5-Deaza-7-desmethylene Analogues of 5,10-Methylene-5,6,7,8-tetrahydrofolic Acid and Related Compounds: Synthesis and *In Vitro* Biological Activity [1]

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1-[4-(*tert*-Butyloxycarbonyl)phenyl]-3-pyrrolidinone and 1-[3-(*tert*-butyloxycarbonyl)phenyl]-4-piperidinone were condensed with ethyl cyanoacetate or malononitrile to form ylidene derivatives, which were then subjected sequentially to (i) catalytic or chemical reduction, (ii) condensation with guanidine, and (iii) gentle trifluoroacetic acid treatment to obtain 3-(2,4-diamino-6(5*H*)-oxopyrimidin-5-yl)-1-(4-carboxyphenyl)pyrrolidine (**27**), 4-(2,4-diamino-6(5*H*)-oxopyrimidin-5-yl)-1-(carboxyphenyl)piperidine (**35**), and 3-(2,4,6-triaminopyrimidin-5-yl)-1-(carboxyphenyl)pyrrolidine (**40**). Condensation of **27**, **35**, and **40** with diethyl or di-*tert*-butyl L-glutamate followed by removal of the ester groups yielded *N*-[4-[3-(2,4-diamino-6(5*H*)-oxopyrimidin-5-yl)pyrrolidino]benzoyl]-L-glutamic acid (**13**), *N*-[4-[4-(2,4-diamino-6-(5*H*)-oxopyrimidin-5-yl)piperidino]benzoyl]-L-glutamic acid (**14**), and *N*-[4-[3-(2,4,6-triaminopyrimidin-5-yl)pyrrolidino]benzoyl]-L-glutamic acid (**15**). Compounds **13** and **14** may be viewed as 5-deaza-7-desmethylene analogues of 5,10-methylene-5,6,7,8-tetrahydrofolic and 5,10-ethylene-5,6,7,8-tetrahydrofolic acid, respectively. Compounds **13** and **15** were good substrates for mouse liver folylpolyglutamate synthetase, with K_m values of 20 and 18 μM and a relative first-order rate constant V_{max}/K_m of 2.2 (aminopterin = 1.0). In contrast, **14** was a very poor substrate, with a K_m of 490 μM and a relative V_{max}/K_m of 0.052. As expected from its structure, **15** was a dihydrofolate reductase inhibitor. However its potency was unexceptional (IC_{50} = 1.2 μM). Compounds **13** and **14** were inactive at concentrations of up to 100 μM, and likewise showed no activity against thymidylate synthase or glycinamide ribotide formyltransferase, two other key enzymes of folate-mediated one-carbon metabolism. Compound **15** was moderately active as an inhibitor of the growth of cultured tumor cells (SCC25 human squamous cell carcinoma), with an IC_{50} of 0.37 μM (72 hour exposure). By comparison the IC_{50} of aminopterin was 0.0069 μM. Thus, even though **15** is a good folylpolyglutamate synthetase substrate, the deep-seated skeletal changes embodied in this structure are unfavorable for DHFR binding and may also be unfavorable for transport into cells.

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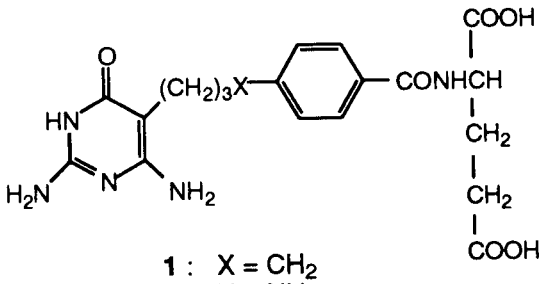
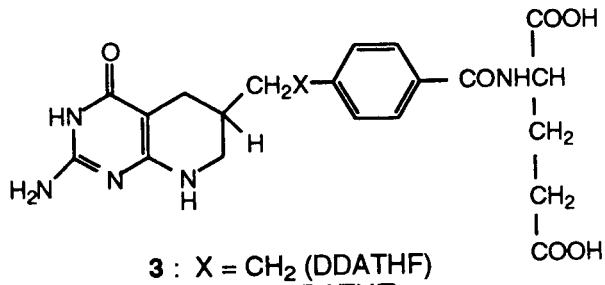
Interest in folate analogues modified in the B-ring and C^9–N^{10} bridge region has been sparked recently by the discovery of several innovative variants of the classical folate structure. Two prominent examples are the ring-opened compounds *N*-[4-(2,4-diamino-6-oxo-1*H*,6*H*-pyrimidin-5-yl)-butyl]benzoyl]-L-glutamic acid (**1**) [3,4] and *N*-[4-[3-(2,4-diamino-6-oxo-1*H*,6*H*-pyrimidin-5-yl)propyl]amino]benzoyl]-L-glutamic acid (**2**) [5,6]. Compounds **1** and **2** possess antitumor activity in cell culture and *in vivo* [5-8], and may be viewed as 7-desmethylene analogues of the potent purine biosynthesis inhibitors 5,10-dideaza-5,6,7,8-tetrahydrofolate (**3**, DDATF) [9,10] and 5-dideaza-5,6,7,8-tetrahydrofolate (**4**, DATHF) [11]. A number of congeners of **1** and **2** further modified in the bridge and phenyl moiety have also been described [3-6,12]. As with DDATHF and DATHF, the primary biochemical target of the 7-desmethylene compounds is considered to be glycinamide ribotide formyltransferase (GARFT) [7,8,13-15]. Another recent group of B-ring analogues is exemplified by the ring-contracted compounds *N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**5**) [16,17] and *N*-[4-[2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl]-L-glutamic acid (**6**) [17], whose potent antitumor activity unexpectedly turned out to be due to inhibition of thymidylate synthetase (TS) rather than GARFT. Other interesting ring-contracted analogues are the 2,4-diamines **7-9** [17,18], whose primary enzyme target (not surprisingly in view of their 2,4-diamino structure) is dihydrofolate reductase (DHFR) rather than TS.

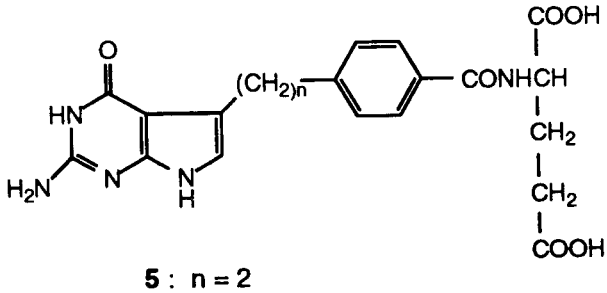
Previous work in this laboratory on folate analogues modified in the B-ring and C^9–N^{10} bridge has included the synthesis of (6*R*,6*S*)-5,8,10-trideaza-5,6,7,8-tetrahydrofolic acid (**10**) [19], 6-aza-5,8,10-trideaza-5,6,7,8-tetrahydrofolic acid (**11**) [20], and 6-aza-5,8,10-trideaza-5,6,7,8-tetrahydroaminopterin (**12**) [20]. In the present paper we describe three other analogues modified in the B-ring/C^9-N^{10} region, namely compounds **13-15**. Structure **13** was formally derived from DATHF (**4**) *via* a disconnection and reconnection strategy wherein one carbon (C^7) was deleted from the B-ring and a new one-carbon bridge was introduced between C^5 and N^{10}. Structure **14** was generated by



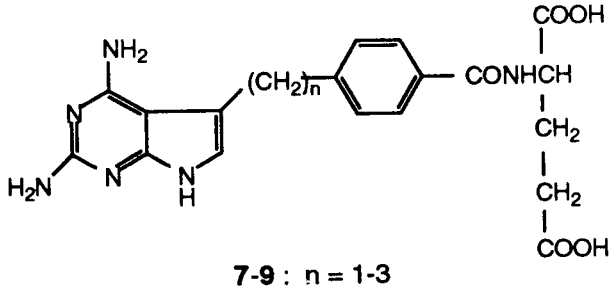
1 : X = CH_2
2 : X = NH



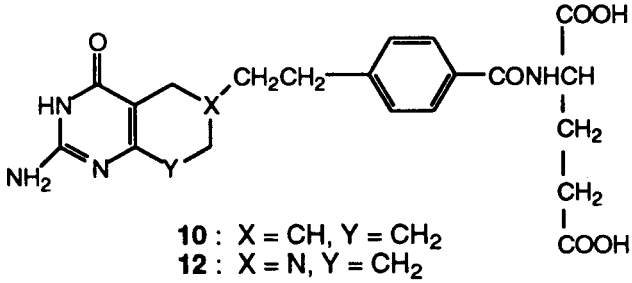
3 : X = CH_2 (DDATHF)
4 : X = NH (DATHF)



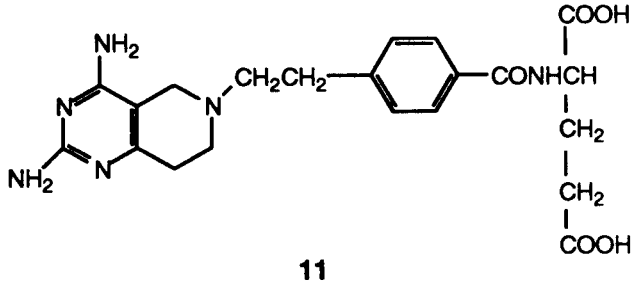
5 : n = 2
6 : n = 3



7-9 : n = 1-3



10 : X = CH, Y = CH_2
12 : X = N, Y = CH_2



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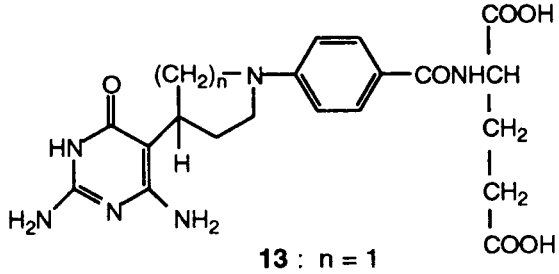
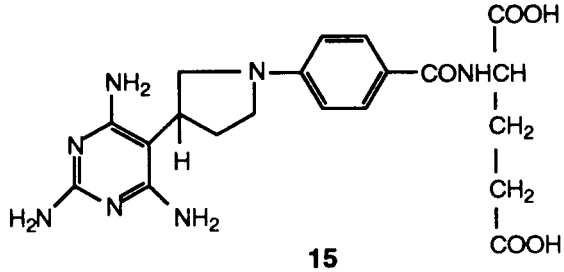


13 : n = 1
14 : n = 2



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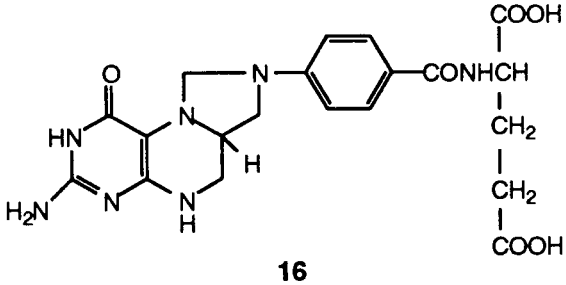

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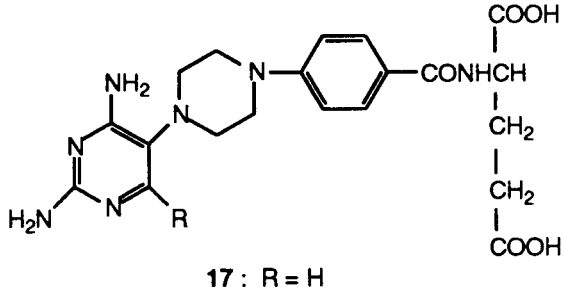


17 : R = H
18 : R = CH_3
19 : R = $NHCH_3$

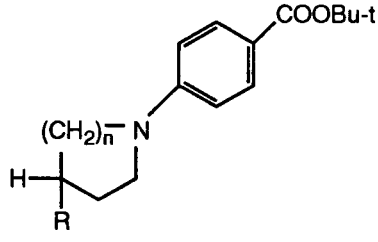
lengthening the bridge to two carbons [21]. We reasoned that **13** and **14** resembled **1** and **2**, respectively, and thus had the potential to interfere with *de novo* purine biosynthesis, especially if they were efficiently converted to polyglutamates in the cell. Polyglutamates of **2** and **3** are known to bind better than the parent monoglutamate to GARFT [5,15]. We also felt that **13** and **14**, and their polyglutamates, had the potential to bind to DHFR depending on how they would align themselves in the active site [22]. Moreover, since **13** may be viewed as a 5-deaza-7-desmethylene analogue of 5,10-methylene-5,6,7,8-tetrahydrofolate (**16**), the normal folate substrate for TS [23], we considered it possible that **10**, or its polyglutamates, might bind to TS as well as to GARFT and/or DHFR. Thus there was the enticing prospect that these compounds, or second-generation derivatives thereof, might interfere with more than one step in folate-mediated one-carbon metabolism.

Chemistry.

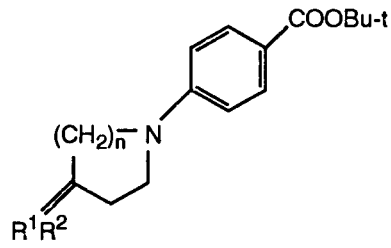
Condensation of 4-hydroxypyrrolidine with *tert*-butyl 4-fluorobenzoate followed by oxidation with dicyclohexylcarbodiimide/dimethyl sulfoxide, using the reported procedures [24] with minor modifications afforded improved yields of *N*-[4-(*tert*-butyloxycarbonyl)phenyl]-3-pyrrolidinol (**20**, 91%) and *N*-[4-(*tert*-butyloxycarbonyl)phenyl]-3-pyrrolidinone (**21**, 74%) respectively. Similarly, 4-hydroxypiperidine was converted to *N*-[4-(*tert*-butyloxycarbonyl)-phenyl]-3-piperidinol (**22**) and *N*-[4-(*tert*-butyloxycarbonyl)-phenyl]-3-piperidinone (**23**) as reported [25]. Attempted reaction of **21** with ethyl cyanoacetate under typical Cope reaction conditions [26] resulted in extensive decomposition. Shorter reaction times, or the use of a basic catalyst at room temperature [27], afforded some conversion to the desired product **24**, along with considerable tar and a significant amount of unchanged **20** from which it could not be separated on silica gel. After considerable experimentation we found it expedient to reduce the crude mixture of **21** and **24** with sodium borohydride in methanol [28] to form a mixture of **20** and **25**, the latter arising *via* concomitant reduction of the double bond and transesterification. That transesterification had occurred was evident from the ^{1}H-nmr spectrum of **25**, which contained a singlet at δ 3.73 (OCH_3). Because the polarity difference between **20** and **25** was greater than between **21** and **24**, pure **25** was easily separated by chromatography on silica gel. Unfortunately the yield of **25** was only 9%, and even the amount of **21** recovered for recycling was only 13%. Heating **25** with guanidine carbonate in refluxing 2-methoxyethanol afforded pyrimidine **26**, but again the yield was low (10%). Treatment of **26** with 1:2 trifluoroacetic acid in methylene chloride at 20° for 3 hours afforded **27** (80%), which on coupling to diethyl L-glutamate in the presence of diethyl phosphorocyanidate [29], followed by saponification yielded the diester **28** (53%) and the diacid **13** (82%).

Since reduction of **24** with sodium borohydride would not be expected to be enantiospecific, **13** is probably an indeterminate mixture of 3*R* and 3*S* isomers (corresponding to 6*R* and 6*S* in tetrahydrofolates with an intact B-ring).

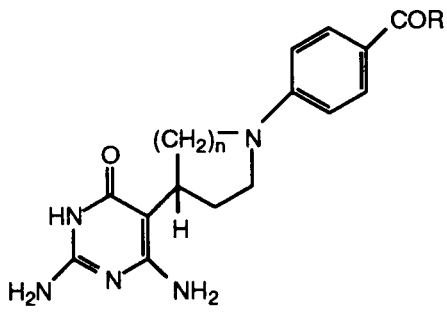
A partial explanation for the unsatisfactory results of the Cope reaction of **21** with ethyl cyanoacetate was suggested by the isolation of a second low-yield product identified as 1-[4-(*tert*-butyloxycarbonyl)phenyl]-3-[cyano(methoxycarbonyl)methyl]pyrrole (**29**, 5%). The structure of **29** was evident from its ^{1}H-nmr spectrum, which displayed three prominent pyrrole signals at 6.4, 7.0, and 7.3, in addition to the characteristic doublets at 7.4 (J = 4.5 Hz) and δ 8.1 (J = 4.5 Hz) for the 4-aminobenzoyl group. We assume that **29** formed when the initial Cope adduct rearranged to a dihydropyrrole which upon loss of two hydrogens, possibly with disproportionation to pyrrolidine **25**, was converted to the aromatic pyrrole. Further elaboration of **29** was of potential interest, as it would yield another novel analogue with a planar five-membered ring between the pyrimidine and 4-aminobenzoyl moiety [30]. Heating **29** with guanidine carbonate, followed by gentle acidolysis with trifluoroacetic acid, afforded the ester **30** and acid **31**, the latter as a partial trifluoroacetate salt. However, reaction of **31** with diethyl L-glutamate by the mixed anhydride method yielded only a unknown product which lacked ester groups and was highly insoluble in organic solvents. Since the amount of **31** available at this stage was very small, further work along this line was not pursued.



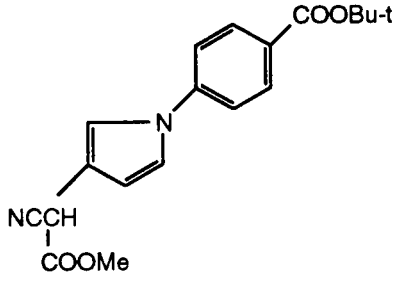
20,22: n = 1,2; R = OH
25: n = 1, R = CH(CN)COOMe
33: n = 2, R = CH(CN)COOEt
38: n = 1, R = $CH(CN)_2$



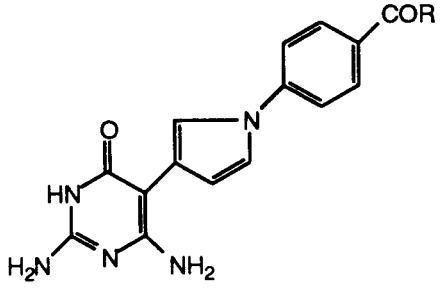
21,23: n = 1,2; R^1R^2 = O
24,32: n = 1,2; R^1R^2 = CH(CN)COOEt
37: n = 1, R^1R^2 = $C(CN)_2$



26,34 : n = 1,2; R = OBu-t
27,35 : n = 1,2; R = OH
28 : n = 1, R = NHCH(COOEt)CH_2CH_2COOEt
36 : n = 2, R = NHCH(COOBu-t)CH_2CH_2COOBu-t



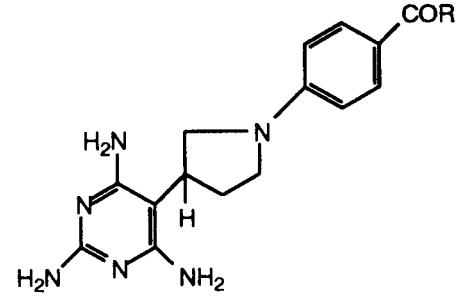
29



30 : R = OBu-t
31 : R = OH

In contrast to the reaction of **21**, condensation of 1-[4-(*tert*-butyloxycarbonyl)phenyl]-4-piperidinone (**23**) [25] with ethyl cyanoacetate under typical Cope conditions proceeded uneventfully, yielding the ylidene derivative **32** (82%), which on catalytic hydrogenation was converted to **33** (100%). The reason for the better behavior of **23** in the Cope reaction may be that it is a β-aminoketone whereas **21** is an α-aminoketone and thus is inductively deactivated by the close proximity of the electron-withdrawing nitrogen. Heating **33** with guanidine in refluxing ethanol for 29 hours afforded only a modest yield of the pyrimidine **34** (13%), which on acidolysis with trifluoroacetic acid was converted to **35** (74%). Further reaction of **35** with di-*tert*-butyl L-glutamate in the presence of diethyl phosphorocyanidate, followed by acidolysis with trifluoroacetic acid, then yielded diester **36** (89%) and diacid **14** (72%) respectively. The low yield obtained in the reactions of guanidine with cyanoesters **25** and **33** invites comment. While it was in marked contrast to the results obtained with simpler α-(ω-aralkyl)-α-cyanoesters [3,4,31], and in one case an α-(4-aryl-4-tosylamino)butyl-α-cyanoester in which the nitrogen was non-basic [6], this low yield may reflect deactivation of the ester group because of an unfavorable long-range dipole effect of the C-N bond in the conformationally defined pyrrolidine or piperidine ring.

Having determined that 2,6-diaminopyrimidin-4(3*H*)-one analogs of DATHF could be made in this manner we also made 2,4,6-triaminopyrimidine **15**, reasoning that it would almost surely be a DHFR inhibitor and might provide an indication of how well this general type of molecule gets into cells. Ketone **21** was condensed with malononitrile, and the resulting ylidene **37** converted uneventfully to **15** *via* intermediates **38-41**. While the Cope reaction proceeded much better with malononitrile than ethyl cyanoacetate, separation of **37** and **21** again proved difficult but could be avoided by reducing the mixture directly to **38** and **20**. In keeping with the chiral nature of C^4 in the pyrrolidine ring of **15**, it is likely that this compound, like **13** and **14**, was a mixture of diastereomers.



39 : R = OBu-t
40 : R = OH
41 : R = NHCH(COOEt)CH_2CH_2COOEt

Biological Evaluation.

Folylpolyglutamate synthetase plays a pivotal role in the cellular pharmacology of classical antifolates with a glutamate side-chain, in that the addition of two or more γ-glutamyl residues greatly prolongs cellular retention and can increase binding to target enzymes by at least two orders of magnitude [32,33]. Thus, a folate analogue may be a potent inhibitor of cell growth even when its non-polyglutamylated from (*i.e.*, the parent drug) is not a particularly good inhibitor of folate-dependent enzymes of one-carbon metabolism. Compounds **13-15** were tested as FPGS substrates under the usual *in vitro* conditions with partly purified mouse liver enzyme in the presence of excess L-gluta-

mate as described earlier [34]. Pyrrolidines **13** and **15** proved to be very good substrates, with K_m values of 20 and 18 μM as compared with 32 μM for the reference compound aminopterin. Since the V_{max} of these compounds was essentially the same as that of aminopterin (AMT), the relative first-order constant k' (V_{max}/K_{max}) was 2.2 in both cases. By contrast, piperidine **14** was a very poor substrate, with a K_m of 490 μM, a relative V_{max} of 0.76 (AMT = 1.0), and a relative k' of 0.052 (AMT = 1.0). Thus, increasing ring size by one carbon resulted in a 40-fold loss of glutamylation efficiency. Still, it was of interest that the K_m of **14** was only two-fold higher than that of homofolic acid [34]. Since **14** is undoubtedly a mixture of 4*R* and 4*S* isomers (corresponding to 6*R* and 6*S* in compounds with an intact B-ring), it is possible that one of these isomers is actually as good a substrate for FPGS as homofolic acid.

It was of interest to compare the FPGS substrate activity of compounds **13-15** with that of another recently described set of acyclo analogues with a hetero ring at the 5-position [35]. It appears from this comparison that a six-membered ring at the 5-position does not necessarily decrease FPGS binding. For example, compound **17**, in which the ring is piperazine and the 6-substituent is H instead of NH_2, has a reported K_m of 17 μM [35]. On the other hand when the 6-substituent is Me (**18**) the K_m becomes 950 μM, and further increases in size of the 6-alkyl group are even more detrimental. Thus, hydrophilic groups appear to be tolerated better than hydrophobic groups at the 6-position of the pyrimidine ring (corresponding to the 8-position in molecules with an intact B-ring). It may be noted that the piperazine analogue **19** with a MeNH group at the 6-position has been synthesized but its FPGS substrate activity is not reported [36]. On the basis of our results and those reported previously for **17** and **18** we would expect **19** to have FPGS substrate activity comparable to **13** and AMT. It would also be of interest to determine the effect of changing the ring at the 5-position in **17** and **18** to pyrrolidine or piperidine, but these compounds are unknown.

As expected, **15** was a DHFR inhibitor, but its potency was unexceptional. In an assay using human recombinant DHFR [37], the IC_{50} of **15** was found to be 1.2 μM as compared with 0.011 μM for methotrexate, indicating a 100-fold difference in binding. Compounds **13** and **14** were also tested as DHFR inhibitors but were inactive at concentrations up to 100 μM. Moreover, neither TS [37] nor GARFT [38] inhibition was observed at this concentration.

Since binding of antifolates to TS [39] and GARFT [15] is known to be increased by as much as two orders of magnitude when the glutamate side chain is extended by polyglutamylation, there was the possibility that **10** and **12** could still inhibit the growth of tumor cells if their polyglutamates become good inhibitors of these enzymes. In a growth inhibition assay in which SCC25 human head-and-neck squamous carcinoma cells were exposed continuously to drug for 72 hours, **12** was found to have an IC_{50} of 0.37 μM. It thus appears that **12** is taken up reasonably well by the cells. However, its potency was 50-fold lower than that of AMT, whose IC_{50} under the same assay conditions was found to be only 0.0069 μM. In contrast to the modest activity of **12**, SCC25 cell proliferation was unaffected by **10** at concentrations of up to 10 μM. Thus replacement of the 4-amino group in **12** by oxygen led to a 30-fold loss of activity consistent with the decrease in DHFR binding. The lack of activity of **10** against intact cells could be due to a number of factors including poor uptake, failure to undergo intracellular polyglutamylation, or the inability of polyglutamates (if they form) to bind significantly better than the parent compound to DHFR, TS, GARFT, or other enzymes of the reduced folate pathway. We have previously documented other examples of folate analogues with favorable FPGS substrate kinetics but little or no biological activity [19,40].

EXPERIMENTAL

Solvents for moisture-sensitive reactions were dried over Linde 4A molecular sieves (Fisher, Boston, MA). Chemicals were purchased from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO). Infrared (ir) spectra were obtained on a Perkin-Elmer Model 781 spectrophotometer in potassium bromide disks unless otherwise specified; peaks below 1600 cm^{-1} are not reported. Ultraviolet (uv) spectra were recorded on a Varian Model 210 instrument, and ^1H nmr spectra on a Varian Model EM360L instrument in deuteriochloroform solution with tetramethylsilane as the reference. The presence of fractional amounts of organic solvents in analytical samples of some of the glutamate esters, which is not unusual in our experience [*cf.*, for example, references 19,41,42], could not be prevented despite careful drying *in vacuo* and was confirmed whenever possible from ^1H nmr spectra. Analytical tlc was performed on fluorescent Baker Si250F silica gel plates or Eastman 13254 cellulose sheets. Spots were visualized under 254 mn uv illumination or with the aid of an iodine chamber. Preparative thin-layer chromatography (tlc) was on Analtech silica GF plates (20 x 20 cm, 1000 μm layer thickness). Column chromatography was on Baker 70-230 mesh silica gel, Baker 'Flash' silica gel (40 μm particle size), or Whatman DE-52 pre-swollen *N,N*-diethylaminoethyl cellulose (DEAE-cellulose). Drying of organic solvents was done over magnesium sulfate unless otherwise specified. Melting points (uncorrected) were obtained on a Fisher-Johns hot stage apparatus. Microanalyses were by Robertson Laboratory, Madison, NJ.

tert-Butyl 4-(3-Hydroxypyrrolidino)benzoate (**20**).

Anhydrous potassium carbonate (5.7 g, 0.041 mole) was added to a stirred solution of 3-hydroxypyrrolidine (4.63 g, 0.049 mole) [43] and *tert*-butyl 4-fluorobenzoate (8.04 g, 0.041 mole) [44] in dimethyl sulfoxide (35 ml), and the reaction mixture was kept at 120-125° under a nitrogen atmosphere for 6.5 hours. The mixture was poured into water (300 ml) with stirring, and the precipitate was filtered, washed with water, and dried by lyophilization to obtain a solid (9.77 g, 91%), mp 168-169° (unchanged on re-

crystallization from 1:1 dichloromethane-hexanes); tlc: R_f 0.71 (silica gel, 95:5 chloroform-methanol); ir: ν 3500, 2960, 2860, 1680, 1615 cm^{-1}; ^1H nmr: δ 1.56 (s, 9H, *t*-Bu), 2.03 (s, 1H, OH), 1.83-2.33 (m, 2H, CH_2), 3.1-3.7 (m, 4H, CH_2N), 4.6 (m, 1H, CH), 6.47 (d, J = 4 Hz, aromatic protons *ortho* to N), 7.83 (d, J = 4 Hz, 2H, aromatic protons *ortho* to ester).

Anal. Calcd. for $C_{15}H_{21}NO_3$: C, 68.41; H, 8.04; N, 5.32. Found: C, 68.35; H, 8.04; N, 5.08.

1-[4-(*tert*-Butyloxycarbonyl)phenyl]-3-pyrrolidinone (**21**).

Pyridine (4.0 ml) and trifluoroacetic acid (1.8 ml) were added dropwise and sequentially to a stirred solution of alcohol **20** (12.8 g, 0.0486 mole) and *N,N*-dicyclohexylcarbodiimide (30.1 g, 0.146 mole) in a mixture of dimethyl sulfoxide (55 ml) and benzene (102 ml) at a rate such that the internal temperature did not exceeded 5°. Stirring at 5° was continued for 3 hours and then at 20° for 15 hours. Ethyl acetate (400 ml) was added, and the solution was washed with water (275 ml, 100 ml, 100 ml). The solid which formed in the organic layer was collected. Several additional crops formed on reducing the volume to 40 ml and cooling to 0°. The combined crops (14.1 g) were resuspended in benzene (110 ml), and the mixture was heated to reflux and filtered while hot to remove *N,N*-dicyclohexylurea (3.82 g). Refrigeration of the filtrate yielded **21** as colorless plates (7.27 g), mp 187-188°. Concentration of the mother liquor afforded a second crop of identical material weighing 2.06 g, total yield 9.33 g (73%); tlc: R_f 0.80 (silica gel, 95:5 chloroform-methanol); ir: ν 3430, 3330, 2980, 2930, 2890, 2850, 1760, 1690, 1610 cm^{-1}.

Anal. Calcd. for $C_{15}H_{19}NO_3$: C, 68.94; H, 7.33; N, 5.36. Found: C, 69.24; H, 7.48; N, 5.59.

1-[4-(*tert*-Butyloxycarbonyl)phenyl]-3-[methoxycarbonyl(cyano)-methyl]pyrrolidine (**25**).

Ammonium acetate (0.38 g, 4.5 mmoles) was added to a solution of ketone **21** (1 g, 3.8 mmoles) [24], ethyl cyanoacetate (0.509 g, 4.5 mmoles), and glacial acetic acid (0.275 g, 4.5 mmoles) in dry benzene (15 ml), and the mixture was stirred under reflux, using a Dean-Stark trap, for 30 minutes. The solution was washed with water and saturated sodium bicarbonate, dried, and evaporated under reduced pressure. The residue was warmed with ether (25 ml), and the mixture left overnight at −20°. Filtration gave a yellow powder (0.60 g) whose ir spectrum indicated it to be a mixture of olefin **24** and unchanged **21**; ir: ν 2230 (nitrile), 1760 (aromatic ester), 1690 (unsaturated ester) cm^{-1}.

A suspension of 0.76 g of the foregoing mixture of **24** and **21** (combined from two experiments) in methanol (20 ml) at 0° was treated with a solution sodium borohydride (106 mg, 2.8 mmoles) in methanol (5 ml). After 15 minutes, the solution was allowed to come to 20° and was left for 2.5 hours. The solution was acidified with a few drops of acetic acid and concentrated to dryness by rotary evaporation. The residue was redissolved in a small volume of 95:5 chloroform-methanol and applied onto a column of 'Flash' silica gel (28 g, 2 x 27 cm), which was eluted with the same solvent mixture. Fractions containing a spot with R_f 0.85 (silica gel, 95:5 chloroform-methanol) were pooled and evaporated to obtain **25** as an oil (131 mg, 9.3%); tlc: R_f 0.35 (silica gel, chloroform); ir (film): ν 3050, 2980, 2940, 2850, 2240, 2750, 1690, 1605 cm^{-1}; ^1H nmr (deuterioacetone): δ 1.53 (s, 9H, *t*-Bu), 2.5-3.7 (m, 7H, CH_2), 3.73 (s, 3H, MeO), 6.53 (d, J = 4.5 Hz, 2H, aromatic protons *ortho* to N), 7.96 (d, J = 4.5 Hz, 2H, aromatic protons *ortho* to ester). An analytical sample was repurified on a column of 'Flash' silica gel by elution with 1:2 ether-hexanes. The fractions with R_f 0.22 (silica gel, 99:1 chloroform-methanol) were combined, evaporated, and kept under vacuum over phosphorous pentoxide at 60° overnight.

Anal. Calcd. for $C_{19}H_{24}N_2O_4 \cdot 0.33(C_2H_5)_2O$: C, 66.16; H, 7.46; N, 7.59. Found: C, 65.94; H, 7.18; N, 7.63.

Continued elution afforded fractions with R_f 0.39 (95:5 chloroform-methanol), which after crystallization from benzene yielded alcohol **20** (132 mg, 13%) of suitable quality for oxidative recycling to **21**.

3-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)-1-[(4-*tert*-butyloxycarbonyl)phenyl]pyrrolidine (**26**).

A mixture of **25** (1.48 g, 43 mmoles) and guanidine carbonate (7.75 g, 43 mmoles) was stirred under reflux in 2-methoxyethanol (65 ml) for 1 hour. The solvent was evaporated under reduced pressure, and the residue dissolved in chloroform (250 ml). The solution was washed with water (200 ml, 100 ml), dried, and evaporated. The residue was adsorbed onto a column of 'Flash' silica gel (50 g, 3 x 19 cm) which was eluted with 85:15:1 chloroform-methanol-concentrated ammonia. Fractions with R_f 0.42 (silica gel, 85:15:1 chloroform-methanol-concentrated ammonia) were combined and evaporated to obtain a tan-colored solid (175 mg, 10%), mp 218-220°; ir: ν 3430, 1700, 1610 cm^{-1}; uv (0.1 *N* hydrochloric acid): λ max 228, 272 nm. The analytical sample was obtained by preparative tlc on a silica gel with 85:15:1 chloroform-methanol-concentrated ammonia as the developing solvent. Extraction of the product spot with the same solvent mixture followed by evaporation under reduced pressure afforded **26** as a colorless solid, mp 195°.

Anal. Calcd. for $C_{19}H_{25}N_5O_3 \cdot 1.75H_2O$: C, 56.62; H, 7.13; N, 17.38. Found: C, 56.57; H, 6.73; N, 17.11.

3-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)-1-(4-carboxyphenyl)pyrrolidine (**27**).

(a) A solution of **26** (39.6 mg, 0.0983 mmole) in 2:1 dichloromethane-trifluoroacetic acid (1 ml) was kept at 20° for 3 hours and concentrated to dryness by rotary evaporation at water aspirator pressure and a bath temperature of 20°. Methanol was added twice and the solution re-evaporated to remove final traces of trifluoroacetic acid. The final residue was dissolved in dilute ammonium hydroxide (4 ml) at *p*H 9.0, the solution was filtered to remove some cloudiness, and the filtrate adjusted to *p*H 5.1 with 10% acetic acid. After cooling to 5°, the precipitated solid was filtered and dried by lyophilization to obtain an off-white powder (28 mg, 80%), mp 265-266°; ir: ν 3400, 3200, 1650, 1600 cm^{-1}.

Anal. Calcd. for $C_{15}H_{17}N_5O_3 \cdot 0.7CH_3COOH$: C, 55.11; H, 5.60; N, 19.60. Found: C, 54.83; H, 5.48; N, 19.74.

(b) A solution of **26** (235 mg, 0.583 mmole) in 2:1 dichloromethane-trifluoroacetic acid was treated as above. The final residue was dissolved in methanol (5 ml) and the solution kept at −20°. The solid was filtered, washed with ether, and dried *in vacuo* over phosphorus pentoxide at 20° to obtain the *trifluoroacetate salt* of **27** as a yellow solid (151 mg, 58%), mp 224-225°; ir: ν 3530, 3410, 3230, 2860, 1735, 1695, 1650, 1630, 1610 cm^{-1}.

Anal. Calcd. for $C_{15}H_{17}N_5O_3 \cdot CF_3CO_2H \cdot 0.5CH_3OH$: C, 47.19; H, 4.52; F, 12.80; N, 15.72. Found: C, 47.56; H, 4.34; F, 13.12; N, 15.72.

Diethyl *N*-[4-[3-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)pyrrolidino]-benzoyl]-L-glutamate (**28**).

Diethyl phosphorocyanidate (133 mg, 0.815 mmole) and triethylamine (0.114 ml, 82.5 mg, 0.815 mmole) were added to a solu-

tion of **27** trifluoroacetate (140 mg, 0.326 mmole) in dry *N,N*-dimethylformamide (25 ml). After 2 hours at 20°, diethyl L-glutamate hydrochloride (195 mg, 0.815 mmole) was added, followed by triethylamine (0.136 ml, 99 mg, 0.978 mmole). The solution was allowed to stand at room temperature for 20 hours, and the solvent was distilled off with the aid of a rotary evaporator and vacuum pump. The residue was dissolved in chloroform, and the solution was washed twice with water, dried, concentrated to a small volume, and applied onto a silca gel column (7.5 g, 1.0 x 20 cm) which was eluted with 9:1 chloroform-methanol. Fractions giving a tlc spot with R_f 0.21 (silica gel, 9:1 chloroform-methanol) were pooled and evaporated. The residue was warmed in ethyl acetate, and the mixture cooled at −20° and filtered. The solid was washed successively with ethyl acetate and ether, and dried *in vacuo* at 80° over phosphorous pentoxide to obtain **28** as a pale-yellow powder (105 mg, 53%), mp 136-138°; ir: ν 3420, 3350, 3200, 2980, 1735, 1645, 1605 cm^{-1}.

Anal. Calcd. for $C_{24}H_{32}N_6O_6 \cdot 0.35H_2O$: C, 56.87; H, 6.50; N, 16.58. Found: C, 57.08; H, 6.59; N, 16.26.

N-[4-[3-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)pyrrolidino]benzoyl]-L-glutamic Acid (**13**).

A solution of **28** (110 mg, 0.217 mmole) in a mixture of 1 *N* sodium hydroxide (1.73 ml) and methanol (5 ml) was kept at room temperature for 4 hours. The solvent was distilled off under reduced pressure, the residue was taken up in water (4 ml), and the solution was filtered. The filtrate was adjusted to *p*H 4.5 with acetic acid and left briefly at 5°. The precipitate was collected, washed with water, and dried by lyophilization. Drying *in vacuo* at 80° over phosphorus pentoxide afforded **13** as a colorless solid (85 mg, 82%), mp 204-206°; tlc: R_f 0.75 (cellulose powder, *p*H 7.4 phosphate buffer); ir: ν 3340, 3200, 2950, 2860, 1710, 1640, 1605 cm^{-1}; uv (0.1 *N* sodium hydroxide): λ max 277 nm (ϵ 18,400), 310 (26,600); (0.1 *N* hydrochloric acid): λ max 226 nm (ϵ 15,900), 271 (21,950), 308 (5,950).

Anal. Calcd. for $C_{20}H_{24}N_6O_6 \cdot 0.5CH_3CO_2H$: C, 53.15; H, 5.52; N, 17.77. Found: 52.92; H, 5.78; N, 17.77.

1-[4-(*tert*-Butyloxycarbonyl)phenyl]-4-[cyano(ethoxycarbonyl)-methylene]piperidinone (**32**).

A mixture of 1-[4-(*tert*-butyloxycarbonyl)phenyl]-4-piperidinone (4.13 g, 0.015 mole) (**23**) [25], ethyl cyanoacetate (1.7 g, 0.015 mole), glacial acetic acid (1.0 ml, 0.015 mole), and ammonium acetate (1.15 g, 0.015 mole) in dry benzene (15 ml) was stirred under reflux for 4 hours in a flask fitted with a Dean-Stark trap. Additional portions of ethyl cyanoacetate (0.5 g), glacial acetic acid (0.25 ml), and ammonium acetate (0.25 g) were then added, and reflux was continued for another 2 hours. The benzene layer was separated, diluted to a volume of 30 ml with benzene, washed with water (3 x 200 ml), dried over magnesium sulfate, and evaporated under reduced pressure. The residue was dissolved in 95:5 chloroform-methanol, and the solution applied onto a column of 'Flash' silica gel (5 x 14 cm). Elution with the same solvent mixture afforded fractions with R_f 0.83 (silica gel, 98:2 chloroform-methanol) which on pooling and evaporation yielded **32** as a pale-yellow powder (4.55 g, 82%), mp 132-134° (methanol-hexanes); ir: ν 3400, 3000, 2980, 2940, 2400, 2220, 1720, 1700, 1610 cm^{-1}; uv (95% ethanol): λ max 227, 305 nm; ^1H nmr: δ 1.40 (t, 3H, CH_3CH_2, 1.57 (s, 9H, *t*-Bu), 2.7-3.7 (m, 8H, CH_2), 4.33 (q, 2H, CH_3CH_2), 6.87 (d, J = 4.5 Hz, 2H, aromatic protons *ortho* to N), 7.93 (d, J = 4.5 Hz, 2H, aromatic protons *ortho* to ester).

Anal. Calcd. for $C_{21}H_{26}N_2O_4$: C, 68.06; H, 7.10; N, 7.56. Found: C, 67.77; H, 7.30; N, 7.82.

1-[4-(*tert*-Butyloxycarbonyl)phenyl]-4-[cyano(ethoxycarbonyl)-methyl]piperidine (**33**).

A solution of **32** (3.25 g, 0.0877 mole) in absolute ethanol (100 ml) was shaken with hydrogen and 10% palladium on charcoal (0.35 g) at an initial pressure of 52 psi. After 2 hours the catalyst was filtered, and the solution evaporated to dryness and left on the rotary evaporator for an additional 2 hours at 60° (bath) to obtain **33** as a thick colorless oil (3.28 g, *ca.* 100%); tlc: R_f 0.25 (silica gel, 99:1 chloroform-methanol); ir (thin film): ν 2240, 1740, 1695, 1600 cm^{-1}; ^1H nmr: δ 1.33 (t, 3H, CH_3CH_2), 1.55 (s, 9H, *t*-Bu), 1.7-2.0 (m, 1H, CH_2CHCH_2), 2.0-4.2 (m, 8H, CH_2), 3.4 (d, 1H, CHCOOEt), 4.26 (q, 2H, CH_3CH_2), 6.83, J = 4.5 Hz, 2H, aromatic protons *ortho* to N), 7.91 (d, J = 4.5 Hz, 2H, aromatic protons *ortho* to ester).

Anal. Calcd. for $C_{21}H_{28}N_2O_4$: C, 67.70; H, 7.57; N, 7.52. Found: C, 67.59; H, 7.63; N, 7.52.

4-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)-1-[4-(*tert*-butyloxycarbonyl)phenyl]piperidine (**34**).

Guanidine hydrochloride (0.143 g, 1.5 mmoles) was added to a solution of sodium ethoxide prepared by dissolving sodium metal (34.5 mg, 1.5 atom equivalents) in absolute ethanol (40 ml). After 30 minutes of refluxing, compound **33** (0.45 g, 1.2 mmoles) was added, refluxing under nitrogen was resumed for another 23 hours, and the solvent was evaporated under reduced pressure. The residue was redissolved in chloroform, and the solution was washed twice with water, dried over magnesium sulfate, concentrated to a small volume, and applied onto a column of 'Flash' silica gel (20 g, 1.5 x 17 cm). The column was eluted with 95:1 and then 9:1 chloroform-methanol, and fractions showing a spot with R_f 0.22 (silica gel, 9:1 chloroform-methanol) were pooled and evaporated. The yellow solid (61 mg, 13%) was washed with a small volume of chloroform to obtain the analytical sample, mp 243-245°; ir: ν 3500, 3460, 3360, 3200, 1705, 1670, 1620, 1600 cm^{-1}.

Anal. Calcd. for $C_{20}H_{27}N_5O_3 \cdot 0.2CHCl_3$: C, 59.27; H, 6.70; N, 17.11. Found: C, 59.09; H, 7.04; N, 16.72.

4-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)-1-(4-carboxyphenyl)piperidine (**35**).

A solution of ester **34** (262 mg, 0.64 mmole) in trifluoroacetic acid (4.5 ml) was kept at 0° for 2 hours and evaporated to dryness at low temperature. Methanol was added and removed twice by rotary evaporation, and the residue was redissolved in water. The *p*H was adjusted to 6.3 with 10% ammonium hydroxide, and the precipitate was filtered, washed with water, and freeze-dried to obtain **35** as a yellow solid (168 mg, 74%), mp >300°; tlc: R_f 0.58 (cellulose, *p*H 7.4 phosphate buffer); R_f 0.22 (silica gel, 10:6:1 chloroform-methanol-concentrated ammonia); ir: ν 3480, 3370, 3100, 2920, 1650, 1605 cm^{-1}. The analytical sample was obtained by dissolving a small portion of the product in dilute ammonia and adding glacial acetic acid dropwise until a yellow solid formed, whose elemental analysis showed it to be a partial acetate salt.

Anal. Calcd. for $C_{16}H_{19}N_5O_3 \cdot 0.45CH_3CO_2H$: C, 56.95; H, 5.88; N, 19.65. Found: C, 56.62; H, 5.91; N, 19.93.

Di-*tert*-butyl *N*-[4-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)piperidino]benzoyl-L-glutamate (**36**).

A suspension of **35** (147 mg, 0.412 mmole) in dry *N,N*-dimethylformamide (25 ml) was treated consecutively with triethylamine (0.115 ml, 83.3 mg, 0.824 mmole) and diethyl phosphocyanidate (0.125 ml, 134 mg, 0.824 mmole). A clear solution formed after 20 minutes. Stirring was continued for a total of 1.5 hours, at which time di-*tert*-butyl L-glutamate hydrochloride (244 mg, 0.824 mmole) and a second portion of triethylamine (0.147 ml, 107 mg, 1.06 mmoles) were added. After 24 hours at room temperature the solvent was removed by rotary evaporation and the residue was washed with ether and taken up in chloroform. The solution was washed with water, dried, concentrated to a small volume, and applied onto a column of silica gel (7.5 g, 1.0 x 20 cm) which was eluted successively with 95:5 and 9:1 chloroform-methanol. Fractions with R_f 0.22 (silica gel, 9:1 chloroform-methanol) were pooled and evaporated to obtain **36** as a pale-yellow solid (216 mg, 89%), mp 137-138°. The analytical sample was recrystallized from 10:1 ether-chloroform and dried *in vacuo* over phosphorus pentoxide; ir: ν 3450-3350, 1730, 1640-1610 cm^{-1}.

Anal Calcd. for $C_{29}H_{42}N_6O_6 \cdot H_2O$: C, 59.16; H, 7.53; N, 14.28. Found: C, 58.90; H, 7.39; N, 14.17.

N-[4-[2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)piperidino]benzoyl-L-glutamic Acid (**14**).

A solution of **36** (173 mg, 0.28 mmole) in 2:1 dichloromethane-trifluoroacetic acid (2 ml) was kept at 20° for 2 hours and then poured into 5% ammonia (20 ml). After extraction with chloroform (20 ml) the aqueous layer was freeze-dried and the residue purified by preparative hplc on C_{18} silica gel with 2% acetonitrile in 0.01 *M* ammonium acetate, *p*H 7.8, as the eluent at a flow rate 10 ml/minute. The main peak (30 minutes) was collected and freeze-dried to constant weight (115 mg, 72%), mp 182° (softening at 174°); ir ν 3370, 3180, 2980, 2820, 1645-1620, 1610 cm^{-1}; uv (0.1 *N* sodium hydroxide): λ max 275 nm (ϵ 27,200), 296 infl (21,900); (0.1 *N* hydrochloric acid): λ max 228 nm (ϵ 19,600), 270 (21,800). The analytical sample was obtained by dissolving a small portion in dilute ammonia, cooling to 0°, and adding acetic acid dropwise until a yellow solid precipitated, whose elemental analysis showed it to be a partial ammonium salt of **14**.

Anal. Calcd. for $C_{21}H_{26}N_6O_6 \cdot 0.33NH_3 \cdot 3.1H_2O$: C, 48.50; H, 6.43; N, 17.05. Found: C, 48.18; H, 5.96; N, 17.03.

1-[4-(*tert*-Butyloxycarbonyl)phenyl]-3-[cyano(ethoxycarbonyl)-methyl]pyrrole (**29**).

A mixture of ketone **21** (8.34 g, 0.0295 mole), ethyl cyanoacetate (3.72 ml, 3.96 g, 0.035 mole), ammonium acetate (2.70 g, 0.035 mole), and glacial acetic acid (2.14 g, 0.035 mole) in benzene (50 ml) was stirred and refluxed in a Dean-Stark apparatus for 6 hours. The solution was washed with water (3 x 20 ml), dried, evaporated to a small volume, and applied onto a silica gel column (10-230 mesh, 300 g, 5 x 59 cm), which was eluted successively with chloroform and 99:1 chloroform-methanol. Fractions containing a tlc spot with R_f 0.56 (silica gel, 99:1 chloroform-methanol) were pooled, evaporated, redissolved in 1:1 ether-hexanes, and applied onto a column of neutral alumina (Woelm activity grade I, 33 g, 1.5 x 23 cm), which was eluted with the same solvent. The first 150 ml of eluate, containing a tlc spot with R_f 0.88 (aluminum oxide, ether), was evaporated and the residue was rechromatographed on the same column with 1:1 cyclohexane-benzene as the eluent. Fractions with R_f 0.88 (aluminum oxide, ether) were pooled and concentrated to an oil by rotary evaporation, yield 0.514 g (4.9%); ir (thin film): ν 2980, 2930, 2240, 1740, 1705, 1610 cm^{-1}; ^1H nmr: δ 1.30 (t, 3H, CH_3CH_2), 1.62 (s, 9H, *t*-Bu), 4.3 (q, 2H, CH_3CH_2), 4.7 (s, 1H, α-CH), 6.4-7.3 (m, 3H, pyrrole protons), 7.4 (d, 2H, J = 4.5 Hz, aromatic protons *ortho* to N), 8.05 (d, 2H, J = 4.5 Hz, aromatic protons *ortho* to ester.

Anal. Calcd. for $C_{20}H_{22}N_2O_4$: C, 67.77; H, 6.16; N, 7.90. Found: C, 67.76; H, 6.33; N, 7.86.

3-(2,4-Diamino-6(5*H*)-oxopyrimidin-5-yl)-1-(4-carboxyphenyl)pyrrole (**31**).

A mixture of **29** (0.49 g, 1.38 mmoles) and guanidine carbonate (2.49 g, 13.8 mmoles) in 2-methoxyethanol (20 ml) was refluxed for 2 hours. The solvent was removed *in vacuo*, the residue was taken up in chloroform (125 ml), and the solution was washed with water (2 x 100 ml), dried, and evaporated. The residue was taken up in a small volume of 1:15:85 concentrated ammonia-methanol-chloroform and applied onto a column of 'Flash' silica gel (20 g, 1.5 x 23 cm), which was eluted with the same solvent mixture. Fractions containing a tlc spot with R_f 0.45 (silica gel, same eluent) were pooled and evaporated, and the residue was warmed in ethyl acetate (1.5 ml). Filtration yielded ester **30** as a colorless solid (81 mg), mp 221-222° dec. Evaporation of the mother liquor and crystallization of the residue from acetone gave another 27 mg of colorless solid, raising the total yield to 108 mg (21%) of material sufficiently pure to be used in the next step; ir: ν 1705, 1605 cm^{-1}; uv (0.1 *N* sodium hydroxide): λ max 276, 297 (infl) nm.

A solution of ester **30** (105 mg, 0.286 mmole) in 2:1 methylene chloride-trifluoroacetic acid (3 ml) was kept at room temperature for 3 hours and removed by rotary evaporation at 25°. After two cycles of methanol addition and evaporation, the residual trifluoroacetic acid in the residue was partially neutralized with dilute ammonium hydroxide. A solid formed immediately, and was collected and dried *in vacuo* at 80° over phosphorous pentoxide to obtain **31** (80 mg) as a partial trifluoroacetate salt, colorless plates, mp 204-206°; tlc: R_f 0.15 (blue-fluorescent, cellulose, *p*H 7.4 phosphate buffer), mp 204-206°; ir; ν 3440, 3340, 3200, 3090, 2900, 2140, 1880, 1660, 1610 cm^{-1}.

Anal. Calcd. for $C_{15}H_{13}N_5O_3 \cdot 0.4CF_3COOH$: C, 53.17; H, 3.78; N, 19.62. Found: C, 53.21; H, 3.82; N, 19.62.

The mother liquor was freeze-dried, the residue was redissolved in water (10 ml), and the *p*H of the solution was adjusted from 6.9 to 4.0 with acetic acid. The precipitated solid was centrifuged, washed with water, lyophilized, and heated *in vacuo* at 90° over phosphorus pentoxide to obtain another 21 mg of colorless material with an ir spectrum almost identical to that of the first crop; total yield 101 mg.

Anal. Calcd. for $C_{15}H_{13}N_5O_3 \cdot 0.175CF_3COOH \cdot 1.45H_2O$: C, 51.59; H, 4.53; N, 19.60. Found: C, 51.61; H, 4.17; N, 19.60.

1-[4-(*tert*-Butyloxycarbonyl)phenyl]-3-(dicyanomethylene)pyrrolidine (**37**).

A mixture of ketone **21** (0.5 g, 1.9 mmoles), malononitrile (0.51 g, 7.7 mmoles), ammonium acetate (0.19 g, 2.5 mmoles), and glacial acetic acid (0.138 g, 2.3 mmoles) in benzene (15 ml) was refluxed in a Dean-Stark apparatus for 30 minutes. The solution was decanted from a small amount of dark oil, then washed with water and saturated sodium bicarbonate, dried, reduced to a small volume by rotary evaporation, and diluted with three volumes of cyclohexane. After standing at 20°, an orange solid precipitated. The solid was filtered, redissolved in 99:1 chloro-

form-methanol (15 ml), and chromatographed on silica gel (70-230 mesh, 18 g, 1.5 x 23 cm). The column was eluted with 99:1 chloroform-methanol, and fractions with a tlc spot at R_f 0.25 (silica gel, same eluent) were pooled. Evaporation and drying *in vacuo* at 60° over phosphorus pentoxide gave **37** as a yellow powder (252 mg, 43%), mp 172-176° after drying *in vacuo* at 60° over phosphorous pentoxide; ir: ν 2240 (nitrile), 1695 (ester), 1610 cm^{-1}; ^1H nmr: δ 1.53 (s, 9H, *t*-Bu), 3.32 (t, 2H, $=CCH_2CH_2$), 3.70 (t, 2H, CH_2CH_2N), 4.41 (s, 2H, $=CCH_2N$), 6.6 (d, 2H, J = 4 Hz, aromatic protons *ortho* to N), 7.39 (d, 2H, J = 4 Hz, aromatic protons *ortho* to ester).

Anal. Calcd. for $C_{18}H_{19}N_3O_2$: C, 69.88; H, 6.19; N, 13.58. Found: C, 69.79; H, 6.10; N, 13.30.

3-(2,4,6-Triaminopyrimidin-5-yl)-1-(4-*tert*-butyloxycarbonylphenyl)pyrrolidine (**39**).

A solution of sodium borohydride (58 mg, 1.53 mmoles) in methanol (5 ml) was added to a stirred suspension of **37** (430 mg, 1.39 mmoles) in methanol (20 ml) at 0°. After 15 minutes the cooling bath was removed and the solution allowed to remain at 20° for 2 hours. A few drops of acetic acid were added, the solution was concentrated to dryness by rotary evaporation, and the residue was chromatographed on a column of 'Flash' silica gel (16 g, 1.5 x 22 cm) with 1:1 chloroform-ether as the eluent. Fractions showing a tlc spot with R_f 0.21 (silica gel, 99:1 chloroform-methanol) were pooled and evaporated to obtain **38** as an off-white solid (306 mg, 71%). Recrystallization from methanol and drying *in vacuo* at 50° over phosphorus pentoxide afforded colorless needles, mp 119-123°; ir: ν 2250, 1680, 1605 cm^{-1}; ^1H nmr: δ 1.56 (s, 9H, *t*-Bu), 1.8-4.0 (m, 7H, pyrrolidine protons), 6.53 (d, 2H, J = 4.5 Hz, aromatic protons *ortho* to N), 7.9 (d, 2H, J = 4.5 Hz, aromatic protons *ortho* to ester).

Guanidine hydrochloride (120 mg, 1.26 mmoles) was added to a solution of sodium (29 mg, 1.26 atom equivalents) in methanol (20 ml), and the solution was refluxed for 30 minutes. A solution of **38** (340 mg, 1.05 mmoles) from the previous step was then added, and refluxing was continued, under nitrogen, for 24 hours. The solvent was removed by rotary evaporation, and the residue taken up in 1:15:85 concentrated ammonia-methanol-chloroform. A small amount of undissolved solid was filtered off, and the solution was chromatographed on a column of 'Flash' silica gel (16 g, 1.5 x 20 cm) with 1:15:85 concentrated ammonia-methanol-chloroform as the eluent. Fractions showing a tlc spot with R_f 0.37 (silica gel, same eluent) were pooled and evaporated. The residue was dried *in vacuo* at 60° to obtain **39** as a beige solid (324 mg, 82%), mp 138-140°. A colorless analytical sample was crystallized from acetone at −20°; ir: ν 3470, 3350, 3190, 2980, 2920, 2850, 1700, 1605 cm^{-1}; uv (95% ethanol): λ max 281 (infl), 305 nm.

Anal. Calcd. for $C_{19}H_{26}N_6O_2 \cdot 0.25H_2O$: C, 60.85; H, 7.06; N, 22.41. Found: C, 60.86; H, 7.24; N, 22.46.

3-(2,4,6-Triaminopyrimidin-5-yl)-1-(4-carboxyphenyl)pyrrolidine (**40**).

Ester **39** (270 mg, 0.72 mmole) was dissolved in 2:1 methylene chloride-trifluoroacetic acid precooled to 0°, and the solution was kept at 0° for 5.5 hours and then concentrated to dryness by rotary evaporation at room temperature. Methanol was added twice to the residue and distilled off, and the resulting solid was taken up in dilute ammonia at *p*H 10. The solution was filtered through Celite, and the filtrate was acidified to *p*H 6.0 with acetic acid and left at 0° overnight. The solid was filtered, washed, and freeze-dried to obtain **40** as a colorless solid (211 mg, 89%), mp 294°; ir: ν 3430, 3340, 3150, 2850, 1650, 1600 cm^{-1}.

Anal. Calcd. for $C_{15}H_{18}N_6O_2 \cdot 0.25CH_3COOH$: C, 56.52; H, 5.81; N, 25.52. Found: C, 56.88; H, 5.64; N, 25.81.

N-[3-(2,4,6-Triaminopyrimidin-5-yl)pyrrolidino]benzoyl-L-glutamic Acid (**15**).

A stirred suspension of **40** (222 mg, 0.67 mmole) in dry *N,N*-dimethylformamide (35 ml) was treated sequentially with triethylamine (0.187 ml, 136 mg, 1.34 mmoles) and diethyl phosphorocyanidate (203 ml, 219 mg, 1.34 mmoles). A clear solution formed within 10 minutes. After 1.5 hours of stirring, diethyl L-glutamate hydrochloride (353 mg, 1.47 mmoles) was added, followed by another portion of triethylamine (205 ml, 149 mg, 1.47 mmoles). The solution was kept at 20° for 20 hours and volatiles were removed by rotary evaporation. The residue was taken up in chloroform, and the solution washed twice with water, dried, reduced to a small volume, and applied onto a column of 'Flash' silica gel (8 g). The column was eluted consecutively with chloroform and 1:15:85 concentrated ammonia-methanol-chloroform. Fractions containing a product with R_f 0.51 (silica gel, same eluent) were pooled and evaporated, and the residue was dissolved in methanol (2 ml) and reprecipitated with ether (40 ml). The solid was isolated by centrifugation and dried at 20° over phosphorus pentoxide to obtain **41** as a cream-colored powder which was used directly in the next step, mp 102-104°; ir: ν 3400, 3200, 2980, 1730, 1640, 1610 cm^{-1}.

A solution of **41** (215 mg, 0.33 mmole) in methanol (5 ml) was treated with 1 *N* sodium hydroxide (1 ml) and the mixture was stirred at room temperature for 5 hours. A small amount of insoluble material was removed by centrifugation and the supernatant was concentrated to dryness by rotary evaporation. The residue was taken up in water (2 ml), the *p*H adjusted to 5.0 by dropwise addition of acetic acid at 0°, and the precipitate isolated by centrifugation and dried by lyophilization, yield 91 mg, mp 235-238°. Freeze-drying of the mother liquor, redissolution of the residue in water, and reprecipitation at *p*H 5 afforded an additional 42 mg of solid, raising the total to 133 mg (76%); ir: ν 3420, 3340, 3150, 2840, 1650, 1600 cm^{-1}; uv (0.1 *N* sodium hydroxide): λ max 219 nm (ϵ 26,100), 284 (24,950), 302 (26,100); (0.1 *N* hydrochloric acid): λ max 288 nm (ϵ 34,100). A sample of this material was further purified by hplc on C_{18} silica gel using 10% acetonitrile in 0.1 *M* ammonium acetate, *p*H 5.5, as the mobile phase at a flow rate of 6 ml/minute. Lyophilization followed by drying over phosphorus pentoxide at 100° afforded the analytical sample of **15** as a partial acetate salt (67 mg).

Anal. Calcd. for $C_{20}H_{25}N_7O_5 \cdot 0.35CH_3COOH \cdot 1.25H_2O$: C, 51.05; H, 5.98; N, 20.13. Found: C, 50.99; H, 5.87; N, 20.18.

DHFR Inhibition.

Human recombinant DHFR was expressed in *E. coli* and purified by affinity chromatography on MTX-Sepharose and by gel filtration as described [37]. Enzyme activity was measured at 22° by following the change in uv absorbance at 340 nm in an assay solution containing 60 μM NADPH, 50 μM dihydrofolate, and 3.00-3.44 mU of enzyme in 50 μM Tris HCl, *p*H 7.0. The reaction was initiated with dihydrofolate after pre-incubating the other components for 2 minutes. The IC_{50} was averaged from three assays performed on different days.

Cell Growth Inhibition.

SCC25 human head-and-neck squamous cell carcinoma cells

[45] were plated at 2 x 10^3 per well in 96-well microtiter plantes and incubated in Dulbecco's Modified Eagle's medium with 10% fetal bovin serum for 24 hours at 37° in a 5% carbon dioxide humidified atmosphere. Drugs were added at ten concentrations between 0.1 n*M* and 100 μM, and incubation was continued for an additional 72 hours. Growth was determined by measurement of the 530 nm absorbance after protein staining with Sulforhodamine B as described [46]. The IC_{50} estimated from semi-log plots were averaged after three or more experiments on different days.

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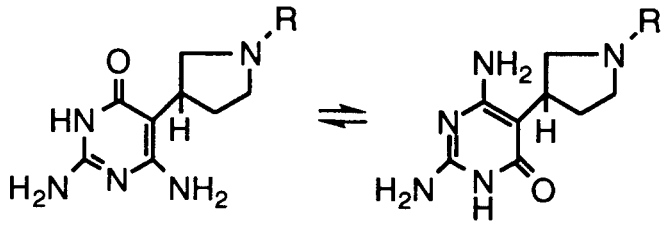

13A **13B**

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