), 7.91 (2 H, d, J = 8.0 Hz); MS (DI-EI) m/z 327 M^+.

***tert*-Butyl 4-[3-(Cyano-3-methoxy-2-cyclopentenyl)propyl]benzoate (10a).** The 3-oxo compound (**8a**) was dissolved in MeOH–CH_3CN (1:1, 150 mL), to this solution were added *N,N*-diisopropylethylamine (0.92 g, 7.1 mmol) and a 10% solution of (trimethylsilyl)diazomethane in hexane (15 g), and the mixture was stirred for 5 h at room temperature. The reaction was quenched with a small amount of AcOH, and the mixture was evaporated to dryness. The residue was dissolved in Et_2O, washed with brine, dried over $MgSO_4$, and evaporated. The resulting residue was purified by chromatography on a silica gel column using hexane–AcOEt (4:1) as the eluent to give the methyl enol ether (**10a**) as a colorless oil: 1.8 g (89%); IR (neat) ν 2978, 2935, 2203, 1712, 1632, 1612, 1457, 1352, 1292, 1167, 1116, 734 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.30–1.41 (1 H, m), 1.41–1.55 (1 H, m), 1.55–1.82 (3 H, m), 1.59 (9 H, s), 2.02–2.12 (2 H, s), 2.42–2.48 (2 H, m), 2.61–2.76 (2 H, m), 2.82–2.91 (1 H, m), 4.03 (3 H, s), 7.22 (2 H, d, J = 8.0 Hz), 7.90 (2 H, d, J = 8.0 Hz); MS (EI-DI) m/z 341 M^+.

***tert*-Butyl 4-[3-(2,4-Diamino-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-5-yl)propyl]benzoate (11a).** The methyl enol ether **10a** (1.43 g, 4.2 mmol) was dissolved in *t*-BuOH (70 mL), and to this solution was added guanidinecarbonate (2.3 g, 12.6 mmol). The mixture was placed in an autoclave and heated at 160 °C for 12 h. After being cooled, the reaction mixture was filtered to remove a precipitate and the filtrate was evaporated. The residue was purified by chromatography on a silica gel column using $CHCl_3$–MeOH (10:1) as the eluent to give the title compound as a colorless powder: 1.23 g (78%); mp 111–113 °C; IR (KBr) ν 3405, 3361, 3325, 3176, 2933, 1714, 1622, 1610, 1588, 1445, 1295, 1168, 1119 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.34–1.47 (1 H, m), 1.56–1.82 (4 H, m), 1.59 (9 H, s), 2.10–2.23 (1 H, s), 2.56–2.86 (4 H, m), 2.95–3.02 (1 H, m), 4.45 (2 H, br s), 4.65 (2 H, br s), 7.21 (2 H, d, J = 8.0 Hz), 7.90 (2 H, d, J = 8.0 Hz); MS (FAB) m/z 369 MH^+. Anal. ($C_{21}H_{28}N_4O_2 \cdot {}^1/_4H_2O$) C, H, N.

4-[3-(2,4-Diamino-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-5-yl)propyl]benzoic Acid (12a). A mixture of **11a** (1.2 g, 3.3 mmol) and 1 N HCl–AcOH (30 mL) was stirred at room temperature over 2 h and evaporated to dryness to yield the carboxylic acid **12a** as a colorless powder: 1.0 g (quant); ^1H NMR (DMSO-d_6) δ 1.16–1.32 (1 H, m), 1.50–1.64 (3 H, m), 1.64–1.74 (1 H, m), 1.94–2.08 (1 H, m), 2.40–2.52 (1 H, m), 2.54–2.74 (3 H, m), 2.92–3.02 (1 H, m), 6.20–6.35 (2 H, br s), 6.60–6.76 (2 H, br s), 7.28 (2 H, d, J = 8.4 Hz), 7.83 (2 H, d, J = 8.4 Hz); MS (FAB) m/z 313 MH^+.

***N*-[4-[3-(2,4-Diamino-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic Acid (3a).** (i) Compound **12a** (1.0 g, 3.2 mmol) was added to a stirred solution of diethyl L-glutamate hydrochloride (1.5 g, 6.2 mmol) in DMF (60 mL) under cooling in an ice bath. To this mixture were added DPPA (1.8 g, 6.5 mmol) and Et_3N (1.3 g, 13 mmol). The reaction mixture was kept stirring for 30 min and then at room temperature for an additional 2 h. A precipitate formed was removed by filtration, and the filtrate was evaporated to dryness. The residue was purified by chromatography on a silica gel column using $CHCl_3$–MeOH (10:1) as the eluent to give the diethyl ester **13a** as a pale yellow solid: MS (FAB) m/z 498 MH^+; a single spot (R_f 0.45) on TLC chromatography (silica, $CHCl_3$/MeOH, 4:1). (ii) To a solution of **13a** in EtOH (40 mL) was added dropwise 1 N NaOH (10 mL), and the mixture was stirred at room temperature for 5 h. This was neutralized with 1 N HCl and evaporated. The residue was purified by chromatography on a silica gel column using $CHCl_3$–MeOH–AcOH (10:10:1) as the eluent to give a white powder. This was further purified by dissolution and reprecipitation using diluted NaOH and diluted HCl. The resulting precipitate was collected and dried over P_2O_5 to give **3a** as a colorless powder: 0.54 g (37%); mp 167–169 °C; ^1H NMR (DMSO-d_6) δ 1.20–1.30 (1 H, m), 1.50–1.72 (4 H, m), 1.88–2.12 (3 H, m), 2.30–2.37 (2 H, s), 2.37–2.50 (2 H, m), 2.55–2.70 (3 H, m), 2.94–3.02 (1 H, m), 4.30–4.42 (1 H, m), 5.77 (2 H, br s), 6.12 (2 H, br s), 7.27 (2 H, d, J = 8.0 Hz), 7.78 (2 H, d, J = 8.0 Hz), 8.36–8.42 (1 H, br); MS (FAB) m/z 442 MH^+. Anal. ($C_{22}H_{27}N_5O_5 \cdot H_2O$) C, H, N.

***tert*-Butyl 4-[2-(2-Cyano-1-oxocyclopentan-3-yl)ethyl]benzoate (8b).** This compound was synthesized from **5** (2.4 g, 22 mmol) by treatment with tributyltin hydride (4.8 g, 16.5 mmol) and **7b** (3.7 g, 11 mmol) in a manner similar to that described for **8a**: yield 0.86 g (25%) as a colorless oil; IR (neat) ν 2977, 2933, 2246, 1760, 1713, 1369, 1312, 1295, 1257, 1167, 1115, 850, 771 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.59 (9 H, s), 1.78–1.96 (1 H, m), 2.02–2.22 (1 H, m), 2.24–2.58 (4 H, m), 2.76–2.94 (3 H, m), 7.26 (2 H, d, J = 8.2 Hz), 7.93 (2 H, d, J = 8.2 Hz); MS (FAB) m/z 314 MH^+.

***tert*-Butyl 4-[2-(2-Cyano-3-methoxy-2-cyclopentenyl)ethyl]benzoate (10b).** This compound was prepared in a manner similar to that described for **10a** with **8b** (0.86 g, 0.27 mmol) instead of **8a**: 0.83 g (94%); IR (neat) ν 2978, 2933, 2203, 1710, 1632, 1612, 1353, 1293, 1166, 1116 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.52–1.72 (2 H, m), 1.59 (9 H, s), 2.02–2.18 (2 H, s), 2.46–2.52 (2 H, m), 2.62–2.82 (2 H, m), 2.82–2.94 (1 H, m), 4.05 (3 H, s), 7.24 (2 H, d, J = 8.2 Hz), 7.90 (2 H, d, J = 8.2 Hz); MS (FAB) m/z 328 MH^+.

***tert*-Butyl 4-[2-(2,4-Diamino-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-5-yl)ethyl]benzoate (11b).** This compound was prepared in a manner similar to that described for **11a** with **10b** (0.5 g, 1.5 mmol) instead of **10a**: 0.42 g (76%); mp 175–178 °C; IR (KBr) ν 3357, 3178, 2976, 2934, 1709, 1632, 1611, 1582, 1450, 1295, 1168, 1117 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.59 (9 H, s), 1.64–1.78 (1 H, s), 1.84–1.98 (2 H, s), 2.16–2.28 (1 H, s), 2.60–2.71 (2 H, m), 2.71–2.80 (1 H, m), 2.80–2.91 (1 H, m), 2.95–3.05 (1 H, m), 4.44 (2 H, br s), 4.67 (2 H, br s), 7.23 (2 H, d, J = 8.0 Hz), 7.91 (2 H, d, J = 8.0 Hz); MS (FAB) m/z 355 MH^+. Anal. ($C_{20}H_{26}N_4O_2$) C, H, N.

4-[2-(2,4-Diamino-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-5-yl)ethyl]benzoic Acid (12b). This compound was prepared in a manner similar to that described for **12a** with **11b** (0.37 g, 1.0 mmol) as a colorless powder: 0.29 g (quant); ^1H NMR (DMSO-d_6) δ 1.66–1.78 (1 H, m), 1.94–2.14 (2 H, m), 2.28–2.42 (1 H, m), 2.70–2.84 (3 H, m), 2.92–3.04 (1 H, m), 3.18–3.26 (1 H, m), 7.31 (2 H, d, J = 8.4 Hz), 7.92 (2 H, d, J = 8.4 Hz); MS (FAB) m/z 299 MH^+.

***N*-[4-[2-(2,4-Diamino-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid (3b).** This compound was prepared from **12b** (0.29 g, 1.0 mmol) via the diethyl ester (**13b**: MS (FAB) m/z 484 MH^+; a single spot (R_f 0.59) on TLC chromatography (silica, $CHCl_3$/MeOH, 4:1)) in a manner similar to that described for **3a** as a colorless powder: 0.1 g (21%); mp 187–189 °C; ^1H NMR (DMSO-d_6) δ 1.45–1.57 (1 H, m), 1.75–2.00 (3 H, m), 2.00–2.13 (2 H, m), 2.33 (3 H, t, J = 7.6 Hz), 2.42–2.50 (1 H, m), 2.64 (2 H, t, J = 8.4 Hz), 2.60–2.79 (1 H, m), 3.00–3.10 (1 H, m), 4.32–4.40 (1 H, m), 6.09 (2 H, br s), 6.42 (2 H, br s), 7.30 (2 H, d, J = 8.0 Hz), 7.78 (2 H, d, J = 8.0 Hz), 8.43 (1 H, d, J = 7.2 Hz); MS (FAB) m/z 428 MH^+. Anal. ($C_{21}H_{25}N_5O_5 \cdot 2.5H_2O$) C, H, N.

***tert*-Butyl 4-[3-[2-(Methoxycarbonyl)-3-oxocyclopentanyl]propyl]benzoate (9a).** This compound was prepared from 2-(methoxycarbonyl)-2-cyclopenten-1-one[11] (**6**, 14 g, 69 mmol) by treatment with **7a** (12 g, 35 mmol) and tributyltin hydride (15 g, 52 mmol) in a manner similar to that described for **8a**: yield 7 g (56%) as a colorless oil; ^1H NMR ($CDCl_3$) δ 1.38–1.75 (5 H, m), 1.59 (9 H, s), 2.18–2.48 (3 H, m), 2.54–2.74 (3 H, m), 2.82 (1 H, d, J = 10.8 Hz), 3.75 (3 H, s), 7.20 (2 H, d, J = 8.0 Hz), 7.90 (2 H, d, J = 8.0 Hz); MS (FAB) m/z 361 MH^+.

***tert*-Butyl 4-[3-(2-Amino-6,7-dihydro-4-oxo-5*H*-cyclopenta[*d*]pyrimidin-5-yl)propyl]benzoate (14a).** To a solution of **9a** (6.9 g, 19.5 mmol) in *t*-BuOH (200 mL) were added guanidine hydrochloride (1.9 g, 20 mmol) and potasium *tert*-butoxide (2.2 g, 20 mmol). The mixture was stirred at reflux for 5 h under N_2 and cooled. A precipitate formed was removed by filtration, and the filtrate was evaporated. The residue was purified by chromatography on a silica gel column using $CHCl_3$–MeOH (20:1) as the eluent to give **14a** as a white powder: 1.6 g (21.4%); mp

223–225 °C; ^{1}H NMR ($CDCl_3$) δ 1.26–1.42 (1 H, m), 1.54–1.74 (2 H, m), 1.57 (9 H, s), 1.80–1.98 (2 H, m), 2.08–2.22 (1 H, m), 2.52–2.80 (4 H, m), 3.01–3.11 (1 H, m), 5.66 (2 H, br s), 7.19 (2 H, d, J = 8.4 Hz), 7.87 (2 H, d, J = 8.4 Hz), 11.8–12.3 (1 H, br); MS (FAB) m/z 370 MH$^+$. Anal. ($C_{21}H_{27}N_3O_3 \cdot {}^1/_5H_2O$) C, H, N.

4-[3-(2-Amino-6,7-dihydro-4-oxo-5*H*-cyclopenta[*d*]pyrimidin-5-yl)propyl]benzoic Acid (15a). Compound **14a** (1.1g, 3 mmol) was saponified in a manner similar to that described for **12a** to yield **15a** as a colorless powder. This showed a single spot (R_f 0.57) on TLC chromatography (silica, $CHCl_3$/MeOH/AcOH, 20:2:1): 1.3 g (quant); MS (FAB) m/z 314 MH$^+$.

***N*-[4-[3-(2-Amino-6,7-dihydro-4-oxo-5*H*-cyclopenta[*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic Acid (4a).** In a manner similar to that described for **3a**, compound **15a** (1.3 g, 3 mmol) was subjected to amidation with L-glutamate followed by saponification gave the title compound: 0.62 g (47%); mp 160–162 °C; ^{1}H NMR (DMSO-d_6) δ 1.15–1.30 (1 H, m), 1.43–1.65 (3 H, m), 1.76–2.10 (4 H, m), 2.20–2.66 (4 H, m), 2.60 (2 H, t, J = 7.2 Hz), 2.84–2.93 (1 H, m), 4.28–4.39 (1 H, m), 6.36 (2 H, br s), 7.26 (2 H, d, J = 7.2 Hz), 7.75 (2 H, d, J = 7.2 Hz), 8.36–8.48 (1 H, br), 10.30–10.75 (1 H, br), 12.3–13.4 (2 H, br); MS (FAB) m/z 443 MH$^+$. Anal. ($C_{22}H_{26}N_4O_6 \cdot H_2O$) C, H, N.

***tert*-Butyl 4-[2-[2-(Methoxycarbonyl)-3-oxocyclopentanyl]ethyl]benzoate (9b).** This compound was prepared from **6** (8.5 g, 42 mmol) by treatment with **7b** (7 g, 21 mmol) and tributyltin hydride (9 g, 31.2 mmol) in a manner similar to that described for **9a**: yield 1.1 g (15%) as a colorless oil; ^{1}H NMR ($CDCl_3$) δ 1.46–1.60 (1 H, m), 1.59 (9 H, s), 1.70–1.82 (1 H, m), 1.86–1.98 (1 H, m), 2.22–2.52 (3 H, m), 2.54–2.82 (3 H, m), 2.90 (1 H, d, J = 10.8 Hz), 3.76 (3 H, s), 7.22 (2 H, d, J = 8.4 Hz), 7.91 (2 H, d, J = 8.4 Hz); MS (FAB) m/z 347 MH$^+$.

***tert*-Butyl 4-[2-(2-Amino-6,7-dihydro-4-oxo-5*H*-cyclopenta[*d*]pyrimidin-5-yl)ethyl]benzoate (14b).** This compound was prepared in a manner similar to that described for **14a** using **9b** (1.0 g, 2.9 mmol) instead of **9a**: 0.68 g (66%); mp 224–226 °C; IR (KBr) ν 3324, 3170, 2977, 2892, 1711, 1668, 1658, 1611, 1495, 1370, 1293, 1165, 1111 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.58 (9 H, s), 1.62–1.84 (2 H, m), 2.12–2.28 (2 H, m), 2.56–2.85 (4 H, m), 3.07–3.17 (1 H, m), 5.38–5.60 (2 H, br), 7.22 (2 H, d, J = 8.4 Hz), 7.87 (2 H, d, J = 8.4 Hz), 11.70–12.10 (1 H, br); MS (FAB) m/z 356 MH$^+$. Anal. ($C_{20}H_{25}N_3O_3$) C, H, N.

4-[2-(2-Amino-6,7-dihydro-4-oxo-5*H*-cyclopenta[*d*]pyrimidin-5-yl)ethyl]benzoic Acid (15b). This compound was prepared in a manner similar to that described for **12a** using **14b** (0.25 g, 0.7 mmol) instead of **11a**: 0.16 g (76.2%); ^{1}H NMR ($CDCl_3$) δ 1.45–1.67 (2 H, m), 2.00–2.18 (2 H, m), 2.38–2.50 (1 H, m), 2.52–2.72 (3 H, m), 2.86–2.96 (1 H, m), 6.24–6.42 (2 H, br), 7.30 (2 H, d, J = 8.4 Hz), 7.82 (2 H, d, J = 8.4 Hz), 10.40–10.60 (1 H, br); MS (FAB) m/z 300 MH$^+$. Anal. ($C_{16}H_{17}N_3O_3 \cdot 0.5H_2O$) C, H, N.

***N*-[4-[2-(2-Amino-6,7-dihydro-4-oxo-5*H*-cyclopenta[*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid (4b).** This compound was prepared in a manner similar to that described for **3a** using **15b** (0.16 g, 0.53 mmol) instead of **12a**: 95 mg (32%); mp 182–184 °C; ^{1}H NMR (DMSO-d_6) δ 1.44–1.70 (2 H, m), 1.86–2.20 (4 H, m), 2.28–2.35 (2 H, m), 2.36–2.72 (4 H, m), 2.85–2.96 (1 H, m), 4.30–4.42 (1 H, m), 6.32 (2 H, br s), 7.28 (2 H, d, J = 8.4 Hz), 7.77 (2 H, d, J = 8.4 Hz), 8.40–8.50 (1 H, m), 10.40–10.60 (1 H, br); MS (FAB) m/z 429 MH$^+$. Anal. ($C_{22}H_{26}N_4O_6 \cdot 0.5H_2O$) C, H, N.

2-Cyano-2-cyclopenten-1-one (5). A cooled (ice–salt bath) solution of toluenethiol (32.6 g, 0.26 mol) in CH_2Cl_2 (100 mL) was stirred under N_2, and trimethylaluminum in hexane (131 mL of a 2.0 M solution, 0.26 mol) was added dropwise over 30 min. Stirring continued for 30 min at 0–5 °C. The mixture was then cooled in a dry ice–acetone bath, and a solution of 2-cyclopenten-1-one (21.5 g, 0.26 mol) in CH_2Cl_2 (80 mL) was added dropwise over 20 min. After 30 min of stirring at −78 °C, the reaction mixture was diluted with THF (200 mL) and a solution of p-toluenesulfonyl cyanide (48 g, 0.26 mol) in THF (60 mL) was added. After another 30 min of stirring at −78 °C and 60 min at room temperature, the reaction was quenched by dropwise addition of MeOH (100 mL). The mixture was diluted with Et_2O (1 L) and washed successively with 3 N HCl, 1 N HCl, saturated $NaHCO_3$, and brine. The organic layer was collected, dried over $MgSO_4$, and evaporated *in vacuo* to leave an oily substance, which on standing for several hours provided 2-cyano-3-[(4-methylphenyl)thio]cyclopentan-1-one (**17**) as a crystalline powder (55 g, 92%). This was dissolved in DMSO and applied to a silica gel column, and the column was eluted with hexane–AcOEt (2:1–1:3). Complete conversion of the 4-methylphenyl thioether to **5** was observed during the course of chromatography. Fractions containing the title compound were combined and evaporated to yield **5** as a pale yellow oil: 18.5 g (73%); IR (neat) ν 3077, 2934, 2238, 1760, 1727, 1608, 1425, 1303, 1257, 1196, 1034, 1002, 925 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.54–2.58 (2 H, m), 2.89–2.93 (2H, m), 8.32 (1 H, t, J = 2.8 Hz); MS (FAB) m/z 107 M$^+$.

3-[4-(*tert*-Butoxycarbonyl)phenyl]propionic Acid (20). To a stirred suspension of NaH (60% dispersion in mineral oil, 6.15 g, 0.154 mol, washed with hexane (20 mL × 2) in THF (100 mL) was added dropwise ethyl (diethylphosphono)acetate (35.3 g, 0.158 mol) in THF (100 mL) over 30 min, on cooling in an ice bath. The mixture was stirred for additional 30 min at room temperature to form a clear solution. To this solution, after being cooled in an ice bath, was added dropwise *tert*-butyl 4-formylbenzoate (**18**, 30.9 g, 0.15 mol) in THF (300 mL) over 30 min. Stirring was continued for an additional 1 h at room temperature, and the reaction mixture was poured into aqueous $NaHCO_3$ (300 mL). The water layer was extracted with Et_2O, and the combined organic layer was washed with brine and dried over $MgSO_4$. This was evaporated *in vacuo* to give ethyl 3-[4-(*tert*-butoxycarbonyl)phenyl]acrylate (**19**) as a pale yellow oil (41 g). This was dissolved in ethanol (100 mL) and hydrogenated in the presence of 10% Pd on charcoal under a H_2 atmosphere. The catalyst was removed by filtration, and the filtrate was diluted with additional ethanol (200 mL). To this solution was added 1 N NaOH (180 mL), and the mixture was stirred at room temperature for 3 h and evaporated to dryness. To this residue were added water (100 mL) and 1 N HCl (180 mL). The resulting white precipitate was filtered, washed with water, and dried over P_2O_5 to give a colorless crystalline powder: 37.1 g (99%); mp 87–89 °C; ^{1}H NMR ($CDCl_3$) δ 1.59 (9 H, s), 2.70 (2 H, t, J = 7.6 Hz), 3.01 (2 H, t, J = 7.6 Hz), 7.26 (2 H, d, J = 8.4 Hz), 7.92 (2 H, d, J = 8.4 Hz).

***tert*-Butyl 4-(3-Hydroxypropyl)benzoate (22a).** To a stirred suspension of $NaBH_4$ (24.6 g, 0.65 mol) in THF (200 mL) was added dropwise $BF_3 \cdot Et_2O$ (123.5 g, 0.87 mol) over 30 min on cooling in a dry ice–acetone bath under N_2. The reaction mixture was stirred for additional 1 h in an ice bath. To this mixture was added dropwise the propionic acid **20** (36.1 g, 0.144 mol) in THF (200 mL) over 1 h. The reaction mixture was allowed to equilibrate to ambient temperature. After the reaction was quenched by adding dropwise MeOH (200 mL), the precipitate that formed was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was dissolved in Et_2O (500 mL), washed with brine, and dried over $MgSO_4$. Evaporation provided **22a** as a slightly colored oil. This showed a single spot (R_f 0.65) on TLC chromatography (silica, hexane–AcOEt, 1:1): 33 g (97%); IR (neat) ν 3150–3050, 2978, 2935, 2870, 1712, 1611, 1309, 1293, 1167, 1116, 1048, 850 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.59 (9 H, s), 1.91 (2H, tt, J = 8.0, 6.4 Hz), 2.76 (2 H, J = 8.0 Hz), 3.67 (2 H, t, J = 6.4 Hz), 7.24 (2 H, d, J = 8.4 Hz), 7.91 (2 H, d, J = 8.4 Hz); MS (FAB) m/z 237 MH$^+$.

***tert*-Butyl 4-(3-Iodopropyl)benzoate (7a).** The 3-hydroxypropyl compound **22a** (32 g, 0.135 mol) and triethylamine (21 g, 0.21 mol) were dissolved in CH_2Cl_2 (300 mL), and the mixture was stirred in an ice bath under N_2. To this solution was added dropwise a solution of methanesulfonyl chloride (21 g, 0.18 mol) in CH_2Cl_2 (50 mL) over 30 min. Stirring continued for an additional 1 h, and then the reaction mixture was poured into 0.5 M $NaHSO_3$ (300 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (200 mL). The organic extracts were combined, dried over $MgSO_4$, and evaporated to give the mesylate of **22a** as colorless crystals (45 g). The mesylate was dissolved in acetone (500 mL), and NaI (31.5 g, 0.21 mol) was added. The mixture was refluxed for 12 h and then cooled. A precipitate was formed, removed by filtration, and then washed with acetone (100 mL). The filtrate and the washing were combined and evaporated *in vacuo* to give a residue. This was purified by chromatography on a silica gel column using hexane–AcOEt (4:1) as eluent to yield a pale yellow oil: 47g (95%); IR (neat) ν 2977, 2933, 1714, 1611, 1368, 1311, 1293, 1168, 1115,

1020, 851 767 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.59 (9 H, s), 2.13 (2 H, tt, J = 7.2, 7.2 Hz), 2.78 (2 H, t, J = 7.2 Hz), 3.15 (2 H, t, J = 7.2 Hz), 7.25 (2 H, d, J = 8.0 Hz), 7.92 (2 H, d, J = 8.0 Hz); MS (FAB) m/z 347 MH^+.

***tert*-Butyl 4-Ethenylbenzoate (21).** To a stirred suspension of methyltriphenylphosphonium bromide (18.2 g, 50.9 mmol) in THF (75 mL) was added dropwise a hexane solution of *n*-butyllithium (31.1 mL of 1.6 M solution, 49.7 mmol) over 25 min with cooling in a dry ice–acetone bath under N_2. After the addition was complete, the bath was changed to an ice bath and stirring was continued for an additional 1 h. A solution of 4-(*tert*-butylcarbonyl)benzaldehyde 18 (10 g, 48.5 mmol) in THF (75 mL) was added dropwise over 10 min. After the addition, stirring was continued for an additional 30 min, and then the reaction was quenched with aqueous NH_4Cl. The mixture was allowed to undergo partition between the organic and water layers, and the organic layer was separated. The water layer was extracted with Et_2O and then combined with the organic layer, which was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by chromatography on a silica gel column using hexane–AcOEt (10:1) as the eluent to give compound 21 as a colorless oil: 8.5 g (87%); IR (neat) ν 2978, 1710, 1608, 1368, 1293, 1166, 1118, 1108, 1016, 916, 861, 783, 713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.59 (9 H, s), 5.36 (1 H, dd, J = 10.8, 0.8 Hz), 5.84 (1 H, dd, J = 17.6, 0.8 Hz), 6.74 (1 H, dd, J = 17.6, 10.8 Hz), 7.43 (2 H, d, J = 8.0 Hz), 7.94 (2 H, d, J = 8.0 Hz).

***tert*-Butyl 4-(2-Hydroxyethyl)benzoate (22b).** To a stirred solution of 21 (8.5 g, 42 mmol) in THF (50 mL) was added dropwise a 0.5 M solution of 9-BBN in THF (100 mL, 50 mmol) over 15 min, and the mixture was stirred for 2 h at room temperature. To this mixture was added H_2O (15 mL) followed by 6 N NaOH (8.3 mL) in one portion. The mixture was treated dropwise with 30% H_2O_2 (17 mL) at a rate such that the internal temperature remained below 50 °C. After being stirred for additional 1 h at room temperature, the reaction mixture was poured into water and extracted with Et_2O. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by chromatography on a silica gel column using hexane–AcOEt (1:1) as the eluent to give the title compound (TLC R_f 0.67, hexane–AcOEt, 1:1): 8.7 g (93%); IR (neat) ν 3150–3700, 2978, 1713, 1611, 1369, 1294, 1167, 1116, 1048, 850 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.41 (t, J = 6.6 Hz), 1.59 (9 H, s), 2.92 (2 H, t, J = 6.6 Hz), 3.88 (2 H, q, J = 8.0 Hz), 7.28 (2 H, d, J = 8.0 Hz), 7.93 (2 H, d, J = 8.0 Hz); MS (FAB) m/z 223 MH^+.

***tert*-Butyl 4-(2-Iodoethyl)benzoate (7b).** The title compound was prepared in a similar manner as described for 7a using the hydroxyethyl compound **22b** (8.7 g, 39 mmol) as slightly brown crystals: 14 g (89%); mp 59–60 °C; IR (KBr) ν 2977, 2929, 1706, 1608, 1367, 1313, 1299, 1254, 1172, 1116, 1016, 851 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.59 (9 H, s), 3.23 (2 H, t, J = 7.6 Hz), 3.36 (2 H, t, J = 7.6 Hz), 7.24 (2 H, d, J = 8.4 Hz), 7.95 (2 H, d, J = 8.4 Hz); MS (FAB) m/z 333 MH^+.

Enzyme Inhibition Assay. Enzyme inhibition activity was determined by spectrophotometric assay, based on the change in molar absorbance at 340 nm due to conversion of dihydrofolate to tetrahydrofolate, essentially according to the method of D. K. Misra.[15] For the measurement partially purified DHFR[16] prepared from P388 mouse leukemia cells and purified enzyme from bovine liver cells (Sigma, D-6385) were used. The IC_{50} values (molar concentration required for 50% inhibition of enzyme activity) for DHFR inhibitors were obtained from graphs of activity versus drug concentration.

Cell Growth Inhibition Assay. (a) P388 leukemia cells, MTX-sensitive and MTX-resistant sublines; colon 26 colorectal carcinoma cells and colon 38 colorectal carcinoma cells; and KB human epidermoid carcinoma cells were used in this test. Cells were seeded at 2.5 × 10^3 cells per well in 96-well culture plates and incubated at 37 °C under a 5% CO_2 atmosphere for 24 h in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), penicillin (100 unit), streptomycin (100 μg/mL), 0.05 mM 2-mercaptoethanol, and 1 mM sodium pyruvate. To this culture was added a solution of drug in the RPMI 1640 medium at final concentrations of 0.1 nM to 1 μM at time zero. The culture was then continued at 37 °C under the 5% CO_2 atmosphere for 72 h. At the end of this period a cell count was made by the MTT colorimetric assay[18] to determine the drug concentration required for 50% cell growth inhibition (IC_{50}). (b) In the short-term exposure type experiments, colon 26 cells were dispensed within 96-well culture plates (initial density 2.5 × 10^3 cells/well), incubated in folate-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum (GIBCO) and 10 nM leucovorin at 37 °C under a 5% CO_2 atmosphere for 24 h, and exposed to various concentration of drugs (0.156–40 μM). Following 4 h of drug exposure, the cells were washed three times and recultured in drug-free medium as described above. After a total period of 72 h of incubation, starting from the drug exposure time, IC_{50} values were evaluated by the MTT assay.

***In Vivo* Antitumor Activity.** (a) Life span assay against P388 leukemia cells:[19] $CD2F_1$ (BALB/c × DBA/2) mice were inoculated ip with 1 × 10^6 P388 cells on day 0. Six mice were used for each treatment group and 10 for the control group. Treatment of the P388 leukemia-bearing mice was started with an ip injection of each drug at doses given in Table 3 on days 1, 2, 3, and 4 (q1d × 4). Drug effectiveness was evaluated on the basis of T/C (%), i.e., 100 × mean life span of treated mice/mean life span of control mice which received only saline. (b) Tumor growth inhibition test on colon 26 mouse colorectal carcinoma in mice: 3 × 10^5 cells of colon 26 tumor were implanted sc in mice (BALB/c) on day 0. Each test sample, after being dissolved at an appropriate concentration in 1–4% $NaHCO_3$ solution, was administered ip on days 1, 3, 5, 7, and 9 (q2d × 5). On day 14, the mice were sacrificed, and the inhibition of tumor growth (T/C, %) was determined by comparing the average tumor weight of the treated mice (n = 6) relative to that of the control mice (n = 10) which received 0.2 mL of 1–4% $NaHCO_3$ solution. (c) Tumor growth inhibition test on LC-6 human non-small cell lung carcinoma in mice: For LC-6 about 2-mm^3 tumor fragments were implanted sc by trocar in nude mice (BALB/c nu/nu). After the tumor grew to a size of 50–150 mm^3, treatments were started. Each test sample dissolved in 1–4% $NaHCO_3$ solution was was administered ip every other day for five times from day 1 to day 9. The inhibition of tumor growth on day 14 was evaluated on the basis of T/C (%) as described above.

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