Structural Studies on Bioactive Compounds. 8.[1] Synthesis, Crystal Structure, and Biological Properties of a New Series of 2,4-Diamino-5-aryl-6-ethylpyrimidine Dihydrofolate Reductase Inhibitors with in Vivo Activity against a Methotrexate-Resistant Tumor Cell Line

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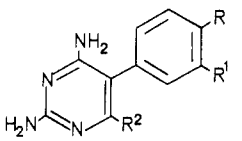
A series of 2,4-diamino-5-aryl-6-ethylpyrimidines embracing basic substituents in the 5-aryl ring was synthesized and evaluated for inhibitory activity against rat liver dihydrofolate reductase (DHFR). Maximal enzyme inhibition was observed for compounds bearing a benzylamino (**19**) or *N*-alkylbenzylamino substituent (**29** and **30**) in the 4-position of the phenyl ring and a nitro group in the 3-position, the corresponding 3-amino, 3-azido, or unsubstituted analogues proving only weakly active or inactive as DHFR inhibitors. Selected compounds were also screened in vivo against a methotrexate-resistant tumor, the M5076 murine reticulosarcoma, and antitumor activity in general paralleled activity against DHFR, the (3,4-dichlorobenzyl)amino analogue **26** proving the least toxic compound to exhibit significant antitumor activity. The X-ray crystal structure of the ethanesulfonic acid salt of the *N*-methylbenzylamino compound **29** has been determined to facilitate future molecular modeling studies in this new series of DHFR inhibitors.

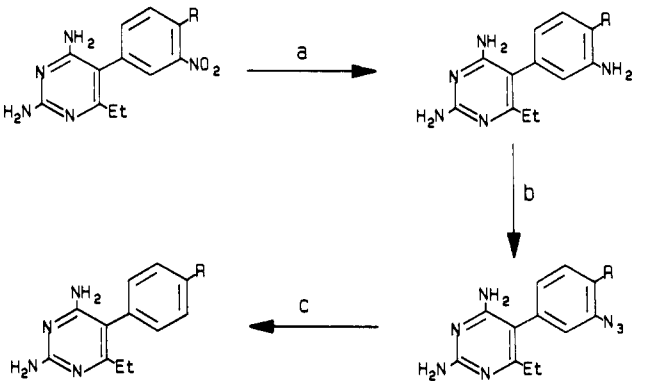
In an earlier paper[2] we reported on the chemical and enzyme-inhibitory properties of 2,4-diamino-2-(azidoaryl)-6-alkylpyrimidines and presented evidence that the lipophilic but biolabile azido group can modulate the properties of this novel type of dihydrofolate reductase (DHFR) inhibitor. One compound, the ethanesulfonic acid salt of 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine (**1**; MZPES)[3] has completed Phase I clinical evaluation as an antitumor agent and been shown to have a biological half-life ($t_{1/2}$) in humans of 35 h, significantly less than the $t_{1/2}$ (>200 h)[4] of the structurally related but extremely toxic agent metoprin [2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine] (**2**).



1: R = Cl; R^1 = N_3; R^2 = Et
2: R = Cl; R^1 = Cl; R^2 = Me
3: R = Cl; R^1 = H; R^2 = Et

As part of a broader study to investigate the nature of the interaction between 2,4-diamino-5-aryl-6-alkylpyrimidines and the active site of DHFR, we set out to prepare and evaluate a series of analogues embracing a variety of groups at the 3- and 4-positions of the 5-aryl ring. Of particular interest were compounds encompassing secondary and tertiary amine substituents on the 5-aryl ring since this structural modification has recently been shown to confer activity against DHFR in two related series of diaminopyrimidines.[5] By adopting this strategy, we hoped to identify novel lipophilic diaminopyrimidines

Scheme I. Synthesis of 2,4-Diamino-5-aryl-6-ethylpyrimidines



[a] Conditions: (a) $SnCl_2$/EtOH, reflux; (b) HNO_2/NaN_3, <5 °C; (c) N_2H_4, reflux. R = 2° or 3° amine substituent.

embracing the potent DHFR-inhibitory activity observed for the prototype antifolate methotrexate (MTX) in tandem with the desirable pharmacokinetic characteristics of MZPES. Such agents may have a clinical role for the treatment of MTX-resistant malignancies and tumors of the CNS which are inaccessible to the polar MTX but should not exhibit the cumulative toxicity observed for metoprin, which is attributable to the protracted biological $t_{1/2}$ of this antifolate.

Chemistry

The starting material for most of the synthetic studies was the readily available antimalarial drug pyrimethamine (**3**). This material was utilized previously[2] for the synthesis of MZPES, a crucial first step involving nitration of pyrimethamine to furnish 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (**4**). Our approach was to exploit the reactivity to nucleophiles imparted upon the 4-chloro substituent by the introduction of a nitro group, to displace the chloro group with a range of amines, and subsequently to modify and ultimately remove the nitro group via the synthetic pathway shown (Scheme I).

4 reacted only slowly with aqueous solutions of methylamine, dimethylamine, and ethylamine, and prolonged reflux times (48 h) were required to effect complete transformation to the nitroamines **6**, **7**, and **8**, respectively. The more hindered aliphatic amines diethylamine, 2-aminopropane, and 2-aminobutane failed to react in aqueous or dimethyl sulfoxide solutions. A more convenient method for the preparation of the nitrodimethylamine

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7 utilized *N,N*-dimethylformamide and ethanolamine as the dimethylaminating agent as described by Yamamoto[6] although the reaction proved unsuccessful when formamide or *N,N*-diethylformamide were substituted for dimethylformamide. An attempt to prepare the nitromethylamine **6** via this method using *N*-methylformamide–ethanolamine furnished only a trace of product (TLC).

Those amines available as pure liquids reacted rapidly with **4** at reflux temperatures (2 h) to give near-quantitative yields of the desired products (**9–18**), although protracted reaction times were necessary with arylalkylamines in order to obtain acceptable yields of the required nitroamines (**19–34**); the use of 2-ethoxyethanol as solvent did not improve upon the yield obtained. Similar reactions between a range of selected high-boiling amines and 2,4-diamino-5-(3-chloro-4-nitrophenyl)-6-ethylpyrimidine (**5**) furnished the series of analogues **35–37**.

Reduction of the nitro group to afford the corresponding (3-aminophenyl)pyrimidine series (**38–50**) was accomplished most satisfactorily by using tin(II) chloride in ethanol[7] and proceeded without incident with the exception of the dibenzylamino pyrimidine **33**, which furnished a mixture of products, presumably due to partial reductive debenzylation, and consequently the required amino dibenzylamine was not obtained. Subsequent diazotization of those (3-aminophenyl)pyrimidines embracing tertiary amino ortho substituents and reaction with hydrazoic acid gave the (3-azidophenyl)pyrimidines **56–60** in high yields, although purification of the azides to analytical standards proved difficult due to decomposition at relatively low temperatures. A detailed examination of these degradations and the formation of benzotriazoles when (aminophenyl)pyrimidines bearing secondary amino ortho substituents are diazotized will be reported separately.

We have demonstrated previously[2] that the azido dimethylamino pyrimidine **56** undergoes a reductive deazidation in boiling hydrazine hydrate to furnish the dimethylamino pyrimidine **61** in good yield, and this approach proved successful for the removal of the azido substituent in compounds **57–59**, thus affording the required diaminopyrimidines embracing a 4-amine substituent but unsubstituted in the 3-position of the aryl ring (**62–64**). Unfortunately, **60** gave only complex mixtures upon treatment with hydrazine and the deazidation products could not be isolated.

The chemical structures and physical properties of diaminopyrimidines are collated in Table I.

Crystal Structure Determination

In order to facilitate future computer graphic modeling of this new type of DHFR inhibitor, the crystal structure determination of the ethanesulfonate salt (2-propanol solvate) of one of the most active compounds (**29**) was accomplished. The structure (PLUTO[8] drawing) with its atom numbering scheme is shown in Figure 1. Where groups are disordered, the positions with the highest occupancy bear the number. The planar rings are so arranged that the pyrimidine ring plane (P1) and central ring plane (P2) intersect at 73.6° while the benzyl ring plane (P3) is nearly parallel to P1 (angle P1–P3 = 6.7°). In the parent compound (**3**) the twist corresponding to P1–P2 is 67° for the hydrobromide salt[9] and perpendicular for the

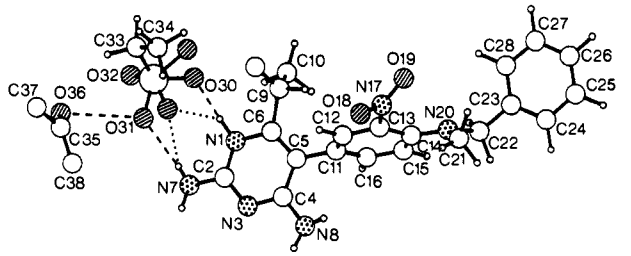

Figure 1. PLUTO drawing of the solvated ion pair. Nitrogen atoms are stippled and oxygen atoms hatched. In the disordered ethyl substituent and ethanesulfonate anion the sites with greater occupancy bear labels, and their hydrogen bonds are dashed. Hydrogen bonds to the alternative site with smaller occupancy are dotted.

complex with *Escherichia coli* DHFR.[10] Donation of protons for hydrogen bonds by protonated N(1) and the amino groups, and acceptance of a hydrogen bond by N(3), are typical of protonated antifolates.[11] However, the placement of the disordered ethanesulfonate ion is unusual. The majority of the anions deploy O(30) and O(31) to accept one hydrogen bond each (Figure 1, dashed lines) at N···O distances of 2.917 (5) and 2.864 (6) Å, respectively, but the alternative location O(31′) enables this single oxygen atom to accept both hydrogen bonds with lengths of 2.73 (2) and 2.80 (2) Å (Figure 1, dotted lines). Comparable alternatives for the counterion have been found in ordered structures of hydrochloride salts of triazine antifolates,[12] where the Cl^- ion may accept a hydrogen bond from the protonated ring,[13] the adjacent amino group,[14] or both.[12] It seems possible that the active-site carboxylate ion of DHFR could similarly utilize just one or both of its oxygen atoms to accept two hydrogen bonds as the inhibitor changed position.

Biological Results

Enzyme-Inhibitory Activity. Compounds were evaluated spectroscopically at 340 nm for inhibitory activity against rat liver DHFR[15] in vitro, and concentrations of inhibitor necessary to reduce enzyme activity by 50% (I_{50}) were determined and compared with those for methotrexate and metoprin as an estimate of inhibitor potency (Table II). Replacement of the chloro group of **4** with a range of aliphatic amines afforded compounds (**6–9**) approximately equiactive with metoprin (**2**), with I_{50} values in the range 0.1–0.3 μM, whereas introduction of bulkier heteroalicyclic groups (**10–14**), cyclohexyl substituents (**15** and **16**), or (aminoalkyl)amino groups (**17** and **18**) had a dyschemotherapeutic effect. In contrast, (3-nitrophenyl)pyrimidines bearing a benzylamino (**19**) or *N*-alkylbenzylamino substituent (**29** and **30**) proved the most active compounds prepared in the series, with I_{50} values of approximately 10 nM, and these compounds were considered sufficiently interesting to warrant a more precise kinetic analysis against rat liver DHFR. Inhibitory constant (K_i) values determined for the *N*-methyl and *N*-

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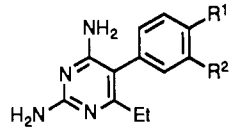
Table I. Synthesis, Structures, and Physicochemical Data for 2,4-Diamino-5-(substituted aryl)-6-ethylpyrimidines



compd no.	R^1	R^2	synthetic route	yield, %	mp, °C	crystn solvent	formula[a]
4[b]	Cl	NO_2		95	203–205	EtOH–H_2O	$C_{12}H_{12}ClN_5O_2$
5[c]	NO_2	Cl		95	266–267	EtOH–H_2O	$C_{12}H_{12}ClN_5O_2$
6	$NHCH_3$	NO_2	A	92	262–265	DMF–H_2O	$C_{13}H_{16}N_6O_2$
7[c]	$N(CH_3)_2$	NO_2	A	68	256–257	DMF–H_2O	$C_{14}H_{18}N_6O_2$
			C	87	256–257	DMF–H_2O	
8	$NHCH_2CH_3$	NO_2	A	97	275–276	DMF–H_2O	$C_{14}H_{18}N_6O_2$
9	$NH(CH_2)_3CH_3$	NO_2	B	98	252–254	2-ethoxyethanol–H_2O	$C_{16}H_{22}N_6O_2$
9 ($EtSO_3H$ salt)[d]				75	250–252	H_2O	$C_{18}H_{28}N_6O_5S$
10	$N(CH_2)_3CH_2$	NO_2	B	89	233–234	DMF–H_2O	$C_{16}H_{20}N_6O_2$
11[b]	$N(CH_2)_4CH_2$	NO_2		90	248–250		
12	$N(CH_2)_2NH(CH_2)_2$	NO_2	B	86	260–261	DMF–H_2O	$C_{16}H_{21}N_7O_2$
13	$N(CH_2)_2NCH_3(CH_2)_2$	NO_2	B	93	233–234	EtOH–H_2O	$C_{17}H_{23}N_7O_2$
14	$N(CH_2)_2O(CH_2)_2$	NO_2	B	97	246–247	2-ethoxyethanol–H_2O	$C_{16}H_{20}N_6O_3$
15	NHC_6H_{11}	NO_2	B	91	252–253	2-ethoxyethanol–H_2O	$C_{18}H_{24}N_6O_2$
16	$N(CH_3)C_6H_{11}$	NO_2	B	67	256–260	EtOH–H_2O	$C_{19}H_{26}N_6O_2$
17	$NH(CH_2)_2NH_2$	NO_2	B	83	240–241	DMF–H_2O	$C_{14}H_{19}N_7O_2$
18	$NH(CH_2)_3NH_2$	NO_2	B	62	238–240	DMF–H_2O	$C_{15}H_{21}N_7O_2$
19	$NHCH_2Ph$	NO_2	B	73	253–255	2-ethoxyethanol–H_2O	$C_{19}H_{20}N_6O_2$
20	$NHCH_2C_6H_4$(2-Cl)	NO_2	B	37	243–244	2-ethoxyethanol–H_2O	$C_{19}H_{19}ClN_6O_2$
21	$NHCH_2C_6H_4$ (4-Cl)	NO_2	B	76	247–248	2-ethoxyethanol–H_2O	$C_{19}H_{19}ClN_6O_2$
22	$NHCH_2C_6H_4$ (4-F)	NO_2	B	61	250–251	2-ethoxyethanol–H_2O	$C_{19}H_{19}FN_6O_2$
23	$NHCH_2C_6H_4$ (4-CH_3)	NO_2	B	86	237–238	2-ethoxyethanol–H_2O	$C_{20}H_{22}N_6O_2$
24	$NHCH_2C_6H_4$ (4-OCH_3)	NO_2	B	35	241–242	2-ethoxyethanol–H_2O	$C_{20}H_{22}N_6O_3$
25	$NHCH_2C_6H_4$ (4-CF_3)	NO_2	B	51	253–254	2-ethoxyethanol–H_2O	$C_{20}H_{19}F_3N_6O_2$
26	$NHCH_2C_6H_3$[3,4-$(Cl)_2$]	NO_2	B	75	244–245	2-ethoxyethanol–H_2O	$C_{19}H_{18}Cl_2N_6O_2$
27	$NHCH_2C_6H_3$[3,4-$(OCH_3)_2$]	NO_2	B	94	232–233	2-ethoxyethanol–H_2O	$C_{21}H_{24}N_6O_4$
28	$NHCH_2C_6H_2$[2,4,6-$(OCH_3)_3$]	NO_2	B	11	265–266	2-ethoxyethanol–H_2O	$C_{22}H_{26}N_6O_5$
29	$N(CH_3)CH_2C_6H_5$	NO_2	B	91	210–211	2-ethoxyethanol–H_2O	$C_{20}H_{22}N_6O_2$
29 ($EtSO_3H$ salt)[d]				66	242–243	2-propanol–H_2O	$C_{22}H_{28}N_6O_5S$
30	$N(CH_2CH_3)CH_2C_6H_5$	NO_2	B	86	214–216	2-ethoxyethanol–H_2O	$C_{21}H_{24}N_6O_2$
31	(±)$NHCH(CH_3)C_6H_5$	NO_2	B	85	208–210	ethyl acetate	$C_{20}H_{22}N_6O_2$
32	$NH(CH_2)_2C_6H_5$	NO_2	B	70	222–225	2-ethoxyethanol–H_2O	$C_{20}H_{22}N_6O_2$
33	$N(CH_2C_6H_5)_2$	NO_2	B	49	197–199	2-ethoxyethanol–H_2O	$C_{26}H_{26}N_6O_2$
34	$N(CH_3)CH_2C_6H_4$ (4-OCH_3)	NO_2	B	25	201–203	2-ethoxyethanol–H_2O	$C_{21}H_{24}N_6O_3$
35	NO_2	$NH(CH_2)_3CH_3$	B	45	271–273	2-ethoxyethanol	$C_{16}H_{22}N_6O_2$
35 ($EtSO_3H$ salt)[d]				56	270	H_2O	$C_{18}H_{28}N_6O_5S$
36	NO_2	$NHCH_2C_6H_5$	D	45	247–248	2-ethoxyethanol–H_2O	$C_{19}H_{20}N_6O_2$
37	NO_2	$NH(CH_3)CH_2C_6H_5$	D	31	189–190	2-ethoxyethanol–H_2O	$C_{20}H_{22}N_6O_2$
38	$NHCH_3$	NH_2	E	94	231–233	EtOH–H_2O	$C_{13}H_{18}N_6$
39[b]	$N(CH_3)_2$	NH_2	E	89	188–189	EtOH–H_2O	$C_{14}H_{20}N_6$
40	$NHCH_2CH_3$	NH_2	E	89	210–211	EtOH	$C_{14}H_{20}N_6$
41	$NH(CH_2)_3CH_3$	NH_2	E	93	191–193	EtOH–H_2O	$C_{16}H_{24}N_6$
42	$N(CH_2)_3CH_2$	NH_2	E	77	208–210	EtOH	$C_{16}H_{22}N_6$
43	$N(CH_2)_4CH_2$	NH_2	E	95	193	EtOH	$C_{17}H_{24}N_6$
44	$N(CH_2)_2NH(CH_2)_2$	NH_2	E	88	210–212[e]	EtOH–H_2O	$C_{16}H_{23}N_7$
45	$N(CH_2)_2NCH_3(CH_2)_2$	NH_2	E	89	241–243	EtOH–H_2O	$C_{17}H_{25}N_7$
46	$N(CH_2)_2O(CH_2)_2$	NH_2	E	77	235–237[e]	2-ethoxyethanol–H_2O	$C_{16}H_{26}N_6O$
47	NHC_6H_{11}	NH_2	E	87	187–188	*f*	$C_{18}H_{26}N_6$[g]
48	$N(CH_3)C_6H_{11}$	NH_2	E	85	192–194	*f*	$C_{19}H_{28}N_6$[g]
49	$NH(CH_2)_2NH_2$	NH_2	E	62	241–243	EtOH–H_2O	$C_{14}H_{21}N_7$
50	$NH(CH_2)_3NH_2$	NH_2	E	69	195–197	EtOH–H_2O	$C_{15}H_{23}N_7$
51	$NHCH_2C_6H_5$	NH_2	E	76	202–204	EtOH–H_2O	$C_{19}H_{22}N_6$
52	$N(CH_3)CH_2C_6H_5$	NH_2	E	94	206–208	EtOH–H_2O	$C_{20}H_{24}N_6$
53	$N(CH_2CH_3)CH_2C_6H_5$	NH_2	E	88	40	*f*	$C_{21}H_{26}N_6$[g]
53 (2 × $EtSO_3H$ salt)[d]			E	85	211–212	EtOH–ethyl acetate	$C_{25}H_{38}N_6O_6S_2$
54	(±)$NHCH(CH_3)C_6H_5$	NH_2	E	87	250–253[e]	EtOH–H_2O	$C_{20}H_{24}N_6$
55	$NH(CH_2)_2C_6H_5$	NH_2	E	85	175–177	EtOH–H_2O	$C_{20}H_{24}N_6$
56[b]	$N(CH_3)_2$	N_3	F	91	158–159	EtOH–H_2O	
57	$N(CH_2)_4CH_2$	N_3	F	82	122–123	EtOH–H_2O	$C_{17}H_{22}N_8$
58	$N(CH_2)_2NH(CH_2)_2$	N_3	F	95	120–121	EtOH–H_2O	$C_{16}H_{21}N_9$
59	$N(CH_2)_2NCH_3(CH_2)_2$	N_3	F	80	128–130	EtOH–H_2O	$C_{17}H_{23}N_9$
60	$N(CH_2)_2O(CH_2)_2$	N_3	F	90	211–213	EtOH–H_2O	$C_{16}H_{20}N_8O$
61[b]	$N(CH_3)_2$	H	G	78	237–239	EtOH	$C_{14}H_{19}N_5$
62	$N(CH_2)_4CH_2$	H	G	85	196–198	EtOH	$C_{17}H_{23}N_5$
63	$N(CH_2)_2NH(CH_2)_2$	H	G	64	250–252[e]	EtOH	$C_{16}H_{22}N_6$
64	$N(CH_2)_2NCH_3(CH_2)_2$	H	G	76	235–236	EtOH	$C_{17}H_{24}N_6$

[a] Anal. C, H, N (except where indicated otherwise). [b] Reference 23. [c] Reference 2. [d] Ethanesulfonic acid salts were prepared by dissolving the amine free bases in boiling water containing ethanesulfonic acid (1.1 mol equiv). [e] Sinters and decomposes. [f] No satisfactory purification solvent could be found; compound unstable on crystallization. [g] Analytical sample unobtainable.

Table II. Dihydrofolate Reductase Inhibitory Activity[a] of 2,4-Diamino-5-(substituted aryl)-6-ethylpyrimidines

compd no.	solvent	I_{50},[b] μM	compd no.	solvent	I_{50},[b] μM
3	A	1.4	**39**	A	67.0
4	A	0.08	**40**	A	>25.0
6	A	0.15	**41**	A	>25.0
7	A	0.25	**42**	A	>25.0
8	A	0.16	**43**	A	>25.0
9	A	0.17	**44**	A	>25.0
10	A	1.7	**45**	A	>25.0
11	A	0.62	**46**	A	>25.0
12	A	3.1	**47**	A	>25.0
13	A	3.9	**48**	A	8.5
14	A	8.0	**49**	A	>25.0
15	A	1.2	**50**	A	1.3
16	A	2.5	**51**	A	0.5
17	A	2.6	**52**	A	0.10
18	A	1.0	**53**	A	4.00
19	A	0.01	**54**	A	35.0
24	A	0.02	**55**	A	1.9
29	A	0.01	**56**	A	1.6
30	A	0.02	**57**	A	8.5
31	A	0.18	**58**	A	>25.0
32	B	0.07	**59**	A	>25.0
33	B	0.26	**60**	A	9.4
34	A	0.38	**61**[b]	A	6.2
36	B	9.2	**62**	A	>25
37	B	0.44	**63**	A	>25
38	A	15.0	**64**	B	>25

[a] Partially purified rat liver DHFR (EC 1.5.1.3) was prepared by the method of Bertino and Fischer[24] and assayed spectrophotometrically by a previously published method.[15] [b] Defined as the final concentration of inhibitor in the assay system necessary to reduce the enzymatic reaction rate to 50% of the uninhibited rate. I_{50} values were determined by conducting assays in duplicate at four inhibitor concentrations estimated to reduce DHFR activity by approximately 20, 40, 60, and 80% of control values. Solvents: (A) 0.1 M hydrochloric acid; (B) 95% ethanol.

ethylbenzylamino analogues (**29** and **30**) by the zone B analysis method[16] appropriate for tight-binding enzyme inhibitors, and assuming a K_m value of 0.2 μM for dihydrofolate,[17] gave figures of 0.009 ± 0.002 (nM) and 0.04 ± 0.03 (nM), respectively. Metoprin (**2**) in comparison gave a K_i of 0.12 ± 0.04 (nM) in the same test. Interestingly, transposing the nitro and *N*-alkylbenzylamino substituents to furnish the isomeric analogues (**36** and **37**) reduced activity considerably.

Reduction of the 3-nitrophenyl substituent to afford the series of compounds **38–55** greatly reduced activity against the enzyme with the exception of the 3-amino-4-[(3-aminopropyl)amino]phenyl derivative **50**, which was equiactive with the parent 3-nitro precursor **18**. The presence of an electron-withdrawing *o*-nitro group would be predicted to reduce the basicity of the adjacent amine substituents and thus favor association with a hydrophobic domain at the active site of the enzyme, a prerequisite for inhibitory activity.[18] However, reduction of the nitro group will abolish the base-weakening effect and indeed gives products substituted in the phenyl ring with two basic substituents, thus profoundly decreasing inhibitory activity as observed. Compounds substituted with a lipophilic azido group in addition to a basic substituent (**56–60**) were poorly active or inactive as DHFR inhibitors, and although removal of the 3-substituent on the 5-aryl residue altogether (**61–64**) restored activity to some degree, these derivatives were inferior to those in the nitro-substituted series.

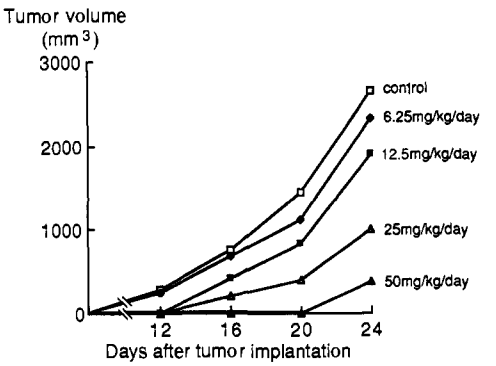


Figure 2. Antitumor dose-response curves for 2,4-diamino-5-[4-(*N*-methylbenzylamino)-3-nitrophenyl]-6-ethylpyrimidine (**29**) versus M5076 murine reticulosarcoma in vivo.

Antitumor Activity. Compounds prepared in the course of this work were screened initially for in vivo antitumor activity against P388 murine leukemia. In general, antitumor activity correlated reasonably with activity against DHFR, the nitropyrimidines proving most active. Compounds **6**, **9**, and **29** were of particular interest in this screen with activity rated (++) on the NCI activity scale[19] and maximum T/C (%) values of 182, 206, and 185, respectively. Interestingly, the benzylamino analogue **19** was inactive against P388 leukemia in contrast to the *N*-methylbenzylamino homologue **29**, notwithstanding identical DHFR-inhibitory activities (I_{50} = 10 nM) observed for these compounds.

In order to evaluate the potential therapeutic utility of these novel lipophilic antifolates for the treatment of MTX-resistant malignancies, selected compounds were screened against the M5076 reticulosarcoma, a murine solid tumor naturally resistant to methotrexate by virtue of a modified transport process.[20] The activity of the *N*-methylbenzylamine **29** is presented as a dose-response relationship in Figure 2, and full details of antitumor evaluation of certain congeners are assembled in Table III. All of the compounds screened were superior to the prototype lipophilic antifolate metoprin (**2**), with the (3,4-dichlorobenzyl)amino pyrimidine **26** exhibiting pronounced antitumor effects at several dose levels with minimal observed toxicity. More comprehensive studies regarding the activity of this series of diaminopyrimidines against a broader spectrum of tumors are under way with a view to selection of a possible clinical candidate, and investigations as to the nature of the potent inhibition of DHFR by these compounds at the molecular level will be reported in future parts of this series.

Experimental Section

Melting points were determined on an Electrothermal instrument and are uncorrected. NMR spectra were recorded on a Perkin-Elmer R34 (200-MHz) or a Bruker WH400 (400-MHz) spectrometer using [$^2H_6$]DMSO as solvent. Infrared spectra were recorded on a Pye Unicam SP8000 spectrometer as potassium chloride disks, and mass spectra were determined on a Micromass 12B single-focusing mass spectrometer. Elemental analyses were obtained from Butterworth Laboratories Ltd., Teddington, Middlesex, U.K. The TLC system employed Kieselgel 60F_{254} (0.25

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Table III. Activity of 2,4-Diamino-5-(substituted aryl)-6-ethylpyrimidines against the M5076 Reticulum Cell Sarcoma in Mice[a]

compd	treatment, mg/kg/day	tumor volumes, mm³; days after tumor implantation 12	16	20	24	T/C, %[b]	weight change[c]	survivors[d] (test/control)
methotrexate	6.25	347	389	dead				0/5
	3.125	411	987	1914	2270	92	+3.9	5/5
	control	511	1000	2086	2483	100	+4.4	5/5
metoprin	25	dead						0/5
	12.5	dead						0/5
	6.25	dead						0/5
	3.125	NM[e]	NM	NM	NM	<8[f]	+4.9	1/5
	control	411	1242	2136	2963	100	+3.3	5/5
6	50	NM	dead					0/5
	25	NM	NM	NM	220	10	+2.1	5/5
	12.5	NM	NM	NM	462	20	+2.1	5/5
	6.25	NM	NM	640	805	35	+3.3	5/5
	3.125	NM	NM	822	1407	61	+4.3	5/5
	control	240	957	1733	2288	100	+1.8	5/5
9	50	NM	NM	NM	368	15	+2.7	5/5
	25	NM	NM	NM	638	22	+3.7	5/5
	12.5	180	283	496	1061	41	+3.6	5/5
	6.25	212	438	913	1920	70	+5.4	5/5
	3.125	271	622	1176	2143	78	+8.2	5/5
	control	283	765	1434	2656	100	+4.4	5/5
19	50	dead						0/5
	25	NM	NM	NM	NM	<8	+1.7	3/5
	12.5	NM	NM	NM	586	21	+1.6	5/5
	6.25	NM	NM	301	932	34	+2.1	5/5
	3.125	NM	NM	443	1454	53	+2.2	5/5
	control	265	1257	1863	2742	100	+4.1	5/5
21	50	dead						0/5
	25	NM	dead					0/5
	12.5	NM	NM	NM	380	13	+2.9	5/5
	6.25	NM	NM	NM	597	20	+3.3	5/5
	3.125	NM	NM	552	873	29	+3.2	5/5
	control	411	1242	2136	2963	100	+3.3	5/5
22	50	NM	dead					0/5
	25	NM	NM	NM	NM	<8	+4.3	0/5
	12.5	NM	NM	NM	386	17	+2.0	5/5
	6.25	NM	NM	NM	448	20	+2.5	5/5
	3.125	NM	337	539	1555	68	+3.8	5/5
	control	240	957	1733	2228	100	+1.8	5/5
23	50	dead						0/5
	25	NM	180	426	514	20	+2.4	5/5
	12.5	NM	198	385	641	24	+2.4	5/5
	6.25	NM	270	704	1099	44	+2.1	5/5
	3.125	238	596	1581	1881	76	+3.0	5/5
	control	511	1000	2086	2483	100	+4.4	5/5
24	50	dead						0/5
	25	NM	NM	NM	NM	<8	+0.2	2/5
	12.5	NM	NM	NM	636	21	+2.3	5/5
	6.25	NM	NM	416	875	30	+3.6	5/5
	3.125	NM	306	831	1482	50	+4.1	5/5
	control	411	1242	2136	2963	100	+3.3	5/5
25	50	dead						0/5
	25	NM	NM	NM	NM	<8	+6.2	1/5
	12.5	NM	NM	NM	265	12	+2.0	5/5
	6.25	NM	NM	NM	454	20	+2.3	5/5
	3.125	NM	291	627	948	40	+3.4	5/5
	control	511	1000	2086	2483	100	+4.4	5/5
26	100	NM	dead					0/5
	50	NM	NM	NM	NM	<8	+2.9	5/5
	25	NM	NM	NM	NM	<8	+2.7	5/5
	12.5	NM	NM	216	395	16	+3.0	5/5
	6.25	NM	256	385	851	36	+3.5	5/5
	3.125	216	498	858	1173	48	+3.6	5/5
	control	511	1000	2086	2483	100	+4.4	5/5
27	50	dead						0/5
	25	dead						0/5
	12.5	NM	NM	NM	dead			0/5
	6.25	NM	NM	NM	324	9	+0.9	0/5
	3.125	NM	NM	NM	371	10	+1.0	5/5
	control	365	1013	1798	3442	100	+3.8	5/5
29	50	NM	NM	NM	384	15	+0.2	4/5
	25	NM	216	405	1000	37	+4.2	5/5
	12.5	NM	409	835	1904	70	+4.1	5/5
	6.25	246	676	1128	2343	85	+5.9	5/5
	3.125	276	661	1486	2557	96	+6.4	5/5

Table III (Continued)

compd	treatment, mg/kg/day	tumor volumes, mm^3; days after tumor implantation 12	16	20	24	T/C, %[b]	weight change[c]	survivors[d] (test/control)
	control	283	765	1436	2656	100	+6.4	5/5
30	50	NM	dead					0/5
	25	NM	NM	NM	NM	<8	+0.8	4/5
	12.5	NM	NM	NM	316	12	+1.1	5/5
	6.25	NM	NM	492	854	31	+1.6	5/5
	3.125	NM	NM	698	1375	50	+2.3	5/5
	control	265	1257	1868	2742	100	+4.1	5/5

[a] 10^6 cells implanted im into the left leg of BDF_1 female mice (groups of five) on day 0. Drug as a solution in 10–20% DMSO in arachis oil was administered ip daily on days 1–17. [b] Antitumor activity expressed as a ratio of tumor volumes of test (T) animals compared to control (C) animals × 100. [c] Mean of weight gain from day 0 to day 24. [d] On day 24. [e] Tumor not measurable. [f] Maximal activity for this system.

mm) as adsorbent and either toluene–acetone–ethanol (7:3:5) or chloroform–methanol (8:2) as solvent.

2,4-Diamino-5-(3-nitroaryl)-6-ethylpyrimidines 6–8. Method A. A suspension of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (4, 10.0 g, 34 mmol) was boiled in 40% aqueous methylamine (200 mL), 40% aqueous dimethylamine (200 mL), or 70% aqueous ethylamine (200 mL) for 48 h with the further addition of amine solution after 24 and 36 h. After cooling, the orange solutions were diluted with water and the appropriate nitroamines (**6–8**) were collected and purified by crystallization from aqueous dimethylformamide (see Table I).

2,4-Diamino-5-(3-nitroaryl)-6-ethylpyrimidines 9–34. Method B. A suspension of 4 (10.0 g, 34 mmol) in the appropriate pure amine (30 mL) was heated under reflux for 4–6 h. The cooled red solutions were diluted with water (for water-miscible amines) or ether (for water-immiscible amines) to liberate the required compounds (**9–37**) (Table I).

2,4-Diamino-5-[4-(dimethylamino)-3-nitrophenyl]-6-ethylpyrimidine (7). Method C. A solution of 4 (2.0 g, 6.8 mmol) in dimethylformamide (10 mL) containing 2-aminoethanol (0.84 g, 13.8 mmol) was stirred at 90 ± 3 °C for 24 h. The red solution was diluted with water and stood at 4 °C for 12 h, whereupon the product crystallized and was collected and recrystallized from aqueous dimethylformamide (Table I).

2,4-Diamino-5-(4-nitroaryl)-6-ethylpyrimidines 35–37. Method D. Treatment of 2,4-diamino-5-(3-chloro-4-nitrophenyl)-6-ethylpyrimidine[2] (**5**, 2.0 g, 6.8 mmol) as for method B above furnished the required isomeric nitroamines (**35–37**) (Table I).

2,4-Diamino-5-(3-aminoaryl)-6-ethylpyrimidines 38–55. Method E. Tin(II) chloride dihydrate (5 mol equiv) was added in small portions (15 min) to a stirred suspension of the appropriate nitro precursor (1.0 g) in ethanol (20 mL) at 50 °C. The coloration was rapidly discharged to form a yellow solution which was heated under reflux for 3 h, cooled, and evaporated under reduced pressure to afford a syrupy residue. The syrup was redissolved in hot water, cooled, and basified to pH 12 with 10 M aqueous sodium hydroxide solution, when the required amine precipitated and was collected, washed with water, and dried (Table I).

2,4-Diamino-5-(3-azidoaryl)-6-ethylpyrimidines 56–60. Method F. A stirred solution of the appropriate amine precursor (2.5 g) in 5 M hydrochloric acid (30 mL) was diazotized at 0 °C by the addition of sodium nitrite (1.1 mol equiv) as a solution in water (3 mL) over 30 min. To the diazonium solution was added sodium azide (4 mol equiv) cautiously in portions over 15 min, and the mixture was stirred at 0 °C for 2 h. After dilution with water (200 mL) the mixture was basified to pH 9 with concentrated aqueous ammonia to afford the appropriate azide as an off-white precipitate (Table I).

2,4-Diamino-6-ethyl-5-(4-piperidinophenyl)pyrimidine (62). Method G. A suspension of **57** (1.0 g, 3 mmol) in hydrazine hydrate (5 mL) was warmed until the vigorous effervescence subsided and then heated under reflux for 30 min. After the addition of ethanol (3 mL) to assist dissolution of sublimed solids the mixture was boiled for a further 15 min, cooled, and diluted with water (25 mL), whereupon the required pyrimidine crystallized from solution and was collected (0.75 g, 85%). Recrystallization from ethanol furnished yellow needles.

Similarly prepared from the azides **58** and **59**, respectively, were **2,4-diamino-6-ethyl-5-(4-piperazin-1-ylphenyl)pyrimidine (63)** and **2,4-diamino-6-ethyl-5-[4-(4-methylpiperazin-1-yl)-phenyl]pyrimidine (64)** (Table I).

X-ray Crystal Structure Determination. The ethanesulfonate salt of compound **29** yielded prismatic crystals from 2-propanol/water. Crystals exhibit triclinic symmetry with space group $P\bar{1}$. Unit cell dimensions were refined by least squares from setting angles of 25 reflections obtained with MoKα radiation (λ = 0.71069 Å) on an Enraf–Nonius CAD4 diffractometer equipped with a graphite monochromator: a = 11.475 (2), b = 12.048 (2), c = 13.030 (1) Å, α = 74.79 (1), β = 64.53 (1), γ = 62.53 (2)°, Z = 2. Intensity data were collected by $\omega - 2\theta$ scans for $2 < \theta < 25°$. Because the samples deteriorated during the experiment, two crystals were used for data collection. Each data set was separately corrected by a linear function of exposure time on the basis of the intensity of three monitor reflections and was assigned a separate scale factor in subsequent refinements. The ion-pair structure was solved by direct methods (MULTAN)[21] and refined by the full-matrix least-squares technique (SHELX).[22] Persistent peaks on difference electron density maps indicated that ethyl carbon C(10) and ethanesulfonate oxygen atoms O(30) and O(31) were disordered. The alternative positions were assigned refinable occupancy factors n and $1 - n$, which converged to 0.78:0.22 for C(10) and 0.73:0.27 for the O atoms. Many hydrogen atoms appeared in difference Fourier syntheses, but in view of the limited accuracy expected, they were placed in calculated positions. However, those in NH^+ and NH_2 groups were freely refined, and methyl groups were refined as rotatable rigid bodies. Additional electron density matched the geometry expected for a molecule of 2-propanol, in which the oxygen atom was distinguishable by its high density and by possible hydrogen bonding to O(31) at a distance of 3.05 (2) Å. Restraints on the CH_3–CH, CH–OH, and $CH_3 \cdots CH_3$ distances facilitated successful refinement. No attempt was made to introduce hydrogen atoms into this molecule. The final refinement included positions and anisotropic thermal parameters for all non-hydrogen atoms and common isotropic temperature factors for chemically similar hydrogen atoms, with weights $w = 1/[\sigma^2(F) + 0.000672F^2]$. Discrepancy indices were R = 0.079, R_w = 0.102 for 3378 unique observed reflections ($F > 3\sigma$). No peak on a final difference electron density map exceeded 0.4 e $Å^{-3}$ except for two of ca. 0.42 e $Å^{-3}$ near S(29) and O(32). These peaks, taken together with the appreciable anisotropy of thermal parameters, suggest that the disorder of the anion may be rotational with C(33) almost stationary, modest effects on S(29) and O(32), and gross changes in O(30) and O(31). Final non-hydrogen fractional coordinates and average U values have been deposited as supplementary material.

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Supplementary Material Available: Table giving final fractional coordinates and average temperature factors for the non-hydrogen atoms of **29** (2 pages). Ordering information is given on any current masthead page.

Pyrido[3,4-*e*]-1,2,4-triazines and Related Heterocycles as Potential Antifungal Agents[1]

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The preparation and biological activities of a series of pyrido[3,4-*e*]-1,2,4-triazines, 1,2,4-triazino[5,6-*c*]quinolines, and related fused triazines are described. Methyl, amino, and acylamino substituents were placed in the pyridyl ring of the former system. Other structural modifications included various alkyl, cycloalkyl, substituted phenyl, and heterocyclic groups in the 3-position of these ring systems. In agar dilution assays, actives in this series inhibited strains of *Candida*, *Aspergillus*, *Mucor*, and *Trychophyton* species at MIC's of ≤16 μg/mL.

The incidence of diseases caused by fungi pathogenic to man has increased significantly over the past 30 years.[2] Superficial infections caused by dermatophytes and *Candida* species may be extremely uncomfortable or disfiguring but are rarely life threatening. Systemic infections are more severe and can often be fatal, due to the involvement of internal organs and the bloodstream. The deep mycoses (blastomycosis, coccidioidomycosis, histoplasmosis) can affect normal individuals, while opportunistic infections (aspergillosis, candidiasis, cryptococcosis) require predisposing factors in the host.[3] These contributing factors include drug treatment (antibiotics, steroids, immunosuppressives, antineoplastics), invasive surgery and associated procedures (parenteral nutrition, indwelling catheters), and various diseases (cancer, AIDS, diabetes). As these have become more prevalent in recent years, so has the incidence of opportunistic mycoses.[4]

In contrast to antibacterial chemotherapy, there are few agents effective against the more serious types of fungal diseases.[5] Although it has severe side effects, amphotericin B is the agent of choice, and sometimes the only effective one, for both deep and opportunistic infections. The imidazoles, miconazole and ketoconazole, are used for both superficial and systemic mycoses, but they also have their limitations as to efficacy and toxicity. There is thus a need for new drugs effective against a variety of fungi, but having low toxicity. The search for such agents has been difficult due to both host and pathogen being eucaryotic organisms with similar metabolism and the lack of detailed biochemical information about the infecting organism.

This paper describes the preparation and biological evaluation of a series of pyrido-1,2,4-triazines and related compounds. Previous efforts in this area have been reported from our laboratory,[6] as well as others.[7-9] In these cases, detailed antifungal data were generally lacking. We have expanded upon the earlier chemical work and also present more extensive in vitro testing results. This has enabled us to draw conclusions about the structure–activity relationships in this class of compounds.

Results and Discussion

Chemical Results. In general, the preparation of 3-substituted pyrido[3,4-*e*]-1,2,4-triazines (**10**) followed previously reported methods[6,7] (Figure 1). These compounds are listed in Table I. Intermediates **7f**, **7g**, **7j**, **7l**, and **7o** could be isolated in pure form by filtration of the reaction mixture, washing with THF and Et_2O, and drying the insoluble product. In all other cases, final products in acceptable yield and purity were obtained without purification of intermediates. Crude **10** was filtered through Magnesol and then recrystallized from an appropriate solvent to obtain analytically pure material.

4-Hydroxypyridine (**1**) was nitrated in a refluxing mixture of red fuming HNO_3 (d = 1.6, Baker) and fuming H_2SO_4 (18–24% SO_3) to give **2**.[10] This was then chlorinated to give **3**.[11] The substituted hydrazide **7** could be

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