oxy]acetanilide, 103789-06-6; 3-(acetylamino)-2,4-pentanedione, 5440-23-3; 4-acetyl-2,5-dimethoxazole, 23000-12-6; 3-(4-nitrophenoxy)propionic acid, 10572-16-4; α-aminopropiophenone hydrochloride, 16735-19-6; α-[[3-(4-nitrophenoxy)propionyl]amino]propiophenone, 141399-55-5; 4-[2-(4-methyl-5-phenyl-2-oxazolyl)ethoxy]nitrobenzene, 107325-00-8; 4-fluorobenzonitrile, 1194-02-1; 4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]benzonitrile, 103789-44-2; 4-hydroxybenzaldehyde, 123-08-0; 4-(2-phenyl-4-thiazolylmethoxy)benzaldehyde, 103789-67-9; β-methyl L-aspartate hydrochloride, 16856-13-6; β-methyl N-benzoyl-L-aspartate, 39741-26-9; methyl 3-(benzoxylamino)-4-oxovalerate, 54819-26-0; methyl 2-(5-methyl-2-phenyl-4-oxazolyl)acetate, 103788-64-3; ethyl 4-chloroacetoacetate, 638-07-3; benzamide, 55-21-0; ethyl 2-(2-phenyl-4-oxazolyl)acetate, 84446-03-7; ethyl 2-(2-cyclohexyl-4-thiazolyl)acetate, 24087-96-5; cyclohexanethiocarboxamide, 7390-42-3.

N-[4-[[(3,4-Dihydro-4-oxo-1,2,3-benzotriazin-6-yl)methyl]amino]benzoyl]-L-glutamic Acid, a Novel A-Ring Analogue of 2-Desamino-5,8-dideazafolic Acid[1]

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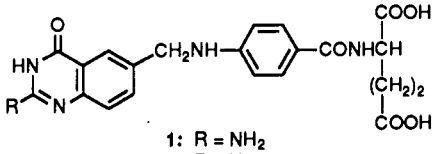
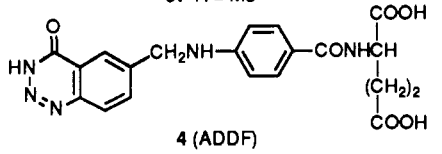
N-[4-[[(3,4-Dihydro-4-oxo-1,2,3-benzotriazin-6-yl)methyl]amino]benzoyl]-L-glutamic acid ("2-aza-2-desamino-5,8-dideazafolic acid", ADDF) was synthesized from 2-amino-5-methylbenzamide via a four-step sequence consisting of diazotization, benzylic bromination, condensation with dimethyl N-(4-aminobenzoyl)-L-glutamate, and ester hydrolysis. ADDF was an inhibitor of recombinant mouse thymidylate synthase; inhibition was competitive with 5,10-methylenetetrahydrofolate as variable substrate (K_i = 2.3 μM). It was a substrate for murine folylpolyglutamate synthetase with kinetic characteristics (K_m = 28 μM) comparable to those of aminopterin, and it inhibited the growth of L1210 cells in culture (IC_{50} = 0.52 μM). The structural modification of the A-ring embodied in ADDF appears to offer a novel, heretofore unexplored approach to the design of TS inhibitors.

The potential therapeutic significance of folic acid analogues targeted against thymidylate synthase (TS) as opposed to dihydrofolate reductase (DHFR) was predicted more than 20 years ago by Borsa and Whitmore.[2] Shortly thereafter, Bird and co-workers[3] reported the potent biological activity of 5,8-dideazafolic acid (1).[4] This led to an extensive program of synthesis of quinazoline analogues substituted at the N^{10} position,[5-7] on the phenyl ring,[8] and in ring B,[9,10] and to selection of N^{10}-propargyl-5,8-dideazafolic acid (CB3717) as a suitable candidate for biochemical[11-13] and clinical[14,15] evaluation. While CB3717 had many desirable pharmacological properties, such as the ability to enter cells by a transport mechanism distinct from that of reduced folates and the classical DHFR inhibitor methotrexate (MTX), its clinical usefulness was hampered by low solubility at physiological pH, which gave rise to hepatic and renal toxicity.[16]



1: R = NH_2
2: R = H
3: R = Me



4 (ADDF)

The toxicity encountered during clinical trials with CB3717 prompted a vigorous search for more soluble congeners, culminating in the discovery of a family of second-generation TS inhibitors that included 2-desamino-5,8-dideazafolic acid (2), 2-desamino-2-methyl-5,8-dideazafolic acid (3), and the corresponding N^{10}-methyl and N^{10}-propargyl analogues.[17-19] Biochemical studies[20-22] revealed that, in general, replacement of the NH_2 group at C^2 by H or Me resulted in weaker binding to purified TS but increased inhibition of the growth of cultured cells.

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(1) Paper 47 in this series. For previous paper, see: Rosowsky, A.; Forsch, R. A.; Reich, V. E.; Freisheim, J. H.; Moran, R. G. Side chain modified 5-deazafolate and 5-deazatetrahydrofolate analogues as mammalian folylpolyglutamate synthetase and glycinamide ribonucleotide formyltransferase inhibitors: synthesis and in vitro biological evaluation. *J. Med. Chem.* **1992**, *35*, 1578–1588.
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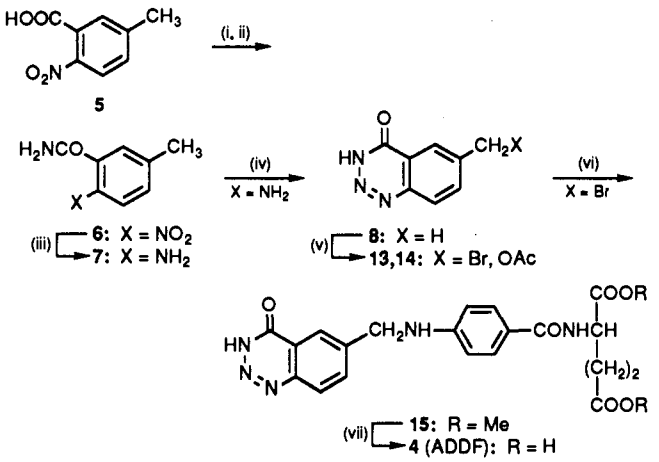
This higher than expected biological potency was rationalized on the basis that (i) substrate activity for folylpolyglutamate synthetase (FPGS) is minimally affected by replacement of the 2-NH_2 group with H or Me, (ii) polyglutamylation increases TS binding by as much as 100-fold, (iii) in contrast to CB3717, 2-desamino and 2-desamino-2-methyl analogues are accepted by the active transport mechanism used by MTX and reduced folates, and (iv) noneffluxing long-chain polyglutamates of these compounds are formed efficiently inside the cell. A large number of analogues of **1–3** modified at the C^2 position,[19] in the benzoyl moiety,[23,24] and in the C^9–C^{10} bridge region[25,26] were synthesized to permit detailed structure–activity analysis. Out of these studies emerged the new TS inhibitor ICI D1694,[27] an exciting third-generation analogue with Me groups at C^2 and N^{10}, and a thiophene ring as the aryl moiety.[28]

Scheme I[a]



[a] Reagents: (i) $SOCl_2$; (ii) NH_3; (iii) H_2/Pd–C; (iv), $NaNO_2$/HCl; (v) *N*-bromosuccinimide/AcOH/*hν*; (vi) 4-$H_2NC_6H_4CONHCH$-(COOMe)CH_2CH_2COOMe; (vii) NaOH.

The absence of a 2-NH_2 group in second- and third-generation analogues of **1** is significant in terms of their ability to interact with TS. According to a recent X-ray crystallographic study of the ternary complex of CB3717 and 2′-deoxyuridylate (dUMP) with TS from *Escherichia coli*,[29] the quinazoline ligand can bind to two different sites in the same domain of the enzyme, and when bound can have two different orientations vis-à-vis the nucleotide ligand. In one orientation, involving the "major" site, the 2-NH_2 group forms an H-bond to the C=O of the penultimate C-terminal residue (position 263, *E. coli* numbering) and to an ordered water molecule which is also H-bonded to N^1; in the other orientation, involving the

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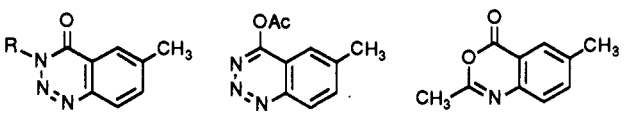
"minor" site, the 2-NH_2 group forms an H-bond to a serine OH (Ser180), and N^1 forms an H-bond to a glutamate COOH (Glu58) which is also H-bonded through an ordered water molecule to Ser180. According to another X-ray crystallographic analysis,[30] this one involving a ternary complex between *E. coli* TS and 5-fluoro-2'-deoxyuridylate (FdUMP) instead of dUMP, the 2-NH_2 group of the quinazoline forms an H-bond to the amide C=O of a residue (Ala263) in the C-terminal region, and N^1 is bound to an ordered water molecule (Wat430) that also interacts with the C=O group of Ala263 and with a highly-conserved arginine residue (Arg21) in the phosphate binding site. The active-site residues with which the 2-NH_2 group of CB3717 interacts in mammalian TS have not yet been described, and may not be exactly the same as those in the *E. coli* enzyme. Moreover, the nature of these interactions may depend on the nucleotide ligand as well as the N^{10} substituent in the quinazoline. However, the evidence reported to date suggests that it is reasonable to suppose that the 2-NH_2 group binds to TS from all species via a network of H-bonds that also involves molecules of ordered water, and that these interactions are probably important not only in correctly aligning the folate cofactor and nucleotide substrate in the active site but also in sequestering them from bulk solvent. When the 2-NH_2 group is replaced by H or Me, binding to the enzyme is diminished because the only H-bonds possible between the quinazoline A-ring and the active site are those involving N^1 as an H-bond acceptor and N^3 as an H-bond donor. The reason the desamino compounds actively inhibit TS in intact cells despite their weaker binding to purified enzyme appears to be that efficient uptake and polyglutamylation compensate for the partial loss of binding caused by removal of the 2-NH_2 group.

In considering possible approaches to improving the solubility of quinazoline inhibitors of TS while preserving their ability to form polyglutamates, we became interested in 2-desamino-4(3*H*)-oxoquinazoline analogues in which C^2 is replaced by a nitrogen atom with its lone pair of electrons. Attractive features of such 2-aza-2-desamino compounds were (i) that removal of H or Me might enable the molecule to extend more deeply into the active-site pocket, and (ii) that N^2 might interact with active-site residues by H-bonding, acting *as an acceptor* either directly or via a molecule of ordered water. Both of these properties ought to favor TS binding. Furthermore, in vivo activity might be anticipated if these compounds are good FPGS substrates and if the polyglutamates show the same increase (100-fold or more) in TS binding as is observed in 2-desamino-5,8-dideazafolates. In this report we describe the synthesis of *N*-[4-[[(3,4-dihydro-4-oxo-1,2,3-benzotriazin-6-yl)methyl]amino]benzoyl]-L-glutamic acid (**4**, "2-aza-2-desamino-5,8-dideazafolic acid", ADDF), which we believe is *the first known example of a 2-azafolate analogue*. We also present data showing that ADDF binds to TS, is a FPGS substrate, and inhibits the growth of tumor cells in culture.

Chemistry

As shown in Scheme I, sequential reactions of 2-nitro-5-methylbenzoic acid (**5**) with $SOCl_2$ and NH_3 afforded 2-nitro-5-methylbenzamide (**6**, 87%), which on catalytic hydrogenation yielded 2-amino-5-methylbenzamide (**7**, 96%).[31] Treatment of **7** with $NaNO_2$/HCl at low temperature[32] then gave 6-methyl-1,2,3-benzotriazin-4(3*H*)-one (**8**, 97%). Confirmation of ring closure to a benzotriazinone came from the ^{1}H-NMR spectrum, in which all three aromatic proton signals were markedly shifted downfield (δ 7.8–8.0), in agreement with the powerful electron-withdrawing character of the triazinone moiety. Several attempts were made to enhance the solubility of **8** by N^3-acylation, with the expectation that this would be needed if subsequent benzylic bromination were done in CCl_4, the traditional solvent for such reactions. Treatment of the Na salt of **8** with (pivaloyloxy)methyl chloride in DMF afforded a single product which appeared to be the desired N^3-derivative **9**. However, in contrast to the facile preparation of the analogous blocked compound in the quinazoline series,[17] all efforts to recrystallize **9** led to loss of the blocking group. We also tried to prepare the N^3-acetyl derivative **10** by reaction of the Ag salt of **8** with acetyl chloride, as has been described for the analogue without a 6-methyl substituent.[33] To our surprise, the reaction of **8** yielded a product whose ^{1}H-NMR spectrum contained two Me groups, but whose UV spectrum differed from that expected on the basis of the literature.[33] Moreover, efforts to purify the product by silica gel chromatography led only to deacylation. Although definitive proof of identity was not obtained, this material was tentatively assigned the *O*-acetyl structure **11**. Finally we tried to prepare **10** from the Na salt of **8** and Ac_2O in DMF, but no reaction occurred at room temperature, and the only product identified after heating was the benzoxazinone **12**.[34]



9: R = *t*-$BuCO_2CH_2$
10: R = Ac

11

12

Bromination of the benzotriazinone **8** without protection at N^3 proved feasible through the use of hot AcOH as the solvent. The presence of the desired product **13** was evident in the ^{1}H-NMR spectrum, which showed a downfield singlet at δ 4.63 as compared with δ 3.30 for the Me group in **8**. Also present were two other singlets, which we believe correspond to **14**, presumably formed by solvolysis of **13**. Heating the crude bromination product (estimated from the ^{1}H-NMR spectrum to contain roughly 40 mol % of **13**) directly with dimethyl *N*-(4-aminobenzoyl)-L-glutamate and $NaHCO_3$ in DMF for 3 days afforded the protected diester **15** (45% crude yield), and further treatment of **15** for a few minutes with NaOH in aqueous MeOH afforded the diacid **4** (ADDF, 81%). The UV absorption spectrum of **4** [λ_{max} (0.1 M HCl) 203, 224, 290 nm; λ_{max} (pH 7.4) 293 nm; λ_{max} (0.1 M NaOH) 298 nm] showed a small bathochromic shift in going from acid to neutral to alkaline pH. IR spectra of both **4** and **15** measured in KBr disks showed strong absorption at 1690 cm^{-1}, indicating that the lactam tautomer **4A** may be present in the solid state. The ^{1}H-NMR was consistent with the benzotriazinone structure, with all three aromatic B-ring protons deshielded (δ 8.10) relative to the 3',5'-protons (δ 6.60) and even the 2',6'-

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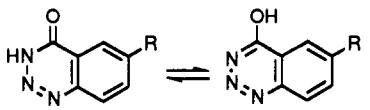
protons (δ 7.57) on the phenyl ring. However, a more interesting feature of the ^{1}H-NMR spectra of diester **15** and diacid **4** was the presence of upfield signals, at δ 1.7 for **15** (in $CDCl_3$ solution) and at δ 2.1 for **4** (in d_6-DMSO solution), which we believe arise from the lactim tautomeric form **4B**. Since the peak areas for these upfield signals in **4** and **15** closely approximated one proton, it appears that very little of the lactam **4A** was present under aprotic conditions. The position of the lactam–lactim equilibrium presumably depends not only on the dielectric constant of the solvent, but also on its protic versus aprotic nature. Whether **4** is in the lactam (**4A**) or lactim (**4B**) tautomeric form when it binds to the TS active site will obviously be worthwhile to examine, since it could be the basis for a fundamental mechanistic difference between 2-desamino-5,8-dideazafolates and 2-aza-2-desamino-5,8-dideazafolates.



4A **4B**

Biological Activity

ADDF was studied as an inhibitor of mouse TS, as an inhibitor of the growth of L1210 cells, and as a substrate for mouse liver FPGS. Affinity column chromatography on 10-formyl-5,8-dideazafolyl-Sepharose was used to purify recombinant mouse fibroblast TS expressed in *E. coli*.[34] Using a spectrophotometric assay, this enzyme had a K_m of 11.3 ± 2.7 μM (n = 4) for (6*R*)-5,10-methylenetetrahydrofolate in excellent agreement with the K_m of 22–27 μM measured using (6*R*,*S*) cofactor with either recombinant or naturally occurring enzyme from LU-37 mouse fibroblasts.[34] ADDF was an inhibitor of this recombinant mouse TS with competitive kinetics against variable cofactor (Figure 1 contained in supplementary material); the K_i for this interaction was 2.3 ± 0.3 μM (n = 3), as compared with published values of 0.067 μM for **1**[27] and 2.0 μM for **2**.[20] Thus, replacement of C^2 and the attached NH_2 group in **1** by a nitrogen atom with a lone pair of electrons had a similar effect on binding to TS as replacement of the 2-NH_2 group by hydrogen. This lends support to our hypothesis that the structural changes embodied in 2-aza-2-desamino antifolates such as ADDF would be a new approach to inhibitors of this enzyme.

The polyglutamate derivatives of CB3717, the 2-desamino analogue of CB3717, and ICI D1694 have been shown to be at least 100-fold more potent inhibitors of TS than the monoglutamate forms of these compounds.[27,28] Because of this clear indication that the potential for polyglutamylation is a major factor in the design of folate analogues inhibitors to TS, the ability of ADDF to serve as a substrate for mouse liver FPGS was studied in vitro. ADDF was a substrate for this enzyme with a K_m of 28 ± 4.3 μM and a V_{max} 1.6 ± 0.1 times that of folic acid (n = 4). These kinetic values are equivalent to those previously observed for aminopterin with this same source of enzyme, and would rank ADDF as a better substrate than CB3717 (K_m = 40 μM) but a less efficiently used substrate than either **1** (K_m = 6.4 μM) or **2** (K_m = 4.8 μM). The fact that the first-order rate constant for the reaction with ADDF as the substrate (V_{max}/K_m = 6.3 ± 0.1 relative to folic acid) was ca. 5-fold less than has been reported for **1** and **2** suggested that the 2-aza-2-desamino compound may be utilized somewhat less efficiently than either the 2-amino or 2-desamino analogues. It should be noted that this in vitro assay measures the conversion of a folyl monoglutamate to a diglutamate, and not to tri- or higher polyglutamate metabolites that efflux from the cell at a slow to nonexistent rate. Nevertheless, it seems reasonable to conclude that ADDF is likely to form noneffluxing polyglutamates, and that the long-chain derivatives of ADDF would be likely to have K_i values in the low nanomolar range.

Continuous exposure of mouse L1210 cells to ADDF for 48 h resulted in complete inhibition of growth at high concentrations and 50% inhibition of growth at a concentration of 0.52 ± 0.08 μM (n = 3). Thus, the potency of ADDF was greater than that reported for **1** (2.7 μM) and similar to that of **2** (0.43 μM).[20]

An interesting feature of the N^{10}-unsubstituted quinazolines **1** and **2** that sets them apart from pure inhibitors of TS is that their inhibitory effect on the growth of L1210 cells is restored to normal levels by a combination of thymidine (dThd) and hypoxanthine (Hx). This observation has been explained on the basis that these quinazolines have a second site of action in the folate pathway, resulting in inhibition of both thymidylate synthesis and purine synthesis. This second site has been identified as DHFR.[20,27] Similar cell culture reversal experiments demonstrated a very interesting pattern with ADDF: when L1210 cells were growth in the presence of 5.6 μM dThd, the IC_{50} of ADDF increased to 0.89 ± 0.09 μM (n = 3), whereas, in the presence of 32 μM Hx, the IC_{50} of ADDF was 0.97 ± 0.18 μM. On the other hand, when both dThd and Hx were present, ADDF was not inhibitory at concentrations as high as 30 μM. Hence, ADDF appeared to inhibit at least two folate-dependent reactions, one of which was TS. The potential for ADDF inhibition of glycinamide ribonucleotide transferase (GARFT) was directly assessed with enzyme purified from mouse L1210 cells: no inhibition was observed at concentrations as high as 30 μM.

In summary, the activity of ADDF against tumor cells, its ability to bind to TS and FPGS, its novel molecular structure, and its relatively easy synthesis all suggest that this compound is a promising lead to the design of cytotoxic antifolates. Manipulations of the structure of ADDF that would result in increased FPGS substrate activity seem particularly interesting at this time.

Experimental Section

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer; only peaks above 1200 cm^{-1} are reported. UV spectra were obtained on a Varian Model 210 instrument. ^{1}H-NMR spectra were obtained on a Varian EM360L spectrometer with Me_4Si or $Me_3Si(CH_2)_3SO_3Na$ as the reference. TLC analyses were done on fluorescent Eastman 13181 silica gel sheets or Eastman 13254 cellulose sheets. Spots were visualized under 254-nm UV illumination. Column chromatography was done on Baker 3405 (60–200 mesh) silica gel or Whatman DE-52 preswollen DEAE-cellulose. Solvents in moisture-sensitive reactions were dried over Linde 4A molecular sieves (Fisher, Boston, MA). HPLC was done on Waters C_{18} radial compression cartridges (analytical: 5 μm particle size, 5 × 100 mm; preparative: 15 μm particle size, 25 × 100 mm). Melting points were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) and are not corrected. Microanalyses were performed by Quantitative Technologies, Inc., Bound Brook, NJ.

6-Methyl-1,2,3-benzotriazin-4(3*H*)-one (8). A mixture of 5-methyl-2-nitrobenzoic acid (**5**) (36.2 g, 0.2 mol) and $SOCl_2$ (50 mL) was heated under reflux for 20 min, during which a homogeneous solution was obtained. After removal of the excess $SOCl_2$ with the aid of a water aspirator, the residue was dissolved in dry THF (40 mL) and the solution was added dropwise with stirring to an ice-cold solution of NaOH (8 g, 0.2 mol) in 28% NH_4OH (300 mL). The precipitate was collected, washed with H_2O, and recrystallized from EtOH–H_2O to obtain 5-methyl-2-nitrobenzamide (**6**) (31.5 g, 87%) as a white solid: mp 176–178 °C (lit.[31]

mp 176–177 °C); IR (KBr) ν 1655 cm^{-1} (amide C=O). A solution of **6** (31.5 g, 0.175 mol) in MeOH (250 mL) was shaken with 5% Pd–C (0.5 g) under 3 atm of H_2 for 24 h in a Parr apparatus. A solid formed after the initial heat of reaction subsided. The mixture was heated to boiling to redissolve the product, and it was filtered while hot. The filtrate was evaporated under reduced pressure, and the residue was dried at 90 °C for 1 h (caution: some sublimation may occur) to obtain 2-amino-5-methylbenzamide (**7**) as a white solid (25.3 g, 96%): mp 173–175 °C (lit.[31] mp 179 °C); ^{1}H-NMR ($CDCl_3$) δ 6.60 (d, 1 H, J = 8 Hz, C_3-H). To an ice-cold suspension of **7** (25.3 g, 0.168 mol) in 3.6 N HCl (260 mL) was added dropwise over 25 min a solution of $NaNO_2$ (12.75 g, 0.185 mol) in H_2O (100 mL), while keeping the internal temperature below 5 °C. After another 20 min of stirring at this temperature, 10 N NaOH (100 mL) was added, causing all the solid to dissolve. The solution was acidified to pH 2 with 12 N HCl and chilled. The solid was filtered and recrystallized from EtOH to obtain off-white needles (26.2 g, 97%): mp 217–218 °C (dec, gas evolution) (lit.[32] mp 219–220 °C); IR (KBr) ν 1680 (lactam C=O); ^{1}H-NMR (d_6-DMSO + D_2O) δ 3.30 (s, 3 H, 6-Me), 8.00 (m, 3 H, aryl); UV (95% EtOH) λ_{max} 208, 225, 282 nm.

Dimethyl *N*-[4-[[(3,4-Dihydro-4-oxo-1,2,3-benzotriazin-6-yl)methyl]amino]benzoyl]-L-glutamate (15). *N*-Bromosuccinimide (1.37 g, 7.7 mol) was added in a single portion to a solution of **8** (1.13 g, 7 mmol) in glacial AcOH (70 mL) in an oil bath at 60 °C. The resulting solution was heated at 70 °C with illumination from a 150-W floodlamp for 2 h. After evaporation of the solvent under reduced pressure, the residue was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was washed with 5% $NaHCO_3$, rinsed with H_2O, and evaporated to obtain a product (0.946 g) which was estimated to consist of a 2:3 mixture of 6-(bromomethyl)-1,2,3-benzotriazin-4(3*H*)-one (**13**) [^{1}H-NMR ($CDCl_3$) δ 4.63] and unchanged **8** [^{1}H-NMR ($CDCl_3$) δ 3.30]. The entire mixture of **13** and **8** from the bromination reaction (estimated to contain 1.97 mmol of **13** from the δ 4.63/δ 3.30 ratio) was added in a single portion to a solution of dimethyl *N*-(4-aminobenzoyl)-L-glutamate (0.588 g, 2 mmol) in dry DMF (10 mL). Then, solid $NaHCO_3$ (0.168 g, 2 mmol) was added, and the mixture was kept in an oil bath at 65 °C for 3 days. The solvent was evaporated under reduced pressure, and the residue was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer (TLC: R_f 0.70, 0.65, 0.4, 0.0; silica gel, 19:1 $CHCl_3$–MeOH) was evaporated, and the residue was chromatographed on a silica gel column (45 g, 2.5 × 40 cm) with 20:1 $CHCl_3$–MeOH as the eluent. Pooled fractions containing the spot with R_f = 0.4 were evaporated in two batches, and the residues were dried separately in vacuo (P_2O_5, 65 °C). The first batch (0.254 g, 28%) was TLC-homogeneous, whereas the other (0.154 g, 17%) contained a small impurity. Rechromatography of the TLC-homogeneous batch afforded a beige powder (0.22 g, 87% recovery): mp 94–102 °C; IR (KBr) ν 3430, 3040, 2960, 1740, 1690, 1640, 1615, 1580, 1515, 1445, 1420, 1335, 1285, 1265 cm^{-1}; NMR ($CDCl_3$) δ 1.71 (br s, 1 H, lactim OH), 2.43 (m, 4 H, CH_2CH_2), 3.63 (s, 3 H, γ-COOMe), 3.75 (s, 3 H, α-COOMe), 4.65 (m, 4 H, CH_2N, NH, α-CH), 6.58 (d, J = 9 Hz, 2 H, 3′- and 5′-H), 7.68 (d, J = 9 Hz, 2 H, 2′- and 6′-H), 7.8–8.4 (m, 3 H, aryl). Anal. ($C_{22}H_{23}N_5O_6$·0.6H_2O) C, H, N.

***N*-[4-[[(3,4-Dihydro-4-oxo-1,2,3-benzotriazin-6-yl)methyl]amino]benzoyl]-L-glutamic Acid (4, ADDF).** A stirred cloudy solution of **15** (195 mg, 0.42 mmol) in MeOH (5 mL) was treated dropwise with 1 N NaOH (5 mL) over 1 min. The solution quickly became homogeneous, and after another 5 min the pH was adjusted to neutrality with HCl. The MeOH was evaporated under reduced pressure, and the product was purified by preparative HPLC (8% MeCN in 0.05 M NH_4OAc, pH 6.9). Pooled pure fractions were concentrated by rotary evaporation and then freeze-dried. The residue was redissolved in H_2O (15 mL), and the solution was lyophilized again to obtain a pale-yellow solid (162 mg, 81%): mp ca. 150 °C (gas evolution after softening at 140–145 °C); IR (KBr) ν 3420, 3150, 3040, 2980, 1690, 1615, 1580, 1555, 1520, 1445, 1405, 1330, 1285, 1270 cm^{-1}; NMR (d_6-DMSO) δ 2.10 (m, 5 H, CH_2CH_2, lactim OH), 4.15 (m, 1 H, α-CH), 4.57 (m, 2 H, CH_2N), 6.60 (d, J = 8 Hz, 2 H, 3′- and 5′-H), 7.02 (m, 1 H, NH), 7.57 (d, J = 8 Hz, 2 H, 2′- and 6′-H, with overlapped m, 1 H, NH), 8.10 (m, 3 H, aryl); UV (0.1 M HCl) λ_{max} 203 nm (ϵ 27 700), 224 (27 600), 290 (12 300); UV (pH 7.4 phosphate buffer) λ_{max} 216–218 nm (plateau, ϵ 32,200), 293 (24,300); UV (0.1 M NaOH) λ_{max} 298 nm (ϵ 25,300). Anal. ($C_{20}H_{19}N_5O_6$·0.75NH_3·2H_2O) C, H, N.

Enzyme Assays. A. Thymidylate Synthase (TS). The plasmid pETSM which allowed expression of mouse fibroblast TS at very high levels was obtained from Dr. Lee F. Johnson (Departments of Biochemistry and Molecular Genetics, Ohio State University). *E. coli* transformed with pETSM were grown in liquid medium, and expression of mouse TS was induced as previously described.[35] Mouse TS extracted from the bacteria was purified to near homogeneity by affinity chromatography on a column of 10-formyl-5,8-dideazafolyl-Sepharose as described by Rode and co-workers[36] with minor modifications.[37] (6*S*)-Tetrahydrofolate was prepared and stored in 1-μmol aliquots as previously described.[38] Immediately prior to enzyme assays, an ampoule of tetrahydrofolate was dissolved in 1% 2-mercaptoethanol (2-ME), and the concentration was determined spectrophotometrically. A solution of 2 mM (6*S*)-tetrahydrofolate was made up in 1% 2-ME containing 25 mM formaldehyde and allowed to stand at room temperature for 10 min to allow formation of (6*R*)-5,10-methylenetetrahydrofolate (CH_2THF). The CH_2THF, along with ADDF and 6 mIU of TS, was added to a cuvette in 950 μL of 3-morpholinopropanesulfonic acid buffer, pH 6.8, and the cuvette was preincubated at 37 °C for 3 min. Both the buffer and the cuvette chamber were preequilibrated at 37 °C. The reaction was initiated by addition of dUMP, in a volume of 50 μL, to the other reactants. Initial rates of reaction were calculated from the change in absorbance at 340 nm. Final concentrations of the reaction components were: CH_2THF, 2.5–20 μM; buffer, 25 mM; formaldehyde, 2.5 mM; 2-ME, 15 mM; dUMP, 100 μM.

B. Folylpolyglutamate Synthetase (FPGS) and Glycinamide Ribonucleotide Formyltransferase (GARFT). FPGS from mouse liver and GARFT from cultured L1210 cells were obtained and assayed as previously described.[37,39]

Cell Growth Inhibition. The origin of the L1210 murine leukemia cells used to measure the effect of ADDF on cell growth, as well as the assay conditions, were described earlier.[40] Three separate experiments using replicate cultures were carried out, and the results were averaged.

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Registry No. **4**, 140410-01-1; **5**, 3113-72-2; **6**, 4315-12-2; **7**, 40545-33-3; **8**, 91532-23-9; **13**, 140410-02-2; **15**, 140410-03-3; TS, 9031-61-2; FPGS, 63363-84-8; GARFT, 9032-02-4; *N*-(4-aminobenzoyl)-L-glutamate, 67278-37-9.

Supplementary Material Available: A Lineweaver–Burk plot for the FPGS-catalyzed addition of glutamic acid to compound **4** (1 page). Ordering information is given on any current masthead page.

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