Quinazoline Antifolates Inhibiting Thymidylate Synthase: 2-Desamino Derivatives with Enhanced Solubility and Potency†,‡

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The poor solubility of the thymidylate synthase (TS) inhibiting antifolate 10-propargyl-5,8-dideazafolic acid has posed problems for its clinical use and is probably responsible for its renal toxicity. The insolubility is caused by the 2-amino-3,4-dihydro-4-oxopyrimidine moiety of the drug which stabilizes the solid state by intermolecular hydrogen bonding. In examining this moiety we have removed the 2-amino group and now report on 2-desamino-10-propargyl-5,8-dideazafolic acid (8e) and four analogues with H, Me, Et, and allyl at N^{10}. 3,4-Dihydro-4-oxo-6-methylquinazoline was solubilized by alkylating the lactam nitrogen with chloromethyl pivalate. Reaction with *N*-bromosuccinimide gave the corresponding 6-bromomethyl compound, which was coupled with diethyl *N*-(4-aminobenzoyl)-L-glutamate or the appropriate N-substituted derivative thereof. The quinazoline N^3 nitrogen and carboxyl groups in the product were simultaneously deprotected by cold alkali in the final step to give the desired five antifolates. These were tested against L1210 TS and it was found that removal of the 2-amino group caused a slight (3–9-fold) loss of TS inhibition. 8e was only 8-fold a lesser TS inhibitor than the parent drug. Inhibition of rat liver dihydrofolate reductase was reduced by over 1 order of magnitude for three compounds tested. All five analogues were more cytotoxic to L1210 cells in culture than their 2-amino counterparts; 8e was 8.5-fold more active with an ID_{50} of 0.4 μM. This remarkable result probably owes to increased cellular penetration. 8e was 5-fold more soluble than 1 at pH 5.0 and >340-fold more soluble at pH 7.4.

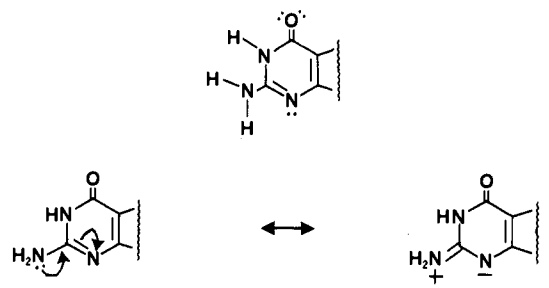
The antifolate 10-propargyl-5,8-dideazafolic acid[1] (1) is cytotoxic to cells by means of its inhibition of the enzyme thymidylate synthase (EC 2.1.1.45, TS).[2,3] In recently reported phase I/II studies this drug had activity against ovarian, liver, and breast cancer with, however, troublesome hepatic toxicity and dose-limiting renal toxicity.[4-10] The drug is poorly soluble at neutral or acidic pH; this has posed problems for its clinical use and is probably responsible for the renal toxicity. It is known that in mice[11] and in rats[12] the drug precipitates in the renal tubule; in rats precipitation in the bile duct has also been observed.[11] In seeking an improved analogue we hypothesized that the renal toxicity of 1, at least, was a physicochemical event directly attributable to its poor aqueous solubility when in the renal tubule at acidic pH.

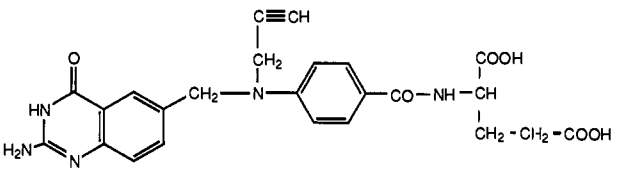
A well-known facet of the behavior of π-deficient heterocycles, such as quinazoline, is the paradox that the introduction of hydrophilic amino, hydroxy, or mercapto substituents far from increasing water solubility actually decreases it. This behavior is caused by the strong intermolecular hydrogen bonds that associate the heterocycles into a stable lattice that water finds difficult to disrupt and solvate.[13] The copresence of an acidic hydroxy group (as the favored lactam tautomer) and a basifying amino group maximizes this association and minimizes the solubility. Such a configuration is found in 1 and consists in all of three electron pairs and three hydrogen atoms seeking electron pairs (Chart I). A fourth electron pair on the 2-amino group involves itself in mesomeric resonance, serving to increase the basicity (and hydrogen bonding capability) of N^1. The 2-amino group thus contributes in no small measure to the intermolecular hydrogen bonding, and we therefore expected that its removal would result in an analogue possessing increased water solubility.

Chemistry

Five new 2-desamino analogues 8a–e containing the substituent series[2] that originally led to the discovery of 1 were synthesized (Scheme I). Fusion of 2-amino-5-

Chart I





1

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(1) 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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Table I. Preparation of Antifolate Triesters 7a–d

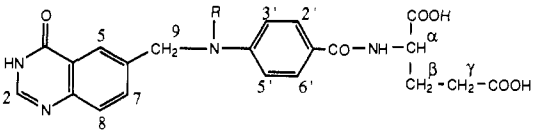
compd	quantity of reactant, mmol: amine 3	quinazoline 4	$CaCO_3$	DMA, mL	reactn temp, °C	reactn time, h	chromatography	yield, %	mp, °C	formula	anal.	M^+
7a	10	10	20	30	60	66	*a*	64.7	63–66	$C_{31}H_{38}N_4O_8$	C,H,N	
7b	6.54	5.95	11.90	20	50	24	*b*	89.3	86.5–90	$C_{32}H_{40}N_4O_8$	C,H,N	
7c	2.50	2.75	5.00	10	50	29	*c*	92.2	gum	$C_{33}H_{42}N_4O_8$	C,H,N	622
7d	1.21	1.21	2.42	5	50	48	*d*	56.3	47-50	$C_{34}H_{42}N_4O_8 \cdot 0.5H_2O$	C,H,N	634

[a] 20–33% EtOAc in CH_2Cl_2. [b] CH_2Cl_2/cyclohexane/EtOAc (3:3:2). [c] Et_2O. [d] 20% EtOAc in CH_2Cl_2.

Table II. Preparation of Antifolate Diacids 8a–d

compd	starting matl 7, mmol	NaOH (aq), mol equiv	reactn time, h	drying temp, °C	yield, %	mp, °C	formula	anal.
8a	0.42	5	20	100	63.6	175–180	$C_{21}H_{20}N_4O_6$	C,H,N
8b	4.35	4.8	24	70	62.1	186.5–189	$C_{22}H_{22}N_4O_6 \cdot H_2O$	C,H,N
8c	1.35	5	18	70	58.5	143.5–145.5	$C_{23}H_{24}N_4O_6 \cdot H_2O$	C,H,N
8d	0.435	5	24	70	77.0	138–143	$C_{24}H_{24}N_4O_6 \cdot 0.75H_2O$	C,H,N

Table III. ^1H NMR Spectral Data of Antifolate Diacids 8a–d[a]



compd	CH_2^β (m)	CH_2^γ (m)	CH^α (m)	CH_2^9 (s)	$H^{3'}$, $H^{5'}$ (d)	H^7 (dd)	H^8 (d)	$H^{2'}$, $H^{6'}$ (d)	H^5 (d)	H^2 (s)	amidic NH (d)	lactam NH (br s)	carboxyls (br s)	R
8a	2.17	2.44	4.80	4.54	6.63	7.85	7.67	7.64	8.22	8.06	nr[f]	nr	nr	*b*
8b	1.99	2.33	4.35	4.81	6.77	7.65	7.65	7.73	7.91	8.06	8.21	12.25	12.35	*c*
8c	1.98	2.32	4.34	4.75	6.71	7.66	7.66	7.70	7.93	8.06	8.17	12.24	12.29	*d*
8d	1.98	2.09	4.34	4.77	6.72	7.67	7.67	7.68	7.94	8.06	8.19	12.25	12.31	*e*

[a] Spectra determined in Me_2SO-d_6 except compound 8a where CD_3OD was used. [b] 4.90, br s, 1 H, $N^{10}H$. [c] 3.13, s, 3 H, CH_3. [d] 1.17, t 6.9 Hz, 3 H, CH_3; 3.58, q, 6.9 Hz, 2 H, CH_2. [e] 4.18, m, 2 H, CH_2C=; 5.18, m, 2 H, CH_2=; 5.83–5.96, m, 1 H, CH. [f] Not recorded.

methylbenzoic acid (**2**) with formamide gave 3,4-dihydro-4-oxo-6-methylquinazoline (**3**).[14,15] This quinazoline was solubilized for the next reaction by N-alkylation with chloromethyl pivalate. The (pivaloyloxy)methyl group, introduced in 1967,[16] has been previously observed to alkylate a lactam nitrogen.[17] In this respect, however, we used it intentionally and it markedly altered the solubility of the heterocyclic base. The derivative **4**, in CCl_4 solution, reacted smoothly with *N*-bromosuccimide to provide the bromomethyl compound **5**. This was coupled with the appropriate amine **6**. The secondary amines **6b–e** were prepared by direct monoalkylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate (**6a**). The allylamine **6d** and the propargylamine **6e** has aleady been synthesized by this method[2] while the methylamine **6b** described by Cosulich and Smith[18] and the ethylamine **6c** described by Montgomery et al.[19] had not. A detailed preparation of the triester **7e** is provided; its congeners **7a–d** were similarly prepared (Table I). The (pivaloyloxy)methyl and ethyl ester functions in the triesters **7a–e** were removed in one step with cold alkali to yield the antifolate diacids **8a–e**. Again, the preparation of the propargyl compound **8e** is described in detail while similar experiments with **8a–d**

Table IV. Ultraviolet Spectral Data of Antifolate Diacids 8a–e[a]

compd	max, nm	ε	min, nm	ε
8a	220	31 100	247	7250
	228	24 100		
8b	222.5	31 600	248	5200
	310	30 200		
8c	224	30 800	250	5500
	312	29 900		
8d	220.5	33 700	250	5700
	310	30 700		
8e	221	34 500	248	6200
	300	29 000		

[a] Spectra determined in 0.1 N NaOH (aqueous).

Table V. Solubilities of Antifolates 1 and 8e[a]

	pH 5.0	pH 7.4
1	0.0086	0.312
8e	0.043	>107

[a] Values in mg mL^{-1}.

Table VI. Biological Properties of Antifolates[a]

compd	R	IC_{50} L1210 TS, μM[b]	K_i rat liver DHFR, nM	ID_{50} L1210 cells in cult., μM
8a	hydrogen	22.72 (2.44)	10.7 (0.58)	0.4 (2.7)
8b	methyl	1.06 (0.36)		0.9 (4.5)
8c	ethyl	0.52 (0.06)	57.8 (3.88)	2.7 (9.0)
8d	allyl	1.12 (0.28)		2.5 (6.0)
8e	propargyl	0.16 (0.02)	2250 (74.5)	0.4 (3.4)

[a] Figures in parentheses are those of the corresponding 2-amino analogues. [b] The ± 5,10-CH_2-FH_4 concentration was 200 μM.

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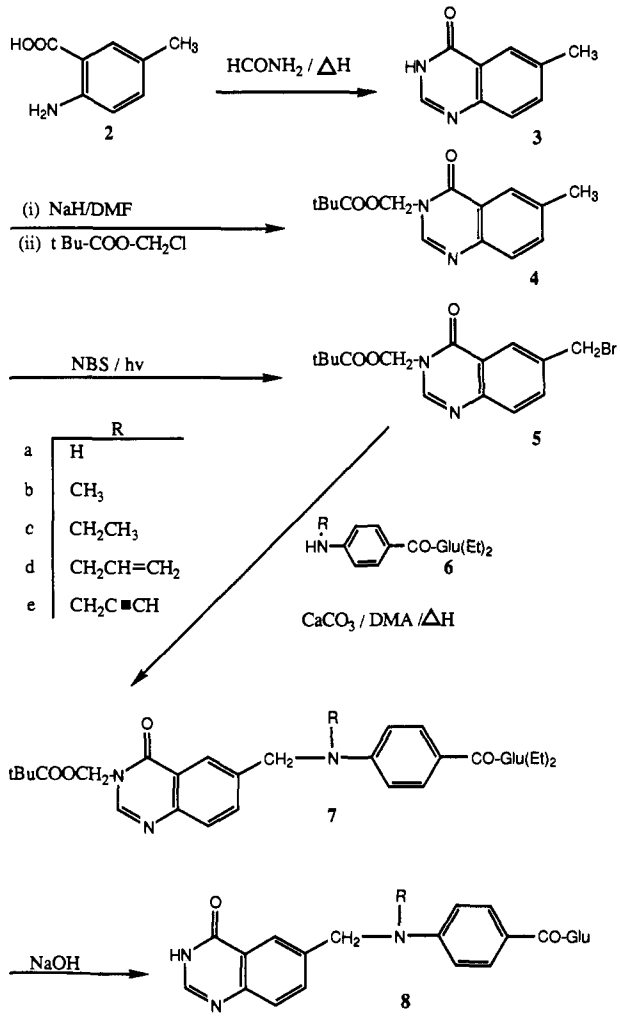
Scheme I



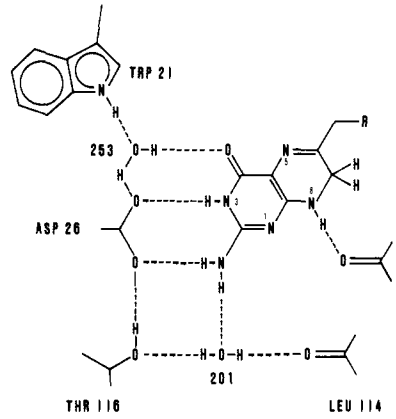
are collected in Table II. The structure and purity of each diacid 8 was upheld by elemental microanalysis, NMR spectroscopy (Table III), and UV spectroscopy (Table IV). The 250-MHz NMR spectra of the triesters **7a–d** were perfectly satisfactory and are not reported.

The solubilities of 10-propargyl-5,8-dideazafolic acid **1** and its desamino analogue **8e** in 0.01 M potassium phosphate buffer at pH 5.0 and at pH 7.4 were determined by a UV method. The results are shown in Table V. The desamino antifolates **8a–e** were tested for their inhibition of partially purified L1210 thymidylate synthase[20,21] and for their inhibition of the growth of L1210 cells in culture;[2] **8a**, **8c**, and **8e** were tested for their inhibition of partially purified rat liver dihydrofolate reductase (DHFR).[22] The results are expressed in Table VI with results for the corresponding 2-amino compounds provided for comparison.

Results and Discussion

The solubility determinations (Table V) on compounds **1** and **8e** were performed with the pH values of the buffers chosen to represent those of human urine (≃5.0) and human plasma (7.4). The desamino compounds **8e** was appreciably more soluble (5-fold) than **1** at pH 5.0. The solubility of **8e** at pH 7.4 was not completely defined since the amount of compound available put a limit on the experiment such was the ease with which it dissolved; **8e** was found to be >340-fold more soluble than **1** at this pH. It was gratifying to observe these enhancements.

Chart II



In Table VI a comparison of the IC_{50} for TS of the 2-desamino analogues **8a–e** with those of their 2-amino counterparts shows that removal of the 2-amino group causes a 3–9-fold loss in TS inhibition. The propargyl compound **8e** was 8-fold less inhibitory than its parent **1**. Despite their poorer inhibition of TS the analogues **8a–e** were more cytotoxic to L1210 cells than the corresponding 2-amino derivatives. In particular, the greatest increment was seen with the propargyl compound **8e**, which was 8.5-fold more active. In the desamino series the N^{10}-propargyl derivative **8e** was the most potent inhibitor of TS, as in the 2-amino series, and the ranking conferred by the N^{10} substituents, propargyl > ethyl > methyl ≃ allyl > hydrogen, was also the same. This parallel suggests that the mode of binding to the enzyme is the same for both series. For the three compounds tested against DHFR, the removal of the amino group caused a loss of inhibition by over 1 order of magnitude. For inhibition of this enzyme the rank order of substituents was hydrogen > ethyl > propargyl, repetitive of the 2-amino series, and again suggesting similar modes of binding. The loss of inhibition with the propargyl compound **8e** was at least 30-fold (2250/74.5) since HPLC revealed about a 1% presence of the stronger inhibitor **8a** in **8e** and this we believe arises in the final saponification during synthesis. Thus, as for the 2-amino series, the N^{10}-propargyl derivative is the strongest TS inhibitor and the weakest DHFR inhibitor, an important property for a compound with such dual inhibition to exert a rate-limiting effect on TS in the thymidylate cycle. Moreover, removal of the amino group increases the specificity toward TS by a small factor in all three cases considered. For the propargyl compound **8e** this factor is $(>2250 \times 0.02)/(74.5 \times 0.16) \geq 4$. The DHFR result is qualitatively explained by the X-ray crystallographically derived model[23] wherein the 2-amino group of dihydrofolate is hydrogen bonded by one H to a conserved carboxylate and by the other to a conserved, ordered water molecule. This is shown in Chart II which details the model for the *Escherichia coli* enzyme. The amino acid sequence for rat liver DHFR is not known but it is highly likely that a glutamate at position 30 takes the place of Asp 27 in the *E. coli.* model since Glu 30 is known to be a conserved residue in five other vertebrate DHFRs, four of them mammalian.[24-28]

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Returning discussion to the targeted enzyme, we expected to lose TS inhibition in the desamino series since we were after all removing an amino group thought by most to be inviolable. At first consideration, were this NH_2 involved in just one hydrogen bond of strength 4.1 kcal mol^{-1}, its removal would reduce binding by 10^3. The small magnitude of the loss was therefore a surprise. However, we later rationalized our finding. If it is assumed that the 2-NH_2 group is providing the hydrogen atom (H–I, below) and a residue B of TS the electron pair toward a presumed hydrogen bond in the enzyme–inhibitor complex, then the following hydrogen bond inventory can be made for the binding process:

$$E\text{–}B\cdots HOH + H_2O\cdots H\text{–}I \rightleftharpoons E\text{–}B\cdots H\text{–}I + HOH\cdots OH_2$$

The hydrated enzyme and the hydrated inhibitor both have to be "dried out" before a hydrogen bond can be formed between them. There are two hydrogen bonds on each side of the equation, and on the good assumption that these are of equal energy, the process is isoenthalpic. This has been pointed out by Fersht and colleagues[29,30] who assert that the contribution of hydrogen bonds to enzyme–ligand binding energy stems from the entropy gained when water molecules previously bonded to enzyme and substrate are released into bulk water. These authors further point out that the deletion of a hydrogen bond in the enzyme–ligand complex does not necessarily lead to the overall loss of the absolute strength of that hydrogen bond. For the desamino series, binding, now represented as

$$E\text{–}B\cdots HOH + [H_2O\ I] \rightleftharpoons [E\text{–}B\ I] + HOH\cdots OH_2$$

is again isoenthalpic with respect to hydrogen bonds and the return of two water molecules to bulk water provides the gain in entropy which favors binding. In reality, although the number of hydrogen bonds on each side of either equation is equal, their strength or enthalpy may not be thus creating a small enthalpic contribution to the free energy of binding. Also a second factor to consider is what, following the removal of the hydrogen, "I" actually represents and presents to B; in our case it is an aromatic hydrogen atom and, possibly, a gap in space. Such an arrangement may not be as stable as the ordered water molecule(s) which was previously held in loose association by dispersion forces with that portion of the inhibitor. These two factors determine the measured difference in binding and not the loss of any hydrogen bond. Fersht and colleagues have undertaken an extensive analysis of hydrogen bonds formed from uncharged species in enzyme–ligand interactions and conclude that the binding energy is relatively low at 0.5–1.8 kcal mol^{-1}, representing a 4–13-fold difference in binding.[29] Another group, who used a complementary approach in which they varied the ligand and not the enzyme, came to the same view.[31] These conclusions harmonize with our own observation as does the recent finding that removal of the 2-amino group from another 2-amino-3,4-dihydro-4-oxo-1,3-diazine, guanosine, accounts for a loss of only 0.7–1.6 kcal mol^{-1} to the free energy of binding of a single Watson–Crick hydrogen bond.[32]

We also recognize that the loss of the electron-donating amino group will have effects on two important properties of the heterocyclic ring. Both involve hydrogen bonding and the above comments on this phenomenon apply equally. First, the lactam NH group (and we have evidence for its involvement in binding)[33] will become more acidic and hence more capable of hydrogen bonding. Second, as already mentioned, the azine nitrogen at N^1 will become appreciably less basic and less capable of donating electron density into a hydrogen bond. It is possible that more than one, or even all, of these hydrogen bonding schematas exist and are modulated by the loss of the amino group to produce the resultant slight decrease in binding which we observe.

The enhanced cytotoxic potency shown by the inferior TS inhibitors **8a–e** is a sharp reminder that binding to a target enzyme is only one of some half a dozen properties needed of an inhibitor for it to qualify as a drug. The classical antifolate 1 is known to undergo intracellular metabolism to polyglutamate forms, some of which are more than 100-fold more inhibitory to TS.[21] It is unlikely that an intrinsic alteration in this metabolism can explain the enhanced potency since **8e** is essentially equiactive with 1 as a substrate for folylpolyglutamate synthetase (FPGS).[34] The enhanced potency we observe probably owes to increased cellular uptake leading to increased substrate concentration for FPGS leading indirectly to increased polyglutamation. In conclusion, having shown that there is scope for maneuver at the 2-position of the quinazoline nucleus, we consider 2-desamino-5,8-dideaza-10-propargylfolic acid **8e** with its enhanced solubility and potency to be an exciting new development in this series of antifolates. It is piquant to note that the only other known desamino folate analogue, that of folic acid itself prepared 35 years ago, was also tested at the Institute of Cancer Research.[35]

Experimental Section

General procedures are as given in ref 36 except as follows. Melting points were determined on a Reichert-Jung Thermovar microscope. Extracts were dried with $MgSO_4$. K_2CO_3 and $CaCO_3$ were dried at 100 °C. Elemental analyses were determined by Butterworth Laboratories, Teddington, Middlesex, England. Results for the elements stated were within 0.4%.

3,4-Dihydro-6-methyl-4-oxoquinazoline (3). 2-Amino-5-methylbenzoic acid (**2**) (30.23 g, 0.2 mol) and formamide (36.03 g, 0.8 mol) were placed in a 400-mL beaker and stirred together to give a homogeneous, thick paste. The beaker was immersed in an oil bath such that the inner temperature came up to 130 °C; a dark-colored liquid was obtained. The mixture was kept at 130 °C for 1 h during which time ammonia gas was evolved followed by extensive solidification. The bath temperature was

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then raised to 180 °C and the mixture held thus for a further 1 h, the large lumps of solid being broken up in an attempt to maintain thermal equilibrium and homogeneity. The mixture was cooled and treated with a mixture of EtOH (20 mL) and H_2O (100 mL), and the lumps of solid were broken up to give a homogeneous slurry. The product (28.8 g) was filtered off and washed thoroughly with H_2O. Recrystallization from EtOH afforded white microneedles (24.76 g, 77.3%), mp 260–261 °C (lit. mp 251 °C[14] and 255 °C[15]).

3,4-Dihydro-6-methyl-4-oxo-3-[(pivaloyloxy)methyl]-quinazoline (4). Sodium hydride 60% oil dispersion (2.50 g, 62.5 mmol, 1.25 equiv) was placed in a 500-mL two-necked flask and rinsed with petroleum ether. DMF (65 mL) was added followed by, with magnetic stirring, **3** (8.00 g, 50 mmol) added over 15 min with occasional ice cooling. The mixture was stirred for a further 30 min in the absence of moisture. To the now almost clear, yellow-gray solution was added chloromethyl pivalate (7.80 g, 50 mmol) during 2 min and the resulting mixture was stirred at 25 °C for 2 h. The mixture, now containing precipitated sodium chloride, was partitioned between ether (1 L) and water (1 L). The ether extract was washed once with saturated brine (1 L), dried, and concentrated in vacuo to give a yellow oil which crystallized spontaneously (13.80 g). This crude product was recrystallized from petroleum ether (ca. 500 mL), filtering off some quinazoline starting material in the process (11.12 g, 81.1%): mp 108.5–109 °C; IR $\nu_{C=O}$ ester 1733 cm^{-1} and $\nu_{C=O}$ lactam 1690 cm^{-1}; NMR ($CDCl_3$, 90 MHz) δ 1.19 (s, 9 H, tBu), 2.50 (s, 3 H, CH_3), 5.95 (s, 2 H, CH_2), 7.60 (dd, 1 H, $J_{7,8}$ = 8.3 Hz, $J_{7,5}$ = 1.8 Hz, H^7), 7.65 (d, 1 H, $J_{8,7}$ = 8.3 Hz, H^8), 8.12 (m, 1 H, H^5), 8.26 (s, 1 H, H^2). Anal. ($C_{15}H_{18}N_2O_3$) C, H, N.

6-(Bromomethyl)-3,4-dihydro-4-oxo-3-[(pivaloyloxy)-methyl]quinazoline (5). Methyl compound **4** (6.86 g, 25 mmol) was placed in a 1-L borosilicate flask and dissolved in CCl_4 (250 mL) with the aid of brief warming. *N*-Bromosuccinimide (4.89 g, 27.5 mmol, 1.1 equiv) was added and the mixture brought to reflux. The flask (entered only 1 cm into the heating mantle) was then irradiated from a distance of 5 cm with a 275-W sun lamp for 20 min. After cooling, the succinimide was filtered off and washed with carbon tetrachloride (2 × 50 mL). The combined filtrate and washings were concentrated to dryness to give a sticky, cream-colored solid (10.69 g). This was recrystallized from petroleum ether (950 mL) during which process an insoluble solid (ca. 5%) was filtered off. Cooling to 2 °C gave unpretty white crystals (7.67 g, mp 104–114 °C) which were recrystallized from petroleum ether (1100 mL) to give more shapely crystals (6.74 g, 76.3%, mp 108–115 °C) of technical grade product suitable for further use. A small portion of this product was recrystallized twice further from petroleum ether to give the analytical sample: mp 121–122 °C; IR $\nu_{C=O}$ (ester) 1730 cm^{-1} and $\nu_{C=O}$ (lactam) 1685 cm^{-1}; NMR ($CDCl_3$, 250 MHz) δ 1.20 (s, 9 H, tBu), 4.60 (s, 2 H, CH_2Br), 5.95 (s, 2 H, CH_2), 7.70 (d, 1 H, $J_{8,7}$ = 8.4 Hz, H^8), 7.82 (dd, 1 H, $J_{7,8}$ = 8.4 Hz, $J_{7,5}$ = 2.2 Hz, H^7), 8.29 (s, 1 H, H^2), 8.32 (d, 1 H, $J_{5,7}$ = 2.2 Hz, H^5). Anal. ($C_{15}H_{17}BrN_2O_3$) C, H, N, Br.

Diethyl *N*-[4-(Methylamino)benzoyl]-L-glutamate (6b). Iodomethane (6.81 g, 48 mmol) was added to a solution of diethyl *N*-(4-aminobenzoyl)-L-glutamate (9.66 g, 30 mmol) in DMF (300 mL) over K_2CO_3 (4.15 g, 30 mmol) and the resulting mixture was stirred vigorously in the absence of moisture for 26 h. The solvent was removed at 1 mm and the residue partitioned between EtOAc (250 mL) and H_2O (250 mL). The organic layer was washed with water (250 mL), dried, and evaporated in vacuo to give a pale brown oil (10.7 g). This was dissolved in 5% EtOAc in CH_2Cl_2 (20 mL) and applied to a column (diameter 5 cm) of silica gel (Merck Art. 7734, 450 g). The column was eluted with cyclohexane/CH_2Cl_2/EtOAc (4:4:2.5), and the appropriate fractions were evaporated under vacuo to give a pale yellow oil (2.32 g, 23%). This solidified on standing to give an off-white crystalline solid. Recrystallization from toluene/hexane (10:1.5, 10 mL) gave fine pure white crystals (2.2 g); mp 89.5–91.5 °C (lit.[18] mp 89.8–91 °C).

Diethyl *N*-[4-(Ethylamino)benzoyl]-L-glutamate (6c). Iodoethane (6.24 g, 40 mmol) was added to a solution of diethyl *N*-(4-aminobenzoyl)-L-glutamate (6.44 g, 20 mmol) in DMF (200 mL) over K_2CO_3 (2.76 g, 20 mmol), and the resulting mixture was stirred vigorously at 100–110 °C for 5 h. The solvent was removed at 1 mm and the residue partitioned between EtOAc (250 mL) and H_2O (500 mL). The organic layer was washed with water (500 mL), dried, and evaporated in vacuo to give a brown oil (8.4 g). This was dissolved in 5% EtOAc in CH_2Cl_2 (10 mL) and applied to a column (diameter 5 cm) of silica gel (Merck Art. 7734, 400 g). The column was eluted with 5% EtOAc in CH_2Cl_2, and the appropriate fractions were concentrated to give a golden-colored oil (4.56 g, 65.1%), which solidified to give a pale yellow solid: mp 77–78 °C (lit.[19] mp 73 °C).

Diethyl *N*-[4-[*N*-[[3,4-Dihydro-4-oxo-3-[(pivaloyloxy)-methyl]-6-quinazolinyl]methyl]prop-2-ynylamino]-benzoyl]-L-glutamate (7e). A mixture of **5** (technical quality, 2.30 g, at least 5 mmol), diethyl *N*-[4-(prop-2-ynylamino)-benzoyl]-L-glutamate[2] (1.80 g, 5 mmol), calcium carbonate (1.00 g, 10 mmol), and DMA (20 mL) was stirred in a stoppered flask at 80 °C for 18 h. The mixture was cooled and filtered through a bed of Celite, and the solids were washed thoroughly with DMA. The clear filtrate was concentrated at 55 °C/4 mm on a rotary evaporator and then at 75 °C/1 mm by direct coupling to an oil pump to give a stiff brown gum (6.78 g). This was dissolved in a mixture of CH_2Cl_2 (13 mL) and ether (20 mL) and applied to a column of silica (Merck Art. 15111, 240 g) made up in ether in a Jobin-Yvon Chromatospac Prep 10 machine. The solution was washed in with a mixture of CH_2Cl_2 (4 mL) and ether (6 mL) and the column then eluted with ether, the effluent passing through a Cecil 212A ultraviolet monitor set at 254 nm. The product eluted last of all and evaporation of the appropriate fractions yielded a gum (2.54 g, 80.3%) which, although pure, could not be induced to solidify. For characterization a portion of the gum was dried at 100 °C/P_2O_5/1 mm/18 h to give a brittle glass: NMR ($CDCl_3$, 250 MHz) δ 1.20 (s, 9 H, tBu), 1.22 (t, 3 H, J = 7.1 Hz, ethyl CH_3), 1.29 (t, 3 H, J = 7.1 Hz, ethyl CH_3), 2.21 (m, 2 H, glu CH_2^β), 2.45 (m, 2 H, glu CH_2^γ), 3.02 (bs, 1 H, ≡CH), 4.10 (q, 2 H, J = 7.1 Hz, ethyl CH_2), 4.16 (d, 2 H, J = 2.3 Hz, $CH_2C\equiv$), 4.22 (q, 2 H, J = 7.1 Hz, ethyl CH_2), 4.74–4.83 (m, 1 H, glu CH^α), 4.76 (s, 2 H, CH_2^9), 5.94 (s, 2 H, OCH_2N), 6.85 (d, 3 H, J = 8.9 Hz, $H^{3'}$ and $H^{5'}$ with amidic NH superimposed), 7.71–7.75 (m, 4 H, H^7, H^8, $H^{2'}$, $H^{6'}$), 8.25 (d, 1 H, $J_{5,7}$ = 1.5 Hz, H^5), 8.26 (s, 1 H, H^2). Anal. ($C_{34}H_{40}N_4O_8$) C, H, N.

***N*-[4-[*N*-[(3,4-Dihydro-4-oxo-6-quinazolinyl)methyl]-prop-2-ynylamino]benzoyl]-L-glutamic Acid (8e).** A mixture of the gummy triester **7e** (0.633 g, 1 mmol), H_2O (18 mL), EtOH (18 mL), and 1.00 N aqueous NaOH (5 mL, 5 mmol) was stirred vigorously for 40 min to give a pale yellow solution. This solution was kept at 22 °C for 18.5 h. It was then filtered through a bed of Celite (carbon treatment) and the clear filtrate acidified to pH 2.5 with 1.00 N aqueous HCl. The resulting white gelatinous precipitate was centrifuged (2500*g*/30 min) and washed by three cycles of resuspension (H_2O: 80 mL, 80 mL, and 40 mL)–centrifugation–decantation. The pellet was dried in vacuo over P_2O_5, first at 22 °C/24 h in a desiccator and then at 80 °C/4 h in a pistol. A pale yellow amorphous solid was obtained (0.312 g, 67.5%): mp 170–173 °C; NMR (Me_2SO-d_6) δ 2.00 (m, 2 H, glu CH_2^β), 2.34 (m, 2 H, glu CH_2^γ), 3.22 (t, 1 H, J = 2.1 Hz, ≡CH), 3.4 (br s, 2 H, water of hydration), 4.36 (m, 3 H, $CH_2C\equiv$ and glu CH^α), 4.81 (s, 2 H, CH_2^9), 6.85 (d, 2 H, J = 8.9 Hz, $H^{3'}$ and $H^{5'}$), 7.65 (d, 1 H, $J_{8,7}$ = 8.4 Hz, H^8), 7.75 (d, 2 H, $H^{2'}$ and $H^{6'}$ superimposed upon dd, 1 H, H^7), 8.03 (d, 1 H, $J_{5,7}$ = 1.8 Hz, H^5), 8.06 (d, 1 H, H^2), 8.27 (d, 1 H, J = 7.7 Hz, amidic NH), 12.24 (br s, 3 H, carboxyls and lactam NH); UV Table IV. Anal. Sample dried P_2O_5/110 °C ($C_{24}H_{22}N_4O_6 \cdot H_2O$) C, H, N.

Solubility Determinations. Experiments were done at 18 °C. A saturated solution of 1 in 0.01 M potassium phosphate buffer was made with the pH adjusted to 7.4 by the dropwise addition of 5 M KOH. This suspension was kept overnight and filtered through a 0.22-μm filter, the pH again adjusted to 7.4, and the suspension refiltered. One portion of the filtrate was diluted with 0.1 M NaOH, the absorbance at 301.5 nm measured, and the solubility calculated with ϵ = 26600).[2] The second portion of the filtrate was adjusted to pH 5.0 with phosphoric acid and filtration and pH readjustment repeated as before; a slightly opalescent solution resulted. High-speed centrifugation of this had no effect but slight dilution with 0.01 M potassium phosphate buffer at pH 5.0 gave a clear solution. This was diluted with 0.1 M NaOH and the absorbance measured as before. These experiments were repeated with **8e**, which, however, was completely soluble at 107 mg mL^{-1} at pH 7.4 and which gave a clear solution at pH 5.0 without event. The concentrations were determined

with ϵ = 29000 at 300 nm. The results are expressed in Table V.

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Registry No. **2**, 2941-78-8; **3**, 19181-53-4; **4**, 106585-52-8; **5**, 106585-53-9; **6a**, 13726-52-8; **6b**, 2378-95-2; **6c**, 70280-71-6; **6d**, 76858-71-4; **6e**, 76858-72-5; **7a**, 106585-62-0; **7b**, 106585-59-5; **7c**, 106585-60-8; **7d**, 106585-61-9; **7e**, 106585-54-0; **8a**, 106585-70-0; **8b**, 106585-67-5; **8c**, 106585-68-6; **8d**, 106585-69-7; **8e**, 106585-65-3; TS, 9031-61-2; DHFR, 9002-03-3; $HCONH_2$, 75-12-7; *t*-$BuCOOCH_2Cl$, 18997-19-8; MeI, 74-88-4; EtI, 75-03-6.

Comparison of the Biological Effects of Selected 5,8-Dideazafolate Analogues with Their 2-Desamino Counterparts[1]

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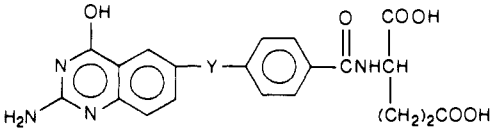
Three new 5,8-dideaza analogues of folic acid devoid of an amino group at position 2 have been prepared by using synthetic routes patterned after earlier methodologies. They were 2-desamino-5,8-dideazaisofolic acid, **2b**, 2-desamino-10-thia-5,8-dideazafolic acid, **2c**, and 2-desamino-10-oxa-5,8-dideazafolic acid, **2d**. These compounds were found to be 4–6-fold more cytoxic toward L1210 leukemia cells than their 2-NH_2 counterparts and to be poor inhibitors of mammalian thymidylate synthase. However, they were only 1.5–3-fold less inhibitory toward dihydrofolate reductase than the analogous compounds containing a 2-NH_2 group. The known thymidylate synthase inhibitors 2-desamino-10-propargyl-5,8-dideazafolic acid and 10-propargyl-5,8-dideazafolic acid were included in this study for purposes of comparison.

Efforts to discover a folate analogue having potent inhibitory activity toward mammalian thymidylate synthase, TS, culminated in the development of 10-propargyl-5,8-dideazafolic acid, **1a**, which became referred to as CB3717.[2] On the basis of its high degree of efficacy against L1210 leukemia in mice, CB3717 was introduced into clinical trials.[3] However, renal toxicity, apparently resulting from poor aqueous solubility, caused the cessation of human studies.[4] Subsequently, it was found that the removal of the 2-amino group of **1a** and related compounds resulted in improved solubility and cytotoxicity. For example, 2-desamino-10-propargyl-5,8-dideazafolic acid, **2a**, was found to be approximately 10-fold more cytotoxic toward L1210 leukemia cells in culture but 8-fold less inhibitory toward TS from L1210 leukemia cells than **1a**.[5,6]



1a: Y = $CH_2N(CH_2C{\equiv}CH)$
b: Y = $NHCH_2$
c: Y = CH_2S
d: Y = CH_2O

The folate analogue 5,8-dideazaisofolic acid, **1b** (IAHQ), has demonstrated activity against the colon tumor 38 in mice, the CX-1 human colon tumor implant in the nude mouse, a human osteogenic sarcoma xenograft in hamsters, as well as a variety of established human colon cell lines in culture.[7-11] However, high doses of IAHQ were required for activity in animal models, apparently due to its slow rate of influx into target cells as recently demonstrated with use of the human colon adenocarcinoma cell line HCT-8.[12]

In spite of the fact that IAHQ has greatly reduced affinity for TS as compared to CB 3717, it appeared to exert its antitumor action by inhibition of TS, since its cytotoxicity toward HCT-8 cells was largely reversed by thymidine.[7] It was of interest, therefore, to prepare the 2-desamino analogue of IAHQ, **2b**, in an effort to achieve enhanced antitumor efficacy. Also, prepared were 2-desamino-10-thia-5,8-dideazafolic acid, **2c**, and 2-desamino-10-oxa-5,8-dideazafolic acid, **2d**, in order to test the generality of cytotoxicity enhancement caused by removal of the 2-NH_2 group. The parent compound of the former, **1c**, was shown to be a reasonably effective inhibitor

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