Quinazoline Antifolates Inhibiting Thymidylate Synthase: Variation of the N^{10} Substituent

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The synthesis of 12 new 5,8-dideazafolates with isopropyl, cyclopropylmethyl, 2-fluoroethyl, carbamoylmethyl, phenacyl, 3-fluorobenzyl, 5-uracilylmethyl, carboxymethyl, 2-carboxyethyl, 3-cyanopropyl, 3-hydroxypropyl, and cyanomethyl substituents at N^{10} is described. In general, the synthetic route involved monoalkylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate, coupling of the resulting secondary amine with 2-amino-6-(bromomethyl)-4-hydroxyquinazoline hydrobromide in *N,N*-dimethylacetamide with calcium carbonate as the base, and deprotection using mild alkali. The cyanomethyl derivative was found to be unexpectedly base labile and was therefore prepared by mild acid deprotection of a di-*tert*-butyl ester. The compounds were tested as inhibitors of purified L1210 thymidylate synthase (TS). Four members of the series were more potent that the N^{10}-hydrogen compound, but none was superior to the previously described N^{10}-propargyl-5,8-dideazafolic acid. Selected compounds were examined as inhibitors of purified L1210 dihydrofolate reductase (DHFR). As desired, N^{10} substitution in general reduced DHFR inhibitory activity; these results are discussed. As a measure of cytotoxicity, the compounds were examined for their inhibition of the growth of L1210 cells in culture. None of the new substituents conferred enhanced potency relative to N^{10}-propargyl-5,8-dideazafolic acid (ID_{50} = 5 μM), which, as the best TS inhibitor and a relatively poor DHFR inhibitor, continues to lead this series.

In seeking an antifolate radically different from methotrexate (MTX), it is necessary to move away from the locus of action of that drug, dihydrofolate reductase (EC 1.5.1.4, DHFR), and to choose as a target some other enzyme in the folate pathway. De novo production of thymidylate is directed solely toward DNA synthesis. The enzyme that mediates it, thymidylate synthase (EC 2.1.1.45, TS), is thus an appropriate focus for a novel antifolate since the biosynthesis of purines, RNA, and protein that are involved in general metabolism would be spared. This specificity of action might preclude some of the side effects seen with DHFR inhibitors such as MTX.[1] Inhibition of TS leading to "thymineless death" as a principle in cancer chemotherapy was put forward over a decade ago.[2-4] This principle promotes selective S-phase cytotoxicity as an efficient way to kill cells. However, selectivity of action toward cancer cells cannot be anticipated since the ability of cells to salvage exogenous thymidine will oppose the effect of a TS inhibitor, and the balance of these two effects in normal and in tumorous tissues is at present largely unknown.[5] The value of thymineless death therefore needed empirical test by putting a specific TS inhibitor to use. 5-Fluorouracil and its derivatives (active as TS inhibitors when metabolized to 5-FdUMP) cannot satisfactorily fulfill this role since their incorporations into RNA lead to additional or alternative cytotoxic events.[6-8]

Thymidylate synthase binds deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate as cosubstrates, thus offering a choice of model from which to develop an inhibitor. We considered the folate cosubstrate to be the better model for the following reasons: (i) Possible polyglutamation excepted, an antifolate would not require metabolic activation (which is the case for pyrimidine analogues and its deletion is a frequent cause of drug resistance in experimental systems[9]). (ii) There are no specific enzymes for folate catabolism in contrast to pyrimidines. (iii) Folate analogues are unlikely to be incorporated into nucleic acids, with associated side effects.[10] (iv) The natural folate cosubstrate for TS lies at a metabolic branch point and is a substrate for three additional enzymes—5,10-methylenetetrahydrofolate reductase, 5,10-methylenetetrahydrofolate dehydrogenase, and serine hydroxymethyltransferase. In the presence of a selective folate-based inhibitor of TS, which is not itself a substrate for these other enzymes, the potential accumulation of the displaced cosubstrate should be limited. The folate cosubstrate is, in any even, present in low concentration.[11,12] These considerations do not apply to a pyrimidine-based TS inhibitor where the accumulated substrate, dUMP, can compete with the inhibitor.[6,13-15]

We recently synthesized N^{10}-propargyl-5,8-dideazafolic acid (**1a**)[16] and showed that it was the most potent antifolate inhibitor of TS yet described (K_i=4.5 nM).[17-19] The

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(16) Synonyms: CB3717; ICI 155,387; NSC 327182; *N*-[4-[*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]-benzoyl]-L-glutamic acid.

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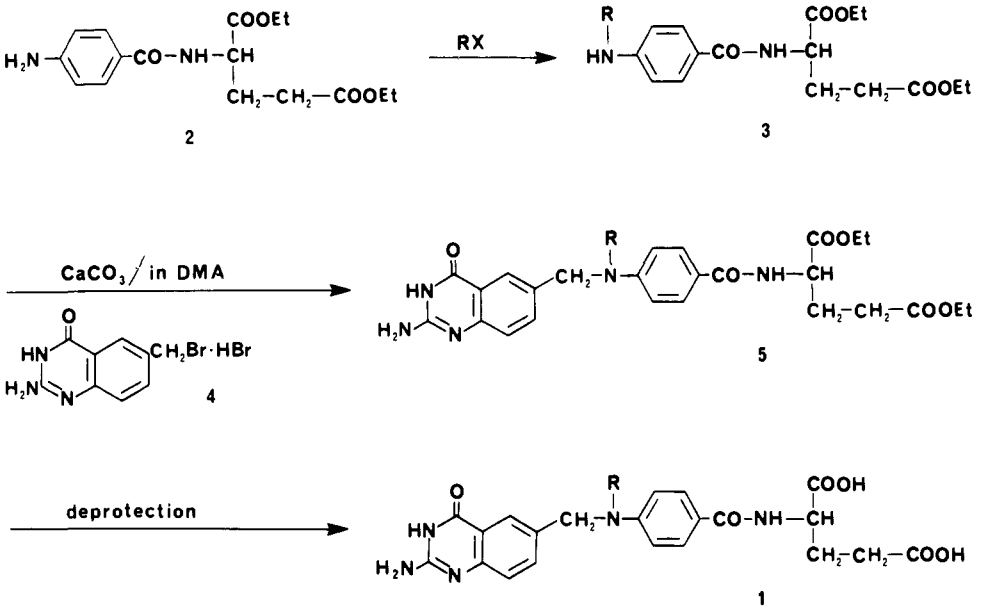
Scheme I



	R			
a	$-CH_2-C\equiv CH$	i	$-CH_2-COOH$	1 only
b	$-CH(CH_3)_2$	j	$-CH_2-CH_2-COOH$	
c	$-CH_2-CH(CH_2)_2$	k	$-CH_2-CH_2-CH_2-CN$	1 and 5 only
d	$-CH_2-CH_2-F$	l	$-CH_2-CH_2-CH_2-OH$	1 only
e	$-CH_2-CONH_2$	m	$-CH_2-CN$	
f	$-CH_2-CO-C_6H_5$	n	$-CH_2-COOEt$	3 and 5 only
g	$-CH_2-C_6H_4F$	o	$-CH_2-CH_2-COOEt$	3 and 5 only
h	$-CH_2-$ uracil	p	$-CH_2-CH_2-CH_2-Br$	3 and 5 only
		q	$-CH_2-CH_2-CH_2-O-COCH_3$	3 only
		r	$-CH_2-CH_3$	
		s	$-H$	

antitumor activity of this quinazoline in vitro and in vivo appears to result from its inhibition of TS alone, with no complicating action at any other locus.[17,19-21] The series that led to CB 3717 was short and encompassed a few N^{10}-alkyl analogues[22-24] and the N^{10}-allyl analogue.[17] The N^{10} substituent is an important determinant of TS inhibition, the parent compound (N^{10}-hydrogen) being a relatively weak inhibitor.[24] Further N^{10} variants are described below.

Chemistry

Twelve new N^{10} analogues (**1b–m**) were synthesized, 10 (**1b–k**) as in Scheme I, one (**1l**) as in Scheme II, and the last (**1m**) as in Scheme III. In Schemes I and II the starting material, diethyl *N*-(4-aminobenzoyl)-L-glutamate (**2**), was N-alkylated to give the seven secondary amines **3b–h** containing the N^{10}-substituent R as desired, the two amines **3n** and **3o** with the N^{10}-substituent in protected form, and the 3-bromopropylamine **3p** with a substituent later to be modified. The amines **3b–h,p** upon further alkylation with 2-amino-6-(bromomethyl)-4-hydroxyquinazoline hydrobromide (**4**)[24] gave the antifolate diesters

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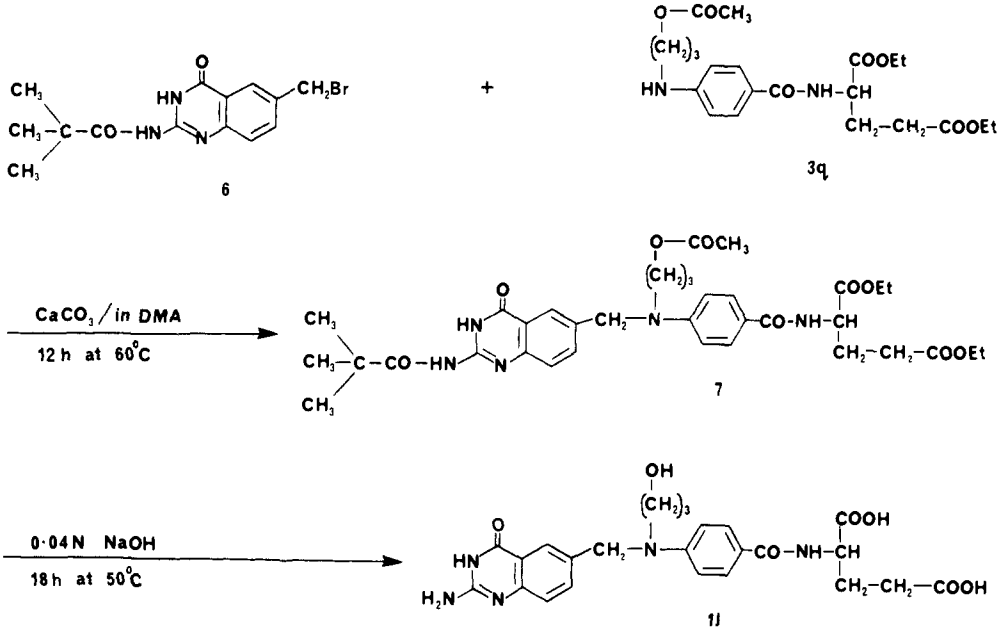
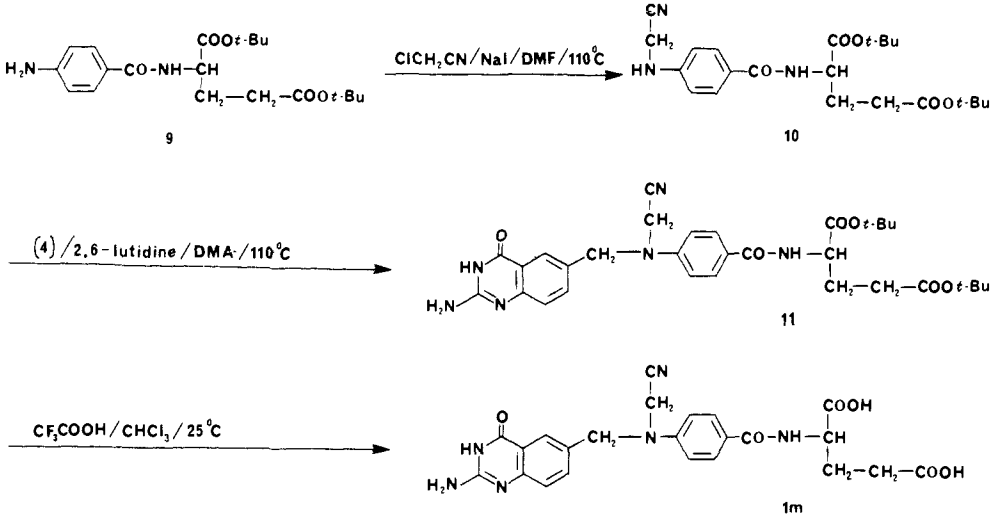
Scheme II



Scheme III



5b–h,p; the amines **3n** and **3o** similarly gave the triesters **5n** and **5o**. The 3-bromopropyl diester **5p** was transformed into the 3-cyanopropyl diester **5k** by treatment with NaCN in Me_2SO. As a variation, the 3-acetoxypropylamine **3q** was alkylated with the pivaloyl derivative **6**[25] to yield the tetraprotected derivative **7** (Scheme II). Deprotection of the products **5b-h,k,n,o** in the last step was accomplished with cold aqueous alkali. The pivalamide **7** required warm alkali. The conversion of **5m** into **1m** could not be achieved because of the unusual lability of the nitrile group in base; **1m** was therefore prepared by other means, as shown in Scheme III.

Alkylation of **2** was generally achieved by heating with the appropriate reagent in a suitable solvent with a base. The reaction was stopped at the optimal time (TLC) and the desired product freed from residual starting amine and byproducts either by chromatography or by extraction with 1 N H_2SO_4 followed by crystallization. In the cyclopropylmethylation of **2** the dialkylated byproduct **8** was isolated. The cyanomethylamine **3m** was prepared from **2** by the reported reaction[26] of weakly basic aromatic amines with formaldehyde and potassium cyanide or preferably by NaI-catalyzed alkylation with chloroacetonitrile. Identical products were obtained, melting sharply at 99.5–100 °C. This intermediate has been reported[27] to melt at 135 °C. The fluoroethylamine **3d** was prepared in low yield from 2-fluoroethyl *p*-toluenesulfonate with $CaCO_3$ as base. Attempts using K_2CO_3 were unsuccessful: the reagent was consumed and the amine left unchanged, suggesting that elimination to vinyl fluoride was taking place.[28]

Details of the second alkylation leadiing to the derivatives **5** (**5h** and **5k** expected) are collected in Table I. We preferred to use the (bromomethyl)quinazoline as the hydrobromide salt **4**[24] made by a variation of the published procedure[25] to the free base. The protonated species **4** is more reactive than the free base, which normally required heating[25] as did the neutral pivalamide **6** in reaction with the acetoxypropylamine **3q**; the proton also precludes self-condensation of the quinazoline. $CaCO_3$, the base of

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Table I. Preparation of Antifolate Diesters 5

compd	quantity of reactant, mmol: amine (3)	quinazoline (4)	$CaCO_3$	DMA, mL	reactn time, h	reactn temp, °C	chromatography[a]	yield, %	mp, °C	formula	anal.
5b	2.5[b]	2.5	10	20	93	25	5% EC	33	120–135	$C_{28}H_{35}N_5O_6$	C, H, N
5c	7.9	8.7	31.6	40	96	25	4% EC	53	107–120	$C_{29}H_{35}N_5O_6$	C, H, N
5d	1	1.2	4	8	45	25	5% EC	46	115–120	$C_{27}H_{32}FN_5O_6$	C, H, N; F[c]
5e	1	1	1	5	60	25	15% MM	30	160 dec	$C_{27}H_{32}N_6O_7 \cdot 0.5HBr \cdot H_2O$	C, H, N
5f	9.3	9.3	9.3[d]	50	168	25	6% MM	33	148–150	$C_{33}H_{35}N_5O_7 \cdot 0.5H_2O$	C, H, N
5g	3	3	9	20	115	25	5% EC	46	113–117	$C_{32}H_{34}FN_5O_6$	C, H, N; F[e]
5m	5	6	20	20	1.5	110[f]	7% EC[g]	33	160–165	$C_{27}H_{30}N_6O_6$	C, H, N
5n	5	5	5	50	60	25	10% MM	25	96–100	$C_{29}H_{35}N_5O_8 \cdot 0.6H_2O$	C, H, N
5o	5	5	5.5	20	60	25	5% MM	69	85–89	$C_{30}H_{37}N_5O_8 \cdot 0.5H_2O$	C, H, N
5p	11.3	11.3	13.5	25	66	25	*h*	65	110 dec	$C_{28}H_{34}BrN_5O_6$	C, H, N, Br

[a] % EC = % EtOH in $CHCl_3$ with Merck Art 15111 or Art 11695 silica; % MM = % MeOH in CH_2Cl_2 with Merck Art 7734 silica. [b] Containing 0.85 mol of DMF. [c] F: calcd, 3.51; found, 2.98. [d] Use of 2,6-lutidine gave an identical product. [e] F: calcd, 3.15; found, 2.55. [f] 25 °C/2.5 h gave no sign of reaction (TLC) nor did 80 °C/0.5 h. [g] This chromatography gave only 21% pure material; a second chromatography of the contaminated fractions with stepwise elution using 3.5, 5, and 7% EC yielded a further 12%. [h] Merck Art 7734 silica, which was eluted first with CH_2Cl_2–EtOAc (19:1) to remove starting amine then with CH_2Cl_2–EtOAc (8:1) to remove the product.

Table II. Preparation of Antifolate Diacids 1

compd	starting matl 5, mmol	NaOH-(aq), mol equiv	reactn time, h	drying temp, °C	yield, %	mp, °C	formula	anal.	HPLC capacity factor (k')[a]
1b	0.68	5	2	35	78	205–212 dec	$C_{24}H_{27}N_5O_6$	C, H, N	6.0
1c	3.43	5	14.5	80	67	resinifies 204	$C_{25}H_{27}N_5O_6$	C, H, N	9.1
1d	0.38	5	2	70	60	245 dec	$C_{23}H_{24}FN_5O_6$	C, H, N; F[b]	1.7
1e	0.16[c]	3	18	freeze-dried	100	225–235	$C_{23}H_{24}N_6O_7 \cdot 1.25H_2O$	C, H, N	0.1
1f	0.39	3	18	freeze-dried	84	170 dec	$C_{29}H_{27}N_5O_7 \cdot 2.75H_2O$	C, N; H[d]	6.0
1g	1.13	5	2	50	79	205–215	$C_{28}H_{26}FN_5O_6$	C, H, N, F	20.6
1h	0.39[e]	8	6	40	76	245–260 dec	$C_{26}H_{25}N_7O_8 \cdot 1.5H_2O$[f]	C, H, N	0.4
1i	2.54	4	18	freeze-dried	90	200 dec	$C_{23}H_{23}N_5O_8 \cdot 1.5H_2O$	C, H, N	0.5
1j	0.49	5	18	freeze-dried	94	194–199	$C_{24}H_{25}N_5O_8 \cdot 0.75H_2O$	C, H, N	0.8
1k	0.50	3	18	freeze-dried	72	197–203	$C_{25}H_{26}N_6O_6 \cdot 1.4H_2O$[g]	C, H, N	1.1
1l	0.74[h]	5[i]	18	freeze-dried	31	220 dec	$C_{24}H_{27}N_5O_7 \cdot 1.2H_2O$	C, H, N	0.8

[a] The propargyl compound (1a) had $k' = 2.3$; the cyanomethyl compound (1m) had $k' = 0.54$. [b] F: calcd, 3.91; found, 3.32, 3.37. [c] Starting material was the diester H_2O–0.5 HBr. [d] H: calcd, 5.4; found, 4.7. [e] Starting material was the diester 1.75 H_2O–0.5 HBr. [f] Following equilibration with the atmosphere for 24 h, this product analyzed (C, H, N) with 2.5 H_2O. [g] The presence of the nitrile group was confirmed by IR spectroscopy (Nujol mull), which showed a band at 2255 cm^{-1}. [h] Starting material was the pivalamide 7. [i] 18.5 mL of 0.2 N NaOH in EtOH (75 mL) at 50 °C.

choice, scavenges the HBr produced in the reaction but only slowly neutralizes the salt. Thus, the hydrobromide 4 was first converted in good yield to the hydrobromide of the product 5, which was further converted to the free base. In some instances the product could be isolated as a mixture of the base and HBr salt (**5e** and **5h**). This could be avoided by using 2,6-lutidine instead of $CaCO_3$. Dimethylacetamide was the preferred aprotic solvent for the reaction, principally for its ability to dissolve the relatively insoluble salt 4 at room temperature. The N^{10}-cyanomethyl diester **5m** was better prepared as shown in Scheme I, but it could also be prepared by treating the N^{10}-unsubstituted diester **5s**[25] with formaldehyde and KCN[26]. The products obtained by these two routes were identical by NMR spectroscopy. Almost all the diesters 5 were purified by column chromatography on silica. Exceptions were the cyanopropyl derivative **5k**, for which chromatography was unnecessary, and the highly polar 5-uracilylmethyl derivative **5h**, which was recrystallized. The structure and purity of these derivatives were established by microanalysis (Table I) and NMR spectroscopy (Table I, supplementary material).

The diacids **1b–l** were prepared by alkaline hydrolysis of the esters **5b–h,k,n,o** and of the pivalamide 7 under the conditions specified in Table II. The gelatinous products were isolated by centrifugation. The structure and purity of each diacid 1 were established by elemental microanalysis (Table II), NMR spectroscopy (Table II, supplementary material), UV spectroscopy (Table III, supplementary material), and analytical HPLC (Table II). The appearance of the NMR signals is not specified but in all cases was as for those shown in Figure 1 (supplementary material), which illustrates the 400-MHz spectrum of **1a**, typical of this series. As expected, the greatest variation in chemical shift with N^{10} substituent occurred in the signals of the C^9 methylene and the 3′,5′-protons of the benzoyl ring. An attempt to prepare the N^{10}-cyanomethyl diacid **1m** from its diethyl ester **5m** using 3 equiv of sodium hydroxide (0.17 N) in aqueous ethanol at 25 °C for 1.25 h gave a mixture of the desired product, in 80% yield by HPLC analysis, and a second product (12%) corresponding in retention time to the known carboxamide **1e**. Several subsequent attempts to achieve selective alkaline hydrolysis of the ester groups in **5m** also failed. For the successful preparation of **1m** we abandoned alkaline hydrolysis of the diethyl ester in favor of mild acid deprotection of the di-*tert*-butyl ester 11 (Scheme III).

The known amino di-*tertbutyl* ester **9**[29] was alkylated with chloroacetonitrile with NaI catalysis to provide the

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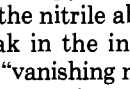
cyanomethylamine **10**. Coupling of this product to the quinazoline moiety using $CaCO_3$ as the base in a similar manner to the preparation of the diethyl ester **5m** gave product contaminated with bromide ion. When 2,6-lutidine was used as the base, this problem was not encountered and the di-*tertbutyl* ester **11** was obtained pure, albeit in low yield. In a small-scale experiment monitored by HPLC the diester **11** in trifluoracetic acid (TFA) solution (10% w/v) had completely reacted after 5 min, giving 92% of the nitrile **1m** accompanied by 3% of the carboxamide **1e**; after 30 min these percentages were 75 and 17%, respectively. Thus, the nitrile group in the diacid **1m** is unstable to both acid and base. Optimal reaction conditions used a mixture of $CHCl_3$ and TFA (2:1), which gave a modest yield of **1m** (96% pure) uncontaminated by the carboxamide **1e**. The structural assignment of **1m** was established by elemental analysis and NMR spectroscopy. Absorption IR spectroscopy was not diagnostic: the nitrile absorption was absent in compound **1m** and weak in the intermediates leading to it. Other examples of "vanishing nitriles" in the literature all contain dipoles opposing that of the nitrile. Our examples may also contain such dipoles, set up by the electron-deficient N^{10} nitrogen atom; this could explain the unexpected hydrolytic activity observed. Against expectation, Raman spectroscopy of **1m** failed to define a nitrile absorption, and this result was attributed to interference by strong hydrogen bonding within the solid. In the NMR spectrum (Table II, supplementary material), the chemical shift of the methylene group of the N^{10} substituent was δ 4.71—a value indentical with that observed in the nitrile diesters **5m** and **11** and distinct from that (δ 4.1) seen in the carboxamides **1e** and **5e**.

While this work was in progress, another research group reported[27] the isolation of **1m** following alkaline hydrolysis of **5m**. However, the microanalytical data that they fit to a monohydrate of **1m** fits the carboxamide **1e** equally. Their choice of TFA as the NMR solvent would probably have prevented observation of the amidic NH_2 group. Furthermore, solutions of the diacid **1m** in TFA are unstable; hydrolysis produces the carboxamide **1e** (see above). Using their reported conditions, we repeated the experiment and showed by HPLC that the product in fact consisted of the carboxamide **1e** (87%) and nitrile **1m** (12%). The NMR spectrum at 250 MHz in Me_2SO-d_6 showed the signals of the carboxamide **1e** described in Table II (supplementary material) matched by chemical shift, appearance, and couplings. The resonance of the CH_2CONH_2 methylene protons was seen at δ 4.07. The amidic NH_2 group could not be clearly seen. Integration of the quinazoline signals at δ 7.15 and 7.50 (H^7 and H^8) gave an area twofold greater than expected, but this discrepancy was resolved when it was found that the addition of D_2O reduced the area of these signals to the theoretical value. The excess area in the initial spectrum was ascribed to the NH_2 protons. The presence of the cyanomethyl compound **1m** in the preparation (12% by HPLC) was confirmed at about this level by the NMR spectrum. The 3′,5′ and 2′,6′ doublets in **1m** and **1e** differ sufficiently in chemical shift to allow detection of the two doublets (at δ 6.95 and 7.78) characteristic of the nitrile **1m**. The experiment of ref 27 in our hands thus gave predominantly the carboxamide **1e** containing the nitrile **1m** only as an impurity.

Biological Evaluation

The diacids **1b–m** were tested as inhibitors of TS from L1210 mouse leukemia. Our previous results in this series were obtained with partially purified TS.[24,17] The assay used in this study incorporated purified enzyme and a higher concentration of substrate.[18] **1a** was included in each assay as a positive control. The ratio of IC_{50}'s (defined as an inverse relative potency) could then be compared. The compounds were also tested for their inhibition of the growth of L1210 cells in culture. The more potent cytotoxic agents were further tested as inhibitors of DHFR. These results are collected in Table III. The stability of the cyanomethyl compound **1m** during its preparation for the TS assay and its stability during the tissue culture assay were both confirmed by HPLC analysis.

Table III. Biological Properties of N^{10}-Substituted 5,8-Dideazafolates

compd	N^{10} substituent	inverse rel potency[a] thymidylate synthase	inverse rel potency[a] dihydrofolate reductase	ID_{50} for L1210 cells in culture, μM
1a	$-CH_2C{\equiv}CH$	1[b]	1.00[c]	5
1d	$-CH_2CH_2F$	3[d]	0.04	16
1l	$-(CH_2)_3OH$	9	0.80	54
1m	$-CH_2CN$	13	1.39	5
1b	$-CH(CH_3)_2$	17		400
1e	$-CH_2CONH_2$	99	0.57	87
1i	$-CH_2COOH$	100	0.30	27
1s	$-H$	120	0.04	5
1k	$-(CH_2)_3CN$	$\simeq$350		>300[e]
1j	$-(CH_2)_2COOH$	1222		>270
1g	$-CH_2C_6H_4F$-*m*	1736	2.05	>200
1h	$-CH_2$(5-uracilyl)	$\simeq$7000		5[f]
1f	$-CH_2COC_6H_5$	>>69	0.02	100
1c		>>86		>100[g]

[a] Defined as IC_{50} (compound)/IC_{50} (1a) determined in the same test. [b] IC_{50} = (2.02 ± 1.20) × 10^{-8} M; range (0.72–4.00) × 10^{-8} M; K_i = 4.5 × 10^{-9} M.[18,19] [c] IC_{50} = (6.60 ± 1.10) × 10^{-6} M; range (5.40–8.00) × 10^{-6} M; K_i = 1.4 × 10^{-8} M.[17] [d] Compound **1r** had inverse relative potency 3 in this assay. [e] 10% inhibition at this concentration. [f] From HPLC analysis this compound in tissue culture medium is degraded to compound **1s**. [g] No inhibition at this concentration.

Results and Discussion

The values of inverse relative potency for the inhibition of purified L1210 TS and purified L1210 DHFR together with the ID_{50} values for the growth inhibition of L1210 cells in culture are shown in Table III ranked in order of TS inhibition. The values obtained for the N^{10}-propargyl compound **1a** and the N^{10}-hydrogen compound **1s** are also given.

As TS inhibitors, certain N^{10} analogues, viz. 2-fluoroethyl (**1d**), 3-hydroxypropyl (**1l**), cyanomethyl (**1m**), and isopropyl (**1b**), were better than the N^{10}-hydrogen analogue (**1s**), but none was more potent than **1a**. The following observations can be made: (i) Comparison of the N^{10}-ethyl compound (**1r**)[24] with **1d** showed that the fluorine substituent did not alter the inhibition. (ii) Small but highly polar substituents such as carbamoylmethyl (**1e**), carboxymethyl (**1i**), and cyanopropyl (**1k**) have activity comparable to that of the N^{10}-hydrogen compound, but homologation of the substituent in **1i** to give the carboxyethyl compound (**1j**) lessened the activity 10-fold. (iii) Large aralkyl groups, polar or not such as phenacyl (**1f**), *m*-fluorobenzyl (**1g**), and 5-uracilylmethyl (**1h**) severely curtail the inhibition. (iv) The cyclopropylmethyl compound (**1c**) is inferior to the isopropyl compound (**1b**), and this decrease in activity is similar to that found previously for the *n*-propyl and *n*-butyl substituents.[24] (v) The cyanomethyl compound (**1m**), arguably very similar in size and structure to the propargyl compound (**1a**) but differing in polarity

(see k' values in Table II), was about 10-fold less active.

The data shown in Table III confirm that these 2-amino-4-hydroxy compounds are DHFR inhibitors, although poor when compared to 2,4-diamino compounds. Against this enzyme the most potent inhibitors were the unsubstituted compound (**1s**), the phenacyl compound (**1f**), and the fluoroethyl compound (**1d**), which were of comparable activity. Compounds **1d** and **1f** apart, N^{10} substitution was detrimental to DHFR inhibition. Although the N^{10}-hydrogen compound (**1s**) inhibits DHFR, published evidence suggests that it is nonetheless functionally a TS inhibitor. Mathematical modeling of folate metabolism has shown that a compound that inhibits both TS and DHFR becomes rate limiting for DHFR only when the ratio $K_i(TS)/K_i(DHFR)$ is greater than about 3000.[19] This ratio for **1s** is 116,[24] indicating that it should function as a TS inhibitor. Moreover, the cytotoxicity of **1s** in tissue culture is reversed by thymidine.[24] All the compounds in Table III that were better TS inhibitors than **1s** were, where tested, either similar to, or less potent than, **1s** as DHFR inhibitors, indicating that they too were functionally TS inhibitors. It has been suggested that binding of TS inhibitors to DHFR is disadvantageous in the treatment of cell lines that are methotrexate resistant by virtue of DHFR overproduction.[19] Such cells are not cross-resistant to **1a** but are partially cross-resistant to the N^{10}-hydrogen compound **1s**.[24] Thus, we considered the low level of DHFR inhibition to be a desirable feature of the compounds in this series.

In tissue culture no compound affected L1210 cells more than **1s** (N^{10}-hydrogen) or **1a** (N^{10}-propargyl), compounds which were equally cytotoxic. The cyanomethyl compound **1m**, however, had the same ID_{50} (5 μM) as **1a** and **1s**. So too did the uracilylmethyl compound **1h**, but further examination showed that it was transformed in the culture medium to a compound with HPLC retention time identical with that of **1s**. This result most probably accounts for the cytotoxicity seen with **1h**.

Conclusion

The N^{10} substituents in the 5,8-dideazafolic acid series have been varied in both size and chemical type. None of the new compounds improved upon the propargyl analogue (**1a**) as a TS inhibitor nor was any more cytotoxic. **1a** thus remains the best compound in this series. It has recently progressed to clinical trials intended to provide an unequivocal evaluation of the efficacy of "thymineless death" in cancer chemotherapy.

Experimental Section

General Procedures. *N,N*-Dimethylformamide (DMF) was dried over activated (250 °C) 3-Å molecular sieves; *N,N*-dimethylacetamide (DMA) (Aldrich Gold Label grade) was similarly dried. Petrol refers to petroleum ether, bp 60–80 °C. Merck silica 60 (particle size 15–40 μM, Art 15111) and silica 60H (average particle size 40 μM, Art 11695) were used in a Jobin-Yvon Chromatospac Prep 10 preparative HPLC machine, with the eluent passing through a Cecil 212A variable-wavelength ultraviolet monitor. Merck silica 60 (Art 7734) was used in gravity columns. TLC was performed on precoated sheets of silica $60F_{254}$ (Merck Art 5735). The Epstein spray test was performed as prescribed.[30] Melting points were determined on a Kofler block or with a Büchi melting point apparatus and are uncorrected. Electron impact mass spectra were determined with a VG 7070H spectrometer and VG 2235 data system using the direct-insertion method, an ionizing voltage of 70 eV and trap current of 100 μA, and an ion-source temperature of 160 °C. NMR spectra were determined on Perkin-Elmer R12B (60-MHz), Bruker HX90E (90-MHz), Bruker WM 250 (250-MHz), and Bruker WH400 (400-MHz) spectrometers. Field strengths are expressed in units of δ (ppm), and peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplets; br s, broad singlet; br, broad signal; m, multiplet. Infrared absorption spectra were taken on a Perkin-Elmer 1310 spectrometer. The Raman infrared spectrum was taken on a solid sample in a thin-walled glass capillary on a Nicolet MX-3600 spectrometer. Elemental analyses were determined by Butterworth Laboratories, Teddington, Middlesex, and at ICI Pharmaceuticals Division.

Diethyl *N*-[4-(Prop-2-ylamino)benzoyl]-L-glutamate (3b). The amine **2** (6.45 g, 20 mmol) in DMF solution (100 mL) over K_2CO_3 (2.76 g, 20 mmol) was heated with isopropyl bromide (9.4 mL, 100 mmol) for 21 h at 110 °C. The solvent was removed at 70 °C in vacuo and the residue partitioned between $CHCl_3$ and H_2O. The organic phase was separated, washed with H_2O, dried ($MgSO_4$), and evaporated to dryness. The crude oil was chromatographed on silica (Merck, Art 15111), eluting with petrol–chloroform (1:1). Although pure, the product (5.20 g, 61%) had 0.85 mol of DMF associated with it (NMR). Upon standing, it crystallized in microneedles. The analytical sample was obtained by washing the crystals three times with petrol and drying: mp 62–65 °C; NMR ($CDCl_3$, 60 MHz) δ 1.25 (d, 6 H, isopropyl), methine not visible. Anal. ($C_{19}H_{28}N_2O_5$) C, H, N.

Diethyl *N*-[4-[(Cyclopropylmethyl)amino]benzoyl]-L-glutamate (3c) and Diethyl *N*-[4-[Bis(cyclopropylmethyl)amino]benzoyl]-L-glutamate (8). A mixture of **2** (4.21 g), (chloromethyl)cyclopropane (3.55 g, 3 equiv), K_2CO_3 (1.80 g, 1 mol equiv), anhydrous NaI (1.96 g, 1 mol equiv), and DMF (60 mL) was stirred at 100 °C for 25 h under a condenser with exclusion of moisture. The DMF was removed at 60 °C in vacuo and the residue worked up by partition between $CHCl_3$ and H_2O as for **3b**. The crude oil was coated onto silica (Merck) and chromatographed on silica (Merck, Art 15111) with petrol–Et_2O (1:1) as the eluent. The faster eluting tertiary amine **8** (1.66 g, 29%) melted at 62–63 °C: NMR ($CDCl_3$, 60 MHz) δ 0.15–0.7 (m, 8 H, cyclopropyl), 0.85–1.4 (m, 2 H, cyclopropyl), 3.3 (d, 4 H, CH_2). Anal. ($C_{24}H_{34}N_2O_5$) C, H, N. The slower eluting secondary amine **3c** (3.27 g, 66%) melted at 85.5–86 °C: NMR ($CDCl_3$, 60 MHz) δ 0.15–0.75 (m, 4 H, cyclopropyl), 0.8–1.4 (m, 1 H, cyclopropyl), 3.0 (d, 2 H, CH_2). Anal. ($C_{20}H_{28}N_2O_5$) C, H, N.

Diethyl *N*-[4-[(2-Fluoroethyl)amino]benzoyl]-L-glutamate (3d). A mixture of **2** (3.22 g, 10 mmol), 2-fluoroethyl *p*-toluenesulfonate[28] (4.36 g, 20 mmol), $CaCO_3$ (3.00 g, 30 mmol), and DMF (40 mL) was heated at 135 °C for 17 h, with additional reagent (4.36 g) being added at 3 h. The mixture was filtered (Celite) and DMF removed at 70 °C (15 mm). The crude product was worked up by partition between $CHCl_3$ and H_2O as for **3b**. The resulting oil was chromatographed on silica (Merck Art 15111) with petrol–chloroform (1:1) as the eluent. The product (0.42 g, 11%) melted at 105.5–106.5 °C: NMR ($CDCl_3$, 60 MHz) δ 3.55 (d of m, J = 26.5 Hz, 2 H, CH_2CH_2F), 4.64 (d of t, J = 47.5 and 5.0 Hz, 2 H, CH_2CH_2F). Anal. ($C_{18}H_{25}FN_2O_5$) C, H, N; F: calcd, 5.16; found, 4.39, 4.41.

Diethyl *N*-[4-[(Carbamoylmethyl)amino]benzoyl]-L-glutamate (3e). A mixture of **2** (3.22 g, 10 mmol), iodoacetamide (1.85 g, 10 mmol), 2,6-lutidine (1.07 g, 10 mmol), and DMA (20 mL) was stirred at 110 °C for 4 h. The mixture was poured into H_2O, the product was extracted with EtOAc, and the organic phase was washed successively with water, 1 N H_2SO_4, and brine. After drying (Na_2SO_4), the solvent was removed in vacuo and the residue recrystallized from ethanol to give the product: 1.2 g (32%); mp 167–169 °C; NMR (Me_2SO-d_6, 90 MHz) δ 3.68 (d, J = 6 Hz, 2 H, CH_2), 6.38 (t, J = 6 Hz, 1 H, aniline NH), 7.08 and 7.32 (2 br s, 2 H, nonequivalent protons of NH_2 group). Anal. ($C_{18}H_{25}N_3O_6$) C, H; N: calcd, 11.08; found, 10.6.

Diethyl *N*-[4-(Phenacylamino)benzoyl]-L-glutamate (3f). A mixture of **2** (9.67 g, 30 mmol), phenacyl bromide (5.97 g, 30 mmol), and 2,6-lutidine (3.21 g, 30 mmol) in DMA (50 mL) was stirred at room temperature for 16 h. The mixture was poured into water (400 mL) and the precipitated solid collected, dried, and recrystallized from EtOH to give the product: 6.9 g (52%); mp 135–137 °C; NMR (Me_2SO-d_6, 90 MHz) δ 4.77 (d, J = 8 Hz, 2 H, CH_2), 6.42 (t, J = 6 Hz, 1 H, aniline NH), 7.67 (m, 3 H, meta and para aromatic protons), 8.13 (m, 2 H, ortho aromatic protons). Anal. ($C_{24}H_{28}N_2O_6$) C, H, N.

(30) Epstein, J.; Rosenthal, R. W.; Ess, R. J. *Anal. Chem.* **1955**, *27*, 1435.

Diethyl *N*-[4-[(3-Fluorobenzyl)amino]benzoyl]-L-glutamate (3g). A mixture of **2** (3.22 g, 10 mmol), 3-fluorobenzyl bromide (4.3 g, 23 mmol), K_2CO_3 (1.38 g, 10 mmol), and EtOH (60 mL) was heated under reflux for 4.75 h. The mixture was filtered and the filtrate evaporated to dryness. Extractive workup with $CHCl_3$–H_2O gave an oil that was purified by chromatography on silica (Merck Art 15111) with $CHCl_3$–petrol (1:1) as the eluent to give a solid (2.3 g, 53%). Recrystallization from aqueous EtOH gave plates: mp 96–106.5 °C; NMR ($CDCl_3$, 60 MHz) δ 4.4 (s, 2 H, CH_2), 6.9–7.4 (m, 4 H, aromatic protons). Anal. ($C_{23}H_{27}FN_2O_5$) C, H, N, F.

Diethyl *N*-[4-[(5-Uracilylmethyl)amino]benzoyl]-L-glutamate (3h). A mixture of **2** (3.22 g, 10 mmol), 5-(chloromethyl)uracil (1.61 g, 10 mmol), $CaCO_3$ (3.00 g, 30 mmol), and DMA (25 mL) was stirred at 25 °C for 90 h, further reagent (0.76 g, 4.7 mmol) being added at 17 h. The jellylike mixture was filtered (Celite) and the filtrate evaporated to dryness at 65 °C (1 mm). The resulting gum was digested with boiling EtOH (800 mL), and the hot digest was filtered under vacuum through a glass fiber pad to remove inorganic salts. Evaporation of the filtrate gave a residue that was recrystallized from EtOH (50 mL). Crystallization took several days at 4 °C, giving an off-white powder: 1.04 g (23%); mp 195–205 °C; NMR (Me_2SO-d_6, 250 MHz) δ 3.9 (d, 2 H, CH_2), 7.3 (s, 1 H, H^6), 10–15 (br, 2 H, lactam protons). Further recrystallization from ethanol gave an analytical sample. Anal. ($C_{21}H_{26}N_4O_7$) C, H, N. This amine reacted positively (blue color) with the Epstein spray reagent.[30]

Diethyl *N*-[4-[(Cyanomethyl)amino]benzoyl]-L-glutamate (3m). Method A.[26] A mixture of **2** (3.22 g, 10 mmol), paraformaldehyde (0.90 g, 30 mmol), KCN (1.95 g, 30 mmol), anhydrous $ZnCl_2$ (5.00 g, 36 mmol), and glacial AcOH (50 mL) was stirred in a stoppered flask at room temperature for 5 h, at which time TLC showed absence of starting material. The mixture was partitioned between $CHCl_3$ (1 L) and H_2O (1 L). The organic phase was separated, washed twice with water, dried ($MgSO_4$), and evaporated to dryness. The crude product was chromatographed on silica (Merck, Art 11695) with 4% EtOH in Et_2O as the eluent. Concentration of the appropriate fractions gave white needles: 2.43 g (67%); mp 99.5–100 °C; NMR ($CDCl_3$, 60 MHz) identical with that of the product obtained below (method B); *m/e* 361 (M^+), 288 (M – COOEt), 159 ($NCCH_2NHArCO$). Anal. ($C_{18}H_{23}N_3O_5$) C, H, N.

Method B. A mixture of **2** (6.45 g, 20 mmol), chloroacetonitrile (7.55 g, 100 mmol), K_2CO_3 (2.76 g, 20 mmol), anhydrous NaI (3.00 g, 20 mmol), and DMF (70 mL) was stirred at 110 °C for 2.5 h under a condenser with exclusion of moisture. The black mixture was partitioned between $CHCl_3$ and H_2O and worked up as for **3b**, but evaporating finally at 80 °C (0.2 mm) to give the crude product, which was chromatographed on silica (Merck Art 15111) with 1% EtOH in CH_2Cl_2 as the eluent. The appropriate fractions were pooled and evaporated to give a product containing two slight impurities at higher R_f (TLC). By slurrying the impure product in Et_2O (in which **3m** is insoluble) and filtering, pure **3m** was obtained (4.36 g, 60%), mp 99.5–100 °C, alone or when mixed with the product from method A. Recrystallization from hexane–CH_2Cl_2 did not raise the melting point: NMR ($CDCl_3$, 250 MHz) δ 4.12 (d, J = 6.8 Hz, collapsing to a singlet with D_2O, 2 H, cyanomethyl), 4.90 (t, J = 6.8 Hz, removed by D_2O, 1 H, aniline NH). Anal. ($C_{18}H_{23}N_3O_5$) C, H, N.

Diethyl *N*-[4-[[(Ethoxycarbonyl)methyl]amino]benzoyl]-L-glutamate (3n). A solution of **2** (6.45 g, 20 mmol), ethyl bromoacetate (3.34 g, 20 mmol), and 2,6-lutidine (2.14 g, 20 mmol) in DMA (50 mL) was heated at 90 °C for 5 h. Workup as for **3e** gave a brown oil that crystallized on trituration with cyclohexane: 6.7 g (82%); mp 84–85 °C; NMR (Me_2SO-d_6, 90 MHz) δ 1.1–1.5 (c, 9 H, ester methyls), 4.0–4.7 (m, 9 H, three ester methylenes, Glu CH^α, CH_2 of substituent), 6.55 (t, J = 6 Hz, 1 H, aniline NH). Anal. ($C_{20}H_{28}N_2O_7$) C, H, N.

Diethyl *N*-[4-[[2-(Ethoxycarbonyl)ethyl]amino]benzoyl]-L-glutamate (3o). A solution of **2** (6.45 g, 20 mmol) ethyl 3-bromopropionate (3.62 g, 20 mmol), and 2,6-lutidine (2.14 g, 20 mmol) in DMA (25 mL) was heated at 90 °C for 20 h. Workup as for 3e gave a brown oil that was chromatographed on silica (Merck, Art 7734) with EtOAc–petrol (1:1) as the eluent. The solvent was removed from the appropriate fractions and the solid residue recrystallized from toluene: 3.88 g (46%); mp 90–91 °C; NMR (Me_2SO-d_6, 90 MHz) δ 1.0–1.3 (c, 9 H, ester methyls), 2.25–2.7 (m, 4 H, 2 CH_2CO), 3.2–3.4 (m, 2 H, NCH_2), 3.9–4.2 (m, 6 H, ester methylenes), 6.2 (t, J = 5 *Hz*, 1 H, aniline NH). Anal. ($C_{21}H_{30}N_2O_7$) C, H, N.

Diethyl *N*-[4-[(3-Bromopropyl)amino]benzoyl]-L-glutamate (3p). A solution of **2** (32.2 g, 100 mmol), 1,3-dibromopropane (10.1 mL, 100 mmol), and 2,6-lutidine (11.6 mL, 100 mmol) in DMA (250 mL) was heated at 100 °C for 12 h. The mixture was concentrated to half-volume and poured into water (800 mL). The aqueous suspension was extracted twice with EtOAc, and the combined organic layers were washed successively with 2 N H_2SO_4, H_2O, and brine and then concentrated to give an oil (40 g). This oil was chromatographed on silica (Merck, Art 7734), which was eluted first with CH_2Cl_2/EtOAc (4:1) to remove a less polar impurity and then with CH_2Cl_2/EtOAc (2:1) to remove the product. Evaporation of the appropriate fractions gave a colorless solid: 7.3 g (16.5%); mp 83.5–87 °C; NMR ($CDCl_3$, 90 MHz) δ 2.0–2.7 (m, 2 H, $CH_2CH_2CH_2Br$), 3.4 (m, 4 H, $CH_2CH_2CH_2Br$). Anal. ($C_{19}H_{27}BrN_2O_5$) C, H, N, Br.

Diethyl *N*-[4-[(3-Acetoxypropyl)amino]benzoyl]-L-glutamate (3q). The amine **2** (6.45 g, 20 mmol) and 3-acetoxypropyl bromide (4.18 g, 23 mmol) were condensed in DMA (20 mL) for 12 h at 100 °C in the presence of K_2CO_3 (2.76 g, 20 mmol). The mixture was poured into H_2O and extracted with EtOAc, the organic phase was washed with H_2O and dried ($MgSO_4$), and the solvent was removed in vacuo. The resulting oil was chromatographed on silica (Merck 60) with CH_2Cl_2–EtOAc (2:1) as the eluent to provide an oil that crystallized on standing: 4.86 g (51%); mp 59–62 °C; NMR ($CDCl_3$, 60 MHz) δ 2.1 (s, 3 H, CH_3CO), 2.4 (m, 2 H, $CH_2CH_2CH_2OAc$), 3.2 (t, 2 H, $CH_2CH_2CH_2OAc$), 4.1 (m, 2 H, $CH_2CH_2CH_2OAc$). Anal. ($C_{21}H_{30}N_2O_7 \cdot 0.66CH_2Cl_2$) C, H, N.

Preparation of Antifolate Diesters 5b–g,m–p. General Method. A mixture of the amine **3**, 2-amino-6-(bromomethyl)-4-hydroxyquinazoline hydrobromide (**4**),[24] dry $CaCO_3$, and DMA was stirred in a stoppered flask. The stopper was lifted occasionally in the first few hours to release gas pressure. TLC (silica gel, 75:20:5 $CHCl_3$–EtOH–AcOH) showed absence of quinazoline starting material (Epstein test[30]), with usually incomplete consumption of amine and a strong product spot at intermediate R_f. The mixture was filtered through Celite and the filter washed with DMA. The combined filtrates were evaporated to dryness at 55 °C (0.2 mm), first on a rotary evaporator and finally by direct coupling to the oil pump via a cold trap. Complete removal of the solvent is important for the subsequent chromatography. Silica column chromatography allowed complete removal of unreacted **3**, which eluted first. The desired product followed, care being taken to exclude those later fractions that contained an increasingly strong blue-fluorescent impurity at lower R_f. Pooling and evaporation of the appropriate fractions gave the pure diester. The structure was confirmed by elemental analysis (Table I) and NMR spectroscopy (Table I, supplementary material). Full details of each preparation are collected in Table I.

Diethyl *N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]-*N*-(5-uracilylmethyl)amino]benzoyl]-L-glutamate (5h). A mixture of **3h** (1.12 g, 2.5 mmol), **4**[24] (1.00 g, 3.0 mmol), $CaCO_3$ (0.75 g, 7.5 mmol), and DMA (10 mL) was stirred in a stoppered flask at 25 °C for 97 h. The mixture was filtered through Celite with washings, and the combined filtrates were evaporated at 60 °C (0.2 mm) to give a gum (3.49 g). This was dissolved in boiling EtOH (150 mL) and the filtered, yellow solution set aside at 4 °C overnight. Centrifugation at 2000*g* for 30 min and decantation gave a gummy residue that was essentially pure. A repeat crystallization from EtOH (150 mL) and centrifugation gave a gelatinous product that was dried for several days over P_2O_5 in vacuo to constant weight: 0.36 g (21%); mp 185–195 °C dec; NMR (Table I, supplementary material). Anal. ($C_{30}H_{33}N_7O_8 \cdot 0.5HBr \cdot 1.75H_2O$) C, H, N; Br: calcd, 5.78; found, 6.32, 6.23.

Diethyl *N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]-*N*-(3-cyanopropyl)amino]benzoyl]-L-glutamate (5k). A solution of diethyl *N*-[4-[*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]-*N*-(3-bromopropyl)amino]benzoyl]-L-glutamate (**5p**) (0.62 g, 1.01 mmol) and NaCN (0.059 g, 1.2 mmol) in Me_2SO (4 mL) was stirred at room temperature for 4 h. The

reaction mixture was slowly added to rapidly stirred water (50 mL). The precipitate was filtered off, washed with water, and dried to give a crystalline solid: 0.50 g (88%); mp 111 °C dec. Anal. ($C_{29}H_{34}N_6O_6 \cdot 0.66H_2O$) C, H, N.

Diethyl *N*-[4-[*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]-*N*-(cyanomethyl)amino]benzoyl]-L-glutamate (5m). Diethyl 5,8-dideazafolate[25] (2.11 g) was dissolved in glacial AcOH (21 mL) and treated with paraformaldehyde (0.38 g, 3 equiv), KCN (0.83 g, 3 equiv), and anhydrous $ZnCl_2$ (2.12 g, 3.65 equiv). The mixture was stirred at 25 °C for 16.5 h in a stoppered flask and then partitioned between $CHCl_3$ (500 mL) and H_2O (500 mL). The organic phase was removed, washed with H_2O, dried ($MgSO_4$), filtered (G3 sinter under light vacuum), and evaporated at 45 °C to give a pale yellow solid (2.01 g). This crude product (in $CHCl_3$–EtOH, 2:1) was adsorbed onto silica (Merck Art 11695, 4.5 g), which was applied to a column of the same silica (180 g). Elution with 6% EtOH in $CHCl_3$ and evaporation of the best fractions gave a solid (1.25 g) that was not quite TLC pure (SiO_2GF–6% EtOH in $CHCl_3$). A portion (0.093 g) rechromatographed as above gave almost quantitative recovery of pure product: mp 145–160 °C; NMR (Me_2SO-d_6, 60 MHz) identical with that of the product made alternatively according to Table I. Anal. ($C_{27}H_{30}N_6O_6$) C, H, N.

Diethyl *N*-[4-[*N*-[[4-Hydroxy-2-(trimethylacetamido)-6-quinazolinyl]methyl]-*N*-(3-acetoxypropyl)amino]benzoyl]-L-glutamate (7). A solution of 6-(bromomethyl)-4-hydroxy-2-(trimethylacetamido)quinazoline (**6**)[25] (2.54 g, 7.5 mmol) in DMA (25 mL) containing $CaCO_3$ (1.5 g, 15 mmol) in suspension was prepared at 60 °C. A solution of the amine **3q** (3.17 g, 7.5 mmol) in DMA (10 mL) was added to the stirred reaction mixture over 1.5 h. After being stirred a further 12 h at 60 °C, the reaction mixture was filtered and the filtrate, after the addition of H_2O (10 volumes), was extracted with EtOAc. The organic phase was washed with H_2O and filtered through phase-separation paper and the solvent removed in vacuo to give an oil. The oil was chromatographed on silica gel (Merck, Art 7734) that was eluted first with EtOAc–CH_2Cl_2 (1:1) to remove some starting amine and then with EtOAc–$CHCl_2$ (3:1) to remove the product. The appropriate fractions were evaporated to dryness under reduced pressure to give an oil that, dried under vacuum, gave a foamlike solid: 3.25 g (64%); mp 61–64 °C; NMR Table I, (supplementary material). Anal. ($C_{35}H_{45}N_5O_9 \cdot 0.4CH_2Cl_2$) C, H, N.

Preparation of Antifolate Diacids 1b–l. General Method. The appropriate derivative **5** was suspended in EtOH–H_2O (20–33%; 15–40 mL/mmol) and treated with 1.00 N aqueous NaOH in the quantity stated (Table II). Brief swirling for 5–10 min gave a solution that was kept at room temperature for the time specified. Filtration gave an opalescent solution that was brought to pH 4.0 with 1 and 0.1 N aqueous HCl. The resulting thick gelatinous mass was centrifuged at 20000*g*. The product was freed from inorganic ions by repeated cycles of aqueous suspension–centrifugation–decantation until the supernatant was free of chloride ion ($AgNO_3$ test). About three such cycles were needed, the last was usually cloudy and represented a significant loss of product. The damp product was either freeze-dried and analyzed directly or was dried in the plastic centrifuge tube over P_2O_5 in vacuo at room temperature until it could be removed without loss to a glass vial in which it was dried further (P_2O_5, 1 mm) at the temperature stated (Table II). The latter samples were finally dried at 80 °C before microanalysis. All the compounds gave poor melting points. Elemental analyses and HPLC data for the products are listed in Table II; NMR and UV data are given in Tables II and III (supplementary material).

Di-*tert*-butyl *N*-(4-Aminobenzoyl)-L-glutamate (9). Di-*tert*-butyl *N*-(4-nitrobenzoyl)-L-glutamate was prepared by a modification of the literature method.[29] Di-*tert*-butyl-L-glutamate was added as its solid hydrochloride salt during 36 min to a mechanically stirred mixture that contained 2 mol equiv of triethylamine. Extractive workup (omitting the first filtration step) gave a crude solid (95%), hydrogenation of which gave the desired amine **9**,[29] crystallizable from EtOH or from 10% EtOH in cyclohexane.

Di-*tert*-butyl *N*-[4-[(Cyanomethyl)amino]benzoyl]-L-glutamate (10). This was prepared from the amine **9** (13 mmol) as for **3m** (method B) but with a reaction time of 1.75 h and the use of $CHCl_3$ as the eluent for chromatography. A pure, snow-white product was obtained (62%). The analytical sample was recrystallized from EtOH: mp 133–134 °C; NMR ($CDCl_3$, 250 MHz) δ 4.17 (d, J = 7.5 Hz, 2 H, CH_2CN), 4.42 (t, 7.5 Hz, 1 H, aniline NH). Anal. ($C_{22}H_{31}N_3O_5$) C, H, N.

Di-*tert*-butyl *N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]-*N*-(cyanomethyl)amino]benzoyl]-L-glutamate (11). A mixture of **10** (2.4 mmol), 4 (2.9 mmol), 2,6-lutidine (5.3 mmol), and DMA (10 mL) was kept (as a solution) in an oil bath at 110 °C for 2.75 h, at which time TLC (SiO_2GF, 20% 2 N HOAc in CH_3CN) showed the absence of quinazoline starting material. The cooled solution was filtered, and the filtrate was poured into H_2O (100 mL) to give a granular white precipitate and a final pH of 6. The suspension was chilled and filtered and the product washed once with H_2O and dried (1.24 g). Chromatography on silica (Art 15111, 235g) with 7% EtOH in $CHCl_3$ gave a pure, glassy, off-white solid: 0.223 g (15.8%); mp 155–158 °C; NMR (Me_2SO-d_6, 250 MHz) δ 1.38 (s, 9 H, *t*-Bu), 1.40 (s, 9 H, *t*-Bu), 4.71 (s, 2 H, CH_2CN, coincident with C^9 protons), 6.95 (d, 2 H, 3′,5′-protons) 7.79 (d, 2 H, 2′,6′-protons). Anal. ($C_{31}H_{38}N_6O_6$) C, H, N.

***N*-[4-[*N*-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]-*N*-(cyanomethyl)amino]benzoyl]-L-glutamic Acid (1m).** The diester 11 (0.209g) was treated with a mixture of TFA (1.3 mL) and $CHCl_3$ (2.7 mL) to give immediately a clear yellow solution. This was stirred vigorously since after 30 min a $CHCl_3$ layer separated out. After 2.75 h the reaction mixture was poured into ether (20 mL) in a centrifuge tube. Centrifugation at 600*g* gave a solid that was subjected to two cycles of resuspension (Et_2O, 25 mL)–centrifugation–decantation; drying in vacuo at 25 °C over P_2O_5/KOH then gave a white solid (0.111 g). HPLC analysis (as described below but with 30% MeOH–70% 0.16 M HOAc as the eluent and injection of the sample in 0.1 M NH_4OAc buffer, pH 7.0) showed 92.5% purity (retention time 3.75 min) with 7.5% of a single, less polar contaminant (retention time 9.35 min). A solution of this solid (0.060 g) in DMA (1 mL) was diluted with water (60 mL) to give a white, gelatinous precipitate. This was isolated and washed (H_2O, 2 × 30 mL) by centrifugation (200*g*/10 min). The resulting white solid was dried over P_2O_5 in vacuo at 80 °C: 0.040 g; mp 195 °C dec; HPLC analysis now showed 95.6% purity with the same single impurity down to 4.4%. Anal. ($C_{22}H_{22}N_6O_6$) C, H, N. NMR: Table II (supplementary material). UV: Table III (supplementary material).

Saponification of the Cyanomethyl Diester (5m) To Provide Impure Carboxamide Diacid 1e.[27] A mixture of the cyanomethyl diester **5m** (0.095 g), 0.1 N NaOH (66.67 mL), and CH_3CN (11.67 mL) was stirred for 16 h at 25 °C. The solution was then concentrated to 50 mL by rotary evaporation at 30 °C. Some slight insoluble matter was filtered off, and the clear filtrate was acidified to pH 3.5 with glacial AcOH. A gelatinous precipitate started forming from pH 5. After being chilled overnight, the mixture was centrifuged (1200*g*/30 min), and the resulting precipitate was subjected to three cycles of suspension (H_2O, 50 mL)–centrifugation–decantation. The pellet was dried over P_2O_5 at 25 °C: 0.059 g (68%); mp 225–235 °C. Anal. Calcd (after drying at 120 °C) for $C_{23}H_{24}N_6O_7 \cdot 0.5H_2O$: C, 54.65; H, 4.98; N, 16.63. Found: C, 54.61, 54.83; H, 4.95, 5.10; N, 16.51, 16.65. HPLC analysis (as described below but with 25% MeOH–75% of 0.16 M HOAc as the eluent revealed two main components, the major of which (86%, k' = 0.5) corresponded to authentic carboxamide **1e** and the minor of which (12%, k' = 2.0) corresponded to authentic nitrile **1m** (k' = 2.0).

Thymidylate Synthase Inhibition. The enzyme was obtained from L1210 cells, purified by affinity chromatography, and assayed as previously described.[18] The (±)-L-5,10-methylenetetrahydrofolate concentration used was 200 μM.

Dihydrofolate Reductase Inhibition. L1210 DHFR was purified by the method of Whitely et al.[31] as modified by Jackson et al.[32] and the IC_{50} values were determined by using the assay described by the latter authors.

Growth Inhibition of L1210 Cells in Culture. The ID_{50} values for the compounds were determined against L1210 cells

(31) Whitely, J. M. Jackson, R. C.; Mell, G. P.; Drais, J. H.; Huennekens, F. M. *Arch. Biochem. Biophys.* **1972**, *150*, 15.
(32) Jackson, R. C.; Hart, L. I.; Harrap, K. R. *Cancer Res.* **1976**, *36*, 1991.

grown in suspension culture as previously described.[17] Briefly, exponentially growing cells in RPMI 1640 medium containing 20 mM *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid buffer and supplemented with 10% horse serum were exposed to varying concentrations of the test compound, and the cell count at 48 h was compared to that of control (untreated) cells.

Analytical High-Pressure Liquid Chromatography. HPLC analyses were performed on a Waters Associates apparatus consisting of two M6000A pumps, a M660 solvent programmer, a WISP autosampler, and a dual-wavelength M440 UV detector operating at 254 and 280 nm. Separations were achieved on a 25 × 0.46 cm μBondapak C_{18} column (Waters Associates). Quinazolines were dissolved in 150 mM $NaHCO_3$ at 0.1 mg·mL^{-1}, 20 μL of the solution was injected, and elution was performed isocratically at room temperature with 35% MeOH–65% 0.16 M AcOH at a flow rate of 2 mL·min^{-1}. Outputs from the M440 detector were recorded on a Trilab integrator (Trivector Ltd.), and the capacity factor, k', was calculated with uracil having been used to determine the void volume [k' = [(retention time of test compound) − (retention time of uracil)]/retention time of uracil]. The results are given in Table II.

In an experiment to determine the stability of 1h in tissue culture medium, the compound was dissolved in 150 mM $NaHCO_3$ at 1 mg·mL^{-1} and diluted into tissue culture medium (see above) to give a final concentration of 20 μM. No cells were present. The solution was incubated at 37 °C under sterile conditions for 47 h during which time aliquots (1 mL) were removed at 0, 6, 21, 30 and 47 h and frozen (−20 °C). Samples were thawed and deproteinated by addition of methanol (2 mL) followed by centrifugation (1000*g*, 10 min, 4 °C). Aliquots (20 μL) of supernatant were analyzed by HPLC as described above, with the mobile phase adjusted to 25% MeOH–75% 0.16 M AcOH to improve the retention of 1h. During the incubation there as a gradual reduction in the peak area of 1h and a parallel increase in that of a component that was indistinguishable from **1s** (synthetic **1s**: k' = 2.1. Decomposition product from 1h: k' = 2.0). By the same method but taking aliquots at 0, 24, and 48 h, the stability of the cyanomethyl compound **1m** in tissue culture medium was studied. All samples showed identical elution profiles, and no decomposition took place.

To examine the stability of the cyanomethyl compound **1m** during its preparation for the TS assay, the compound (0.5 mg) was dissolved in 50 mM $NaHCO_3$, pH 8.1 (1 mL), and the solution was diluted with 50 mM phosphate buffer (pH 7.4) to a volume of 10 mL. This solution was kept at 37 °C for 1 h and then analyzed by injecting 20 μL on the column described above, with 30% MeOH–70% 0.16 M AcOH as the eluent. The elution profile showed 95.6% purity and was invariant with time.

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Registry No. **1b**, 97280-34-7; **1c**, 97280-35-8; **1d**, 80015-07-2; **1e**, 80014-95-5; **1f**, 80014-93-3; **1g**, 97280-36-9; 1h, 80030-17-7; **1l**, 80014-94-4; **1j**, 97280-37-0; **1k**, 97280-38-1; **1l**, 80015-03-8; 1m, 80015-09-4; **2**, 13726-52-8; **3b**, 97280-19-8; **3c**, 97280-20-1; **3d**, 97280-21-2; **3e**, 80014-83-1; **3f**, 80014-84-2; **3g**, 97280-22-3; **3h**, 80015-04-9; **3m**, 51043-64-2; **3n**, 97280-23-4; **3o**, 97280-24-5; **3p**, 97280-25-6; **3q**, 80015-01-6; 4·HBr, 77766-62-2; **5b**, 97280-26-7; **5c**, 97280-27-8; **5d**, 80015-06-1; **5e**·$^1/_2$HBr, 97280-28-9; **5f**, 80014-75-1; **5g**, 97280-29-0; **5h**·$^1/_2$HBr, 97280-30-3; **5k**, 97280-31-4; **5m**, 80015-08-3; **5n**, 80014-76-2; **5o**, 97280-32-5; **5p**, 97280-33-6; **6**, 58677-07-9; **7**, 80015-02-7; **8**, 97280-16-5; **9**, 76282-66-1; **10**, 97280-17-6; 11, 97280-18-7; iodoacetamide, 144-48-9; phenacyl bromide, 70-11-1; 3-fluorobenzyl bromide, 456-41-7; 5-(chloromethyl)uracil, 3590-48-5; paraformaldehyde, 30525-89-4; potassium cyanide, 151-50-8; chloroacetonitrile, 107-14-2; ethyl bromoacetate, 105-36-2; ethyl 3-bromopropionate, 539-74-2; 1,3-dibromopropane, 109-64-8; 3-acetoxypropyl bromide, 592-33-6; isopropyl bromide, 75-26-3; (chloromethyl)cyclopropane, 5911-08-0; 2-fluoroethyl *p*-toluenesulfonate, 383-50-6; diethyl 5,8-dideazafolate, 27069-81-4; di-*tert*-butyl *N*-(4-nitrobenzoyl)-L-glutamate, 88050-23-1; di-*tert* butyl-L-glutamate hydrochloride, 32677-01-3; cyanomethylamine, 540-61-4; thymidylate synthase, 9031-61-2; dihydrofolate reductase, 9002-03-3.

Supplementary Material Available: ^{1}H NMR spectral data of diesters **5b–h,k,m–p** and compound **7** (Table Is) and diacids **1a–m** (Table IIs), and ultraviolet spectral data of diacids **1b–m** (Table IIIs) and 400-MHz FT ^{1}H NMR spectrum of 10-propargyl-5,8-dideazafolic acid (**1a**) (solvent Me_2SO-d_6; probe temperature 297 K; (a) δ 1.3–8.7, (b) δ 6.3–8.4; Figure Is) (4 pages). Ordering information is given on any current masthead page.