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THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF A SERIES OF 2,4-DIAMINOPYRIDO[2,3-*D*]PYRIMIDINE BASED ANTIFOLATES AS ANTINEOPLASTIC AND ANTIARTHRITIC AGENTS[§]

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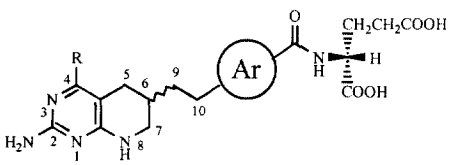
Abstract: A new series of 2,4-diaminopyrido[2,3-*d*]pyrimidine based antifolates **1–3** were synthesized through an efficient conversion of 2-pivaloyl-4-oxo-6-ethynylpyrido[2,3-*d*]pyrimidine **5** to the corresponding 4-amino analog **7** via the activated 1,2,4-triazole intermediate **6**. Compound **7** was used as the key intermediate for the preparation of the final products. The detailed biological evaluation of these compounds both as antineoplastic and antiarthritic agents will be discussed.

As part of the continuing structure activity relationship (SAR) study of the 5,10-dideazapyrido[2,3-*d*]- and pyrrolo[2,3-*d*]pyrimidine classes of deazafolates, we have examined the role of the 4-oxo group on the pyrimidine ring of both 5,10-dideazatetrahydrofolic acid, DDATHF[1] **4**, and LY231514[2]. Previously we have reported[3] a series of antifolates in which the 4-oxo group of the pyrimidine ring portion of both DDATHF and LY231514 was replaced with a hydrogen atom. To our surprise, these analogs proved to be potent inhibitors of dihydrofolate reductase (DHFR) with very minimal inhibitory activity against the enzymes glycinamide ribonucleotide formyltransferase (GARFT) and thymidylate synthase (TS), and this SAR was in sharp contrast to the conventional knowledge on DHFR inhibitors that stressed a 2,4-diaminopyrimidine configuration as a prerequisite structural requirement for potent inhibition of the enzyme. To verify that the 2,4-diamino configuration is indeed also important for eliciting DHFR inhibition for the DDATHF series, we have now developed a very efficient synthesis of a number of 2,4-diamino-5,10-dideazatetrahydropyrido[2,3-*d*]pyrimidine based antifolates, compounds **1-3**. These compounds were made through a common 2-pivaloyl-4-amino-6-ethynylpyrido[2,3-*d*]pyrimidine intermediate **7**, which was in turn prepared in high efficiency from the corresponding 4-oxo precursor via the 4-substituted 1,2,4-triazole intermediate **6**. Compounds **1-3** were evaluated extensively for their activity against various folate requiring enzymes such as DHFR, TS, GARFT, and folylpolyglutamate synthetase (FPGS). They were also evaluated for their cytotoxicity in cell culture, for their antitumor activity in the mouse 6C3HED lymphosarcoma model, and finally for their antiarthritic activity in the rat adjuvant arthritis model.



Ar = (**1**) LY335518, 1,4-phenyl, R = NH_2
(**2**) LY335580, 2,5-thienyl, R = NH_2
(**3**) LY335738, 2,5-furanyl, R = NH_2
(**4**) DDATHF, 1,4-phenyl, R = OH

The synthesis of **1** is the representative example for all 3 compounds (Scheme 1). First, 2-pivaloyl-6-ethynylpyrido[2,3-*d*]pyrimidine[4] **5** was reacted with 1,2,4-triazole[5] to give compound **6** in 59% yield, which was then reacted with concentrated NH_4OH to give 2-pivaloyl-4-amino-6-ethynylpyrido[2,3-*d*]pyrimidine[4] **7** in 74%

[§]Dedicated to the memory of my father Charles R. Gossett, September 13, 1927 to May 6, 1998

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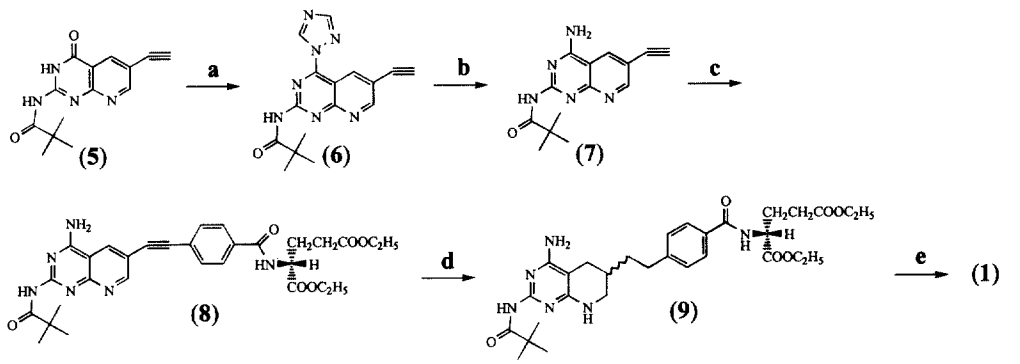
yield. Next a Heck reaction between **7** and 4-iodobenzoyl-L-glutamic acid diethyl ester gave the key intermediate **8** in 64% yield. The ethynyl bridge and the pyridine ring were reduced using hydrogen with 10% Pd/C to give compound **9** in 78% yield. The protecting groups were then removed in 1 N NaOH to give N-[1-[2,4-diamino-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine-6-yl)ethyl]benzo-4-yl]-L-glutamic acid **1** in 70% yield. Similarly, N-(2-bromofuran-5-yl)-L-glutamic acid diethyl ester or N-(2-bromothienyl-5-yl)-L-glutamic acid diethyl ester were used in the Heck coupling (step **c**) to give N-[(2-[(2,4-diamino-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine-6-yl)ethyl]thien-5-yl]-L-glutamic acid **2** or N-[2-[2-(2,4-diamino-5,6,7,8-tetrahydohydropyrido-[2,3-*d*]pyrimidine-6-yl)ethyl]furan-5-yl]-L-glutamic acid **3**. This convergent synthetic approach avoids the problematic guanidine cyclization route which often requires the use of intermediates that have low solubility. The use of **6**, which is efficiently obtained from **5** in high yields, provides a valuable intermediate that is quite soluble and easy to handle.

Scheme 1



(a) 1,2,4-triazole, 4-chlorophenyl dichlorophosphate, pyridine (b) conc NH_4OH, 1,4-dioxane (c) 4-iodobenzoyl-L-glutamic acid diethyl ester, $[(C_6H_5)_3P]_4Pd$, CuI, Et_3N, DMF (d) 10% Pd/C, H_2, 1/1 $EtOH/CH_2Cl_2$ (e) 1 N NaOH.

Compounds **1**, **2**, and **3** were evaluated for their ability to inhibit the growth of CCRF-CEM human lymphoblastic leukemia cells in culture for 72 h at 37° C. Two of the new analogs **1** and **2** possessed essentially the same high level of cytotoxicity with IC_{50} values of 6.3 nM and 2.7 nM respectively. Compound **3** however, displayed only moderate cytotoxicity with an IC_{50} value of 1503 nM, about 250 fold less cytotoxic than **2**.

Table 1 Measured K_i's Against Various Folate Requiring Enzymes

Compound	DHFR (nM)[a]	TS (μM)[b]	GARFT (μM)[c]
(1) LY335518	0.29	> 5	56
(2) LY335580	0.69	> 5	9.20
(3) LY335738	9.87	> 5	0.71

(a) recombinant human DHFR, *Biochemistry*, **1989**, *27*, 3664, (b) recombinant human TS, *Biochemistry*, **1993**, *32*, 10283, (c) murine trifunctional GARFT, *Biochemistry*, **1991**, *30*, 1997

The capacity of compounds **1**, **2**, and **3** in their monoglutamated forms to inhibit the folate requiring enzymes DHFR, TS, and GARFT is shown in Table 1. Interestingly, not all three new 2,4-diaminopyrido[2,3-*d*]pyrimidine analogs exhibited potent inhibition against DHFR. While compounds **1** and **2** were highly potent with subnanomolar K_i values of 0.29 nM and 0.69 nM respectively, **3** possessed a K_i of 9.87 nM which is approximately 15 fold less potent than **2** and 35 fold less potent than **1**. Cell culture end product reversal studies

indicated that the primary target of inhibition for these compounds was DHFR since both thymidine (5 μM) and hypoxanthine (100 μM) were required to reverse the cytoxicity (data not shown). While **1** and **2** possessed only weak inhibition against GARFT, **3** showed surprisingly good inhibition with a K_i value of 0.71 μM -just ten fold less potent than the GARFT inhibitor DDATHF **4**.

These three new analogs were evaluated as potential substrates for hog liver folylpolyglutamate synthetase (FPGS) (Table 2). A good substrate for FPGS could lead to increased cellular retention and the formation of more effective inhibitors against various folate requiring enzymes. All of the new analogs have good K_m values, measuring 28.2 μM and 11.6 μM for compounds **1** and **2**. Compound **3** has less substrate affinity with a K_m of 75.3 μM. Compounds **1** and **2** possess excellent first order rate constants with V_{max}/K_m values of 40 and 98 respectively. With a V_{max}/K_m value of only 9.2, compound **3** is a less efficient substrate for FPGS, and its first order rate constant is similar to methotrexate. Compound **3** is also significantly less cytotoxic in vitro when compared to compounds **2** and **3**. This lower toxicity can be better understood now when considering both its relatively poor DHFR potency and weak FPGS substrate activity.

Table 2 Substrate Activity of Various Compounds For FPGS[a]

Compound	K_m (μM)	V_{max}[b]	V_{max}/K_m
(**1**) LY335518	28.2	1189	40
(**2**) LY335580	11.6	1141	98
(**3**) LY335738	75.3	696	9.2
(**4**) DDATHF	16.4	977	60
Methotrexate	116	498	4.3

(a) isolated from hog liver, *Mol. Pharmacol.*, **1995**, *48*, 326 (b) nmol/h·mg

Compound **2** was also evaluated in vivo in mice against 6C3HED lymphosarcoma with a dosage from 1.0 to 16.0 mg/kg qd x 8. This compound was quite efficacious, and at 4.0 mg/kg displayed 100% inhibition of tumor growth with no toxicity. However, **2** had a narrow therapeutic index with the LD_{30} measured at 8.0 mg/kg.

Table 3 Adjuvant Arthritic Rat Model[a]

Treatment	Net Swelling in mL of Displaced Water X ± SEM (%Inhibition)	
	Acute Phase	Delayed Phase
Vehicle, po (10 mL/kg x 5)	0.77 ± 0.34	0.14 ± 0.06
(**1**), ip (0.125 mg/kg x 5)	0.55 ± 0.22 (35%)	0.12 ± 0.05 (14%)
(**1**), ip (0.06 mg/kg x 5)	0.82 ± 0.37 (0%)	0.15 ± 0.07 (0%)
Hydrocortisone, po (30 mg/kg x 5)	0.52 ± 0.23 (32%)	0.09 ± 0.04 (36%)

(a) Groups of 5 Wistar derived male rats weighing 130-150 g were used. On Day 1, the animals were injected with 0.1 mL of Complete Freund's Adjuvant in the subplantar region of the right hindpaw. In the acute phase the net swelling in the injected paws is compared between measurements on Day 5 vs. Day 1, and in the delayed phase the net swelling in the contralateral uninjected paws is compared between Day 18 vs. Day 14. Swelling was measured by water displacement. Vehicle or test compounds were administered daily for 5 consecutive days beginning on Day 1.

Finally, compounds **1**, **2**, and **3** were evaluated in the rat adjuvant arthritis model[6] (Table 3). In this initial assay, these compounds were found to be highly toxic, and no activity was observed at nontoxic po doses (data not shown). When **1** was given ip at does of 0.125 mg/kg x 5, a moderate level of rat paw swelling inhibition was seen at the acute phase (35%), which is comparable to that observed with hydrocortisone (32%). Less inhibition

was measured at the delayed phase (14%), while hydrocortisone showed a 36% inhibition. However, both compounds **2** and **3** proved to be too toxic in this study.

In summary, compounds **1** and **2** behaved quite predictably in their biological profile. Both compounds were quite potent cytotoxic agents against CCRF-CEM human leukemia cells with IC_{50} values less than 10 nM. Likewise both of these analogs were excellent substrates for FPGS with values similar to DDATHF **4**. Also both **1** and **2** were highly potent DHFR inhibitors with K_i values of 0.29 and 0.69 nM respectively. However, compound **3** proved to possess quite a different biological profile. In comparison, **3** was about 250 times less cytotoxic than **2**. Also there was about a 20-fold decrease in DHFR inhibition. Still, **3** showed substantial and unpredicted GARFT inhibition of 0.71 μM, just tenfold less than DDATHF **4**. Compound **3** proved to be a weak substrate for FPGS, similar to methotrexate. Consequently, the relatively poor inhibition towards DHFR plus the fact that **3** is less polyglutamated may collectively contribute to its poorer cytotoxicity against CCRF-CEM cells. Finally, **1** did display moderate inhibition of rat paw swelling (35%) in the acute phase of an initial rat adjuvant arthritis assay.

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