6-Aza-5,8,10-trideaza Analogues of Tetrahydrofolic Acid and Tetrahydroaminopterin: Synthesis and Biological Studies [1]

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6-Aza-5,8,10-trideaza-5,6,7,8-tetrahydrofolic acid (**3**) and 6-aza-5,8,10-trideaza-5,6,7,8-tetrahydroaminopterin (**4**) were synthesized from 6-aza-5,8,10-trideaza-5,6,7,8-tetrahydropteroic acid (**5**) and 4-amino-6-aza-5,8,10-trideaza-4-deoxy-5,6,7,8-tetrahydropteroic acid (**6**), respectively, by mixed carboxylic-carbonic anhydride condensation with dimethyl L-glutamate followed by ester hydrolysis. The pteroic acid analogues **5** and **6** were prepared in several steps from 1-benzyl-3-carbethoxypiperidin-4-one *via* 2-amino-6-benzyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one (**7**). Compound **3** did not inhibit the growth of L1210 mouse leukemia cells in culture, and was not an inhibitor of dihydrofolate reductase (DHFR) or thymidylate synthase (TS). It was a very poor substrate for mouse liver folylpolyglutamate synthetase (FPGS). The 2,4-diamino analogue **4** was only a marginal substrate for FPGS, yet showed activity comparable to methotrexate as a DHFR inhibitor and as an inhibitor of tumor cell growth. The cytotoxicity of **4** is noteworthy because this compound is, to our knowledge, the first example of a classical antifolate which forms polyglutamates poorly even though it contains an intact *p*-aminobenzoyl-L-glutamic acid side-chain. The inability of **3** and **4** to form polyglutamates indicates that a basic nitrogen at position 6 is highly unfavorable for binding to FPGS.

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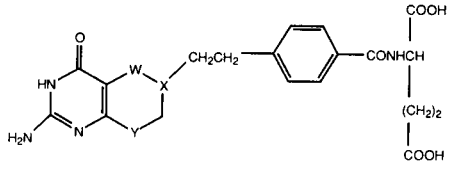
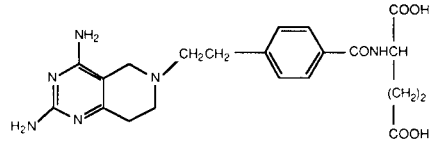
Carbon for nitrogen and nitrogen for carbon substitutions in the pteridine ring and 9,10-bridge region have been investigated in a number of laboratories as a means of identifying the essential structural features required in folate analogues of the 2,4-diamino and 2-amino-4(3*H*)-oxo type for antifolate activity and inhibition of tumor cell growth [2]. Replacement of a single nitrogen by carbon has included 1-deaza [3a,b], 3-deaza [3a,3b], 5-deaza [4a-d], 8-deaza [5a-d], and 10-deaza [6a-g] compounds, while replacement of a single carbon by nitrogen has been limited to 7-aza analogues [7a,b]. In some instances two or more substitutions have also been made, as in 5,8-dideaza [8a-j], 5,10-dideaza [9a-c], 8,10-dideaza [10a-c], 5,8,10-trideaza [11a-c], 9-aza-10-deaza [12a,b], and 9-aza-5,8,10-trideaza [8e,i] compounds, respectively. This bioisosteric replacement approach, with further refinements involving 10-substitution and, in some instances, reduction to a 5,6,7,8-tetrahydro derivative, has led to intensive preclinical and clinical evaluation of three promising new antifolates. One of these, 10-ethyl-10-deazaaminopterin (EDAM) [13a-f], inhibits dihydrofolate reductase (DHFR), while the other two, 10-propargyl-5,8-dideazafolate (PDDF) [14a-i] and 5,10-dideaza-5,6,7,8-tetrahydrofolate (**1**, DDATHF) [15a,b], act at the level of thymidylate synthase (TS) and glycinamide ribotide transformylase (GAR TFase), respectively. A notable aspect PDDF and DDATHF is the possibility of combining these drugs with lipophilic DHFR inhibitors to achieve therapeutic synergy [16,17]. These developments continue to stimulate interest in new antifolates as potential chemotherapeutic agents.

In a previous paper in this series [11c], we demonstrated that 5,8,10-trideaza-5,6,7,8-tetrahydrofolate (**2**), like **1**, was an excellent substrate for folylpolyglutamate synthetase (FPGS) but was inactive as an inhibitor of tumor cell growth in culture. We concluded that replacement of NH by CH_2 at position 8 was highly detrimental to biological activity, either because the NH was required for GAR TFase binding or, as a less likely reason, because it was important for transport. To further explore the role of nitrogen substitution in ring B, we were interested in examining compounds wherein carbon has been replaced by nitrogen at position 6, *i.e.*, pyrido[4,3-*d*]pyrimidine derivatives. Although members of this ring system designed as lipid-soluble small-molecule antifolates were reported in 1972 [18], analogues containing the classical *p*-aminobenzoyl-L-glutamate side chain required for active transport and polyglutamylation have not been described. This paper reports the synthesis and *in vitro* biological evaluation of the glutamate-containing pyrido[4,3-*d*]pyrimidine analogues, 6-aza-5,8,10-trideaza-5,6,7,8-tetrahydrofolate (**3**) and 6-aza-5,8,10-trideaza-5,6,7,8-tetrahydroaminopterin (**4**). Unlike **1**, **3** was inactive as an inhibitor of tumor cell growth in culture. Moreover, **3** differed from **2** in being a very poor substrate for FPGS. Compound **4**, on the other hand, was a potent inhibitor of DHFR and was active against cultured cells even though it was a substrate for

polyglutamylation only at very high concentrations. These results indicate that the introduction of a basic nitrogen at position 6 fails to restore the biological activity lost on replacement of NH at position 8 in **1** by CH_2. However, despite the fact that the introduction of a basic center at position 6 in the 2,4-diamino compound **4** results in great loss of binding to FPGS, this structure modification is well tolerated for binding to DHFR. As a result, **4** shows activity comparable to the classical antifolate, methotrexate, as an inhibitor of tumor cell growth in culture.



1: W = CH_2, X = CH, Y = NH
2: W = Y = CH_2, X = CH
3: W = Y = CH_2, X = N



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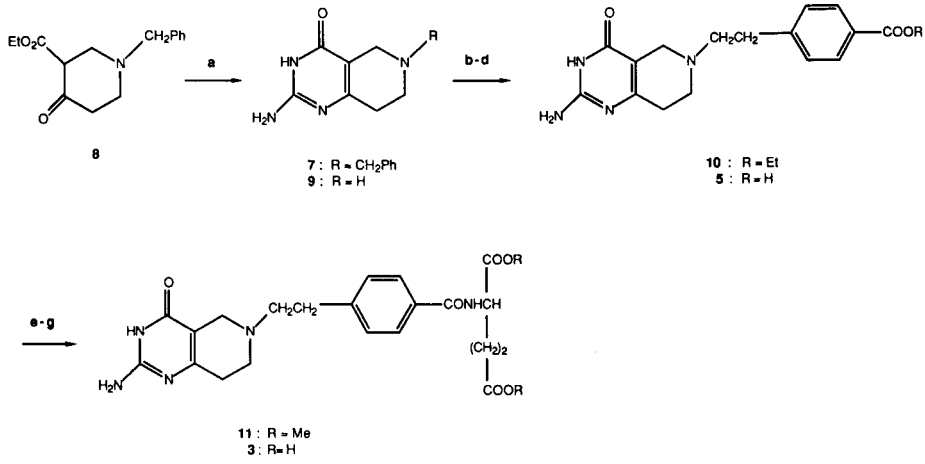
Chemistry.

After considering several possible approaches to the synthesis of the desired compounds, we chose the routes outlined in Schemes 1 and 2. Key intermediates in our approach were the heretofore undescribed compounds, 2-amino-5,6,7,8-tetrahydro-6-[2-(4-carboxyphenyl)ethyl]-pyrido[4,3-*d*]pyrimidin-4(3*H*)-one (**5**) and 2,4-diamino-5,6,7,8-tetrahydro-6-[2-(4-carboxyphenyl)ethyl]pyrido-[4,3-*d*]pyrimidine (**6**), which may be viewed as 6-aza-5,8,10-trideaza-5,6,7,8-tetrahydro analogues of pteroic and 4-amino-4-deoxypteroic acid, respectively. Further elaboration of **5** to **3** and of **4** and **6** would be achieved by coupling to dimethyl L-glutamate followed by ester hydrolysis.

2-Amino-6-benzyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one (**7**) was prepared from ethyl 1-benzyl-4-oxopiperidine-3-carboxylate (**8**) and guanidine as described in the literature [19a,b], and it was debenzylated catalytically in the presence of acid to obtain **9**·2HCl (99% yield). Direct condensation of **9** with 4-(2-bromoethyl)benzoic acid in either ethanol or dimethylsulfoxide in the presence of *N*-ethyl-*N*,*N*-diisopropylamine and potassium iodide gave only small yields of impure alkylation product **10**. The reaction proceeded in better yield with ethyl 4-(2-bromoethyl)benzoate, which has been claimed to react with amines by intermediate formation of ethyl 4-vinylbenzoate followed by Michael addition [20]. However, conversion to **10** remained less than complete (30-46% yield), even after prolonged reaction and with the bromo compound added in excess. On the other hand, since **10** could be prepared on a large scale and subsequent steps afforded good yields, the alkylation reaction was satisfactory for our needs. Alkaline hydrolysis of ester **10** at room temperature afforded the acid **5** (94% yield), and coupling of **5** with dimethyl L-glutamate *via* the mixed carboxylic-carbonic anhydride method gave the diester **11** (85%). The coupling reaction was performed with several cycles of activation, as previously described [21]. Mild alkaline hydrolysis of **11** in aqueous methanol followed by purification of the product by anion-exchange chromatography yielded **3** (95%).

Scheme 1



a: $H_2NC(=NH)NH_2$, **b**: H_2/Pd-C, **c**: $BrCH_2CH_2C_6H_4(4\text{-}COOEt)$, **d**: NaOH, **e**: *i*-BuOCOCl/Et_3N, **f**: $H_2NCH(COOMe)CH_2CH_2COOMe$, **g**: NaOH

The starting material required for the preparation of **6** was 2,4-diamino-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine (**12**), which Elslager and coworkers [18] obtained from 4-amino-3-cyano-1,2,5,6-tetrahydropyridine (**13**) and guanidine carbonate. To our surprise, attempts to duplicate this synthesis under the reported conditions (heating in 1,2-diethoxyethane at 125° for 18 hours) afforded a complex mixture of products from which no more than a trace of **12** was recoverable. We attributed this to lack of solubility of the reactants in 1,2-diethoxyethane, and therefore examined other solvents. When the reaction was performed at the same temperature and for the same length of time (125°, 18 hours) in diethylene glycol dimethyl ether, which increased solubility, a 13% yield of **12** was obtained. The yield increased when a refluxing (131°) 3:1 mixture of 2-methoxyethanol and diethylene glycol dimethyl ether was used, but only to 19%. Extensive purification had to be carried out in order to remove numerous byproducts, and alternative approaches to the synthesis of **12** were therefore investigated. In one of these, 1-benzyl-4-oxopiperidine (**14**) was condensed with cyanoguanidine, both under fusion conditions [22a] and in dimethyl sulfoxide [22b], and the resulting product (**15**) was catalytically debenzylated as in the case of **7**. A variety of conditions were examined for the cyanoguanidine reaction, ranging from 4.5 hours at 150° to 10 minutes at 190°, but the yield of **12** after catalytic debenzylation proved again to be very low (< 10%). In a third attempt to increase the yield of **12**, the 4-oxo compound **7** was converted to 2-amino-6-benzyl-4-chloro-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine (**16**, 72%) by treatment with phosphorus oxychloride in refluxing *N,N*-dimethylformamide, and **16** was converted smoothly to the corresponding 4-hydrazino derivative **17** (71%) by reaction with ethanolic hydrazine at room temperature. Hydrogenolysis of **17** over 10% palladium-on-carbon in the presence of acid gave the heretofore unknown 4-hydrazino derivative **18** (81%), while further hydrogenolysis of **18** in the presence of Davison sponge nickel yielded **12** (92%). Although it involves more steps than the other two methods, this final route appears to be the best one presently available.

Condensation of **12** with ethyl 4-(2-bromoethyl)benzoate under conditions analogous to those employed for the preparation of **10** afforded the 2,4-diamino ester **19** (26% yield), and alkaline hydrolysis of **19** gave the acid **6** (93%). Attempts to couple **6** to dimethyl L-glutamate *via* the mixed anhydride method as in the synthesis of **11** proved unsuccessful, but when coupling was performed with the aid of diethyl phosphorocyanidate [21] the desired 2,4-diamino diester **20** (41%) was formed. Mild alkaline hydrolysis followed by anion-exchange chromatography then gave the diacid **4** (93%).

Biological Evaluation.

Compounds **3** and **4** were tested for the ability to inhibit the growth of L1210 mouse leukemia cells, to act as substrates for partially purified FPGS from mouse liver, and to inhibit purified DHFR and TS from human an murine leukemic cells, respectively (Table 1). The methods employed for these assays have been described earlier [23].

Scheme 2

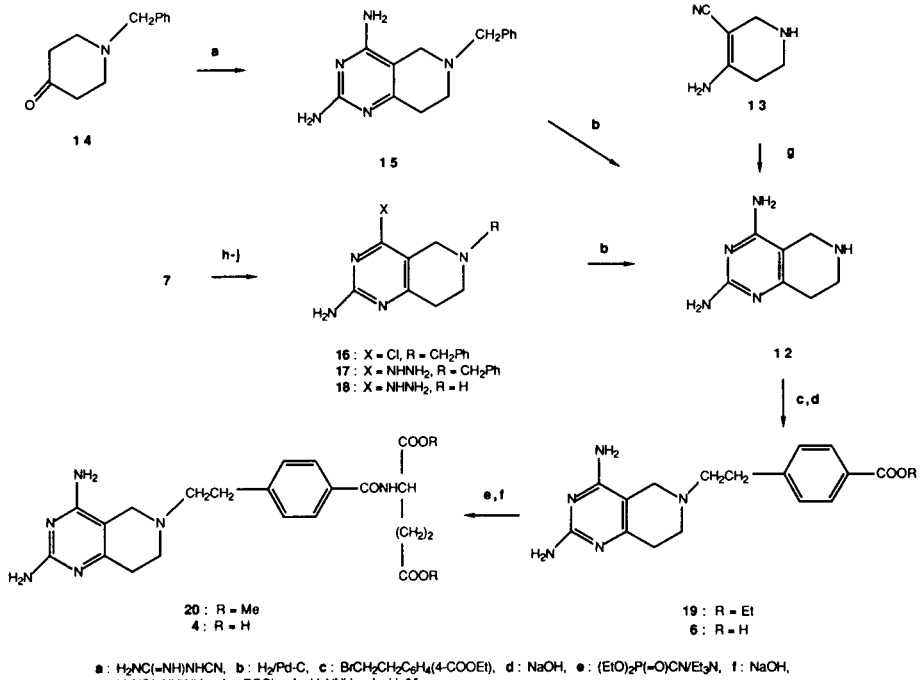


Table 1

Biological Activity of 6-Aza-5,8,10-tridea-5,6,7,8-tetrahydroafolic Acid (**3**) and 6-Aza-5,8,10-trideaza-5,6,7,8-tetrahydroaminopterin (**4**)

Compound	Cells, IC_{50} (μM)		Enzyme Inhibition		FPGS Substrate Activity [a]		
	L1210	L1210/R81	DHFR, IC_{50} (μM)	TS, IC_{50} (μM)	$K_{m,app}$ (μM)	V_{max} (rel)	k' (rel)
3	>130	>130	>20	>190	500 ± 18	1.4 ± 0.01	0.32 ± 0.01
4	0.0038	>50	0.035	>100	970 ± 120	1.7 ± 0.11	0.16 ± 0.01
MTX	0.0046	200	0.025	20	166 ± 49	0.99 ± 0.16	0.90 ± 0.05

[a] V_{max} (rel) and k'(rel) values are relative to folic acid (= 1.0), in the same experiment. The first-order rate constant (k') is calculated from the expression $k' = V_{max}/K_{m,app}$. The data represent means of two separate experiments on **3** and four separate experiments on **4**. Kinetic data for MTX were reported previously [24a].

The 2-amino-4(3*H*)-oxo analogue **3** was found to be inactive as an inhibitor of tumor cell growth at concentrations of up to 140 μM, which was $>10^5$-fold higher than the 50% inhibitory concentration (IC_{50}) of methotrexate (MTX). Lack of activity was observed regardless of whether the growth medium contained dialyzed or non-dialyzed fetal bovine serum, indicating that failure to inhibit growth was not due to protection of the cells by exogenous thymidine or hypoxanthine. Compound **3** was also not an inhibitor of purified TS at concentrations of up to 250 μM or of purified DHFR at concentrations of up to 20 μM. Moreover, in assays of substrate activity toward partially purified FPGS from mouse liver, **3** had a K_m of 500 ± 18 μM as compared with the value of 15.4 ± 6.9 μM which we have reported for **2** [11c]. Thus, in addition to lacking anti-DHFR and anti-TS activity, **3** was a very poor substrate for polyglutamylation. The fact that the FPGS substrate activity of **3** was approximately one-thirtieth of that of **2** showed that a basic nitrogen at position 6 is detrimental to binding. Analyses of the structural requirements for FPGS substrate activity have been confined thus far to molecules containing a carbon at this position [24a,b]. The lack of cytotoxicity of **3** in comparison with DDATHF (**1**), from which it differs only in that nitrogen is located at position 6 instead of 8, was also noteworthy, and was consistent with our finding [11c] that the NH at position 8 of **1** is important for biological activity.

The 2,4-diamino analogue **4**, in contrast to **3**, was a potent DHFR inhibitor, with an IC_{50} of 35 nM as compared with 25 nM for MTX, and was toxic to cultured L1210 cells, with an IC_{50} of 3.8 nM as compared with 4.6 nM for MTX. Inhibition of TS was negligible (IC_{50} >125 μM) in comparison with inhibition of DHFR, and therefore could not be a significant factor in cell growth inhibition. L1210/R81 cells, a subline which is highly resistant to MTX and owes its resistance mainly to a profound defect in active transport [25], was cross-resistant to **4**, providing evidence that the two drugs are taken up similarly by cells. Interestingly, the *in vitro* FPGS substrate activity of **4** was very low, suggesting that intracellular polyglutamylation is unlikely to play a role in cell-killing by this compound. To our knowledge, **4** is the first reported example of a cytotoxic 2,4-diamino antifolate with (a) an intact glutamate side chain and (b) little or no FGPS substrate activity at therapeutically relevant concentrations. While this lack of polyglutamylation might be useful in certain situations [26], studies directed toward analogues of **4** with greater FGPS substrate activity would also be of interest.

EXPERIMENTAL

Solvents used for moisture-sensitive reactions were dried over Linde 4A molecular sieves (Fisher, Boston, MA). Ethyl 1-benzyl-4-oxopiperidine-3-carboxylate (**8**), 1-benzyl-4-oxopiperidine (**14**), and cyanoguanidine were purchased from Aldrich, Milwaukee, WI. Ethyl 4-(2-bromoethyl)benzoate was synthesized according to Foreman and McElvain [20], and 4-amino-3-cyano-1,2,5,6-tetrahydropyridine (**13**) according to Taub and coworkers [27]. The ir spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer, and uv spectra on a Varian Model 210 instrument. The ^{1}H nmr spectra were recorded on a Varian T60 instrument with tetramethylsilane as the reference. Tlc was performed on fluorescent Baker Si250F silica gel plates, with spots being visualized under 254 nm uv illumination in a viewing chamber. Column chromatography was on Baker 3405 (60-200 mesh) silica gel or Whatman DE-52 pre-swollen *N,N*-dimethylaminoethylcellulose (DEAE-cellulose). Hplc was on a Waters C_{18} radial compression cartridge column (5 μm particles, 0.5 × 10 cm) connected to a Waters Model 400 instrument equipped with a Model 490 multiwavelength detector and Model 660 programmable solvent gradient system. Samples for microanalysis were dried *in vacuo* over phosphorus pentoxide at the indicated temperatures. Melting temperatures were obtained on a Fisher-Johns hot stage apparatus. Microanalyses were by Galbraith Laboratories, Knoxville, TN, or MultiChem Laboratories, Lowell, MA. Fractional moles of water or organic solvents in some analytical samples, which we have frequently observed among folic analogues and their protected intermediates (see, for example, references 23 and 26), could not be prevented in spite of rigorous drying *in vacuo* and was confirmed wherever possible by nmr spectral evidence.

2-Amino-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one Dihydrochloride (**9**·2HCl).

A solution of **7** [19a,b] (3.0 g, 0.012 mole) in a mixture of 1 *N* hydrochloric acid (35 ml), 2-propanol (25 ml), and water (25 ml) was shaken with 10% palladium-on-charcoal (0.25 g) in a low-

pressure Parr apparatus under a nitrogen atmosphere for 7 hours. The catalyst was filtered, the filtrate evaporated under reduced pressure, and the residue dried azeotropically (benzene/ethanol) and redissolved in absolute ethanol. A small amount of insoluble material was filtered off, the solution evaporated to dryness, and the residue dried *in vacuo* at 100° to obtain a colorless solid (2.8 g, 99%); mp 300° dec (after softening at 282° and resolidifying at 287°); ir (potassium bromide): ν 3300, 1710, 1682, 1655 cm^{-1}; uv (0.1 *N* hydrochloric acid): λ max 219 nm (ϵ 10,450), 269 (6.850); (0.1 *N* sodium hydroxide): λ max 228 nm (ϵ 8,350), 276 (6,500); ^1H nmr (trifluoroacetic acid): δ 2.4 (m, 2H, C_8-CH_2), 3.23 (m, 2H, C_7-CH_2), 3.4 (t, 2H, protonated amine), 4.1 (m, 2H, C_5-CH_2), 7.6 and 8.2 (m, 4H, protonated amine).

Anal. Calcd. for $C_7H_{12}Cl_2N_4O$: C, 35.16; H, 5.06; N, 23.43. Found: C, 35.44; H, 5.07; N, 23.24.

2-Amino-6-[2-(4-carbethoxyphenyl)ethyl]-5,6,7,8-tetrahydropyrido-[4,3-*d*]pyrimidin-4-(3*H*)-one (**10**).

A solution of **9**·2HCl (0.251 g, 1 mmole) and sodium acetate (0.164 g, 2 mmoles) in dimethyl sulfoxide (25 ml) was obtained by warming to 100°, and after partial cooling of this solution, ethyl 4-(2-bromoethyl)benzoate [20] (0.394 g, 1.55 mmole) and potassium iodide (16 mg, 0.1 mmole) were added. The reaction mixture was kept at 75° for 4 days and monitored by tlc (silica gel, 10:6:1 chloroform-methanol-28% ammonium hydroxide) for the disappearance of starting material (R_f 0.25) and the formation of product (R_f 0.78). The solvent was removed by vacuum distillation, the residue taken up in 5% acetic acid (50 ml), and the resulting solution extracted twice with ether. The combined organic layers were dried over magnesium sulfate and evaporated to dryness to recover unchanged bromo ester (0.134 g, 34% recovery). The aqueous layer was adjusted to *p*H 9.0 with 10% ammonia, left at 0°, and filtered. Drying of the filtered solid *in vacuo* at 80° gave **10** as a colorless solid (0.16 g, 46%), mp 259° dec; ir (potassium bromide): ν 3400, 3130, 1715 (ester C=O), 1645, 1610 cm^{-1}.

Anal. Calcd. for $C_{18}H_{22}N_4O_3 \cdot H_2O$: C, 59.98; H, 6.71; N, 15.55. Found: C, 60.09; H, 6.69; N, 15.53.

2-Amino-6-[2-(4-carboxyphenyl)ethyl]-5,6,7,8-tetrahydropyrido-[4,3-*d*]pyrimidin-4(3*H*)-one (**5**).

A suspension of **10** (1.1 g, 3.1 mmole) in 0.5 *N* sodium hydroxide (20 ml) was stirred until most of the solid dissolved, and was then kept at 20° for 3.5 hours. Undissolved material was removed by filtration, and the filtrate was acidified to *p*H 4.5 with 10% acetic acid and stored at 0° until a granular solid formed. Filtration and drying, first in a lyophilizer and then *in vacuo* at 100°, gave a colorless powder (0.95 g, 94%), dec 281°; tlc: R_f 0.32 (silica gel, 15:5:1 chloroform-methanol-acetic acid); ir (potassium bromide): ν 3400, 3150, 1655, 1610 cm^{-1}; uv (0.1 *N* hydrochloric acid): λ max 233, 263 (infl) nm; (0.1 *N* sodium hydroxide): λ max 233, 274 nm.

Anal. Calcd. for $C_{16}H_{18}N_4O_3 \cdot 0.5H_2O$: C, 59.43; H, 5.92; N, 17.33. Found: C, 59.51; H, 5.91; N, 17.17.

Dimethyl *N*-[4-[2-(2-Amino-4(3*H*)-oxopyrido[4,3-*d*]pyrimidin-6-yl)-ethyl]benzoyl]-L-glutamate (**11**).

Isobutyl chloroformate (65 μl, 0.5 mmole) was added at room temperature to a stirred suspension of **10** (162 mg, 0.5 mmole) in dry *N,N*-dimethylformamide (7.5 ml) containing triethylamine (0.25 g, 2.5 mmoles). After 15 minutes, dimethyl L-glutamate hydrochloride (0.11 g, 0.5 mmole) was added, followed 10 minutes later by another portion of isobutyl chloroformate (33 μl, 0.25 mmole). After another 15 minutes, a second portion of diester (53 mg, 0.25 mmole) was added, followed 10 minutes later by isobutyl chloroformate (16 μl, 0.13 mmole). After 15 minutes, a third portion of diester (27 mg, 0.13 mmole) was added, followed 10 minutes later by isobutyl chloroformate (16 μl, 0.13 mmole). Finally, after 15 minutes a fourth portion of diester (27 mg, 0.13 mmole) was added. The reaction was monitored by tlc (silica gel, 5:1 chloroform-methanol) for the disappearance of **10** (R_f 0.02) and formation of **11** (R_f 0.33). After evaporation under reduced pressure, the residue was washed twice with ether and redissolved in 10% acetic acid. The solution was adjusted to *p*H 8.0 with 28% ammonia, and the precipitated solid was collected, stirred in water, and filtered. Drying, first in a lyophilizer and finally *in vacuo* at 100° gave a colorless powder (0.21 g, 85%), mp 192° dec; ir (potassium bromide): ν 3400, 1740 (ester C=O), 1660, 1630, 1610 cm^{-1}; tlc: R_f 0.78 (silica gel, 5:4:1 chloroform-methanol-28% ammonium hydroxide).

Anal. Calcd. for $C_{23}H_{29}N_5O_6 \cdot H_2O$: C, 56.43; H, 6.38; N, 14.31. Found: C, 56.18; H, 6.28; N, 14.13.

N-[4-[2-(2-Amino-4(3*H*)-oxopyrido[4,3-*d*]pyrimidin-6-yl)ethyl]benzoyl]-L-glutamic Acid (**3**).

A solution of **11** (0.21 g, 0.43 mmole) in a mixture of methanol (4 ml) and 1 *N* sodium hydroxide (2.13 ml) was kept at 25° for 3 hours and monitored for the product by tlc (R_f 0.92, cellulose, *p*H 7.4 phosphate buffer; R_f 0.12, silica gel, 5:4:1 chloroform-methanol-28% ammonium hydroxide). The solution was adjusted to *p*H 7.0 with 6 *N* hydrochloric acid and evaporated to dryness. The residue was taken up in water (2 ml) and applied onto a DEAE-cellulose column (40 g, 2.0 × 28 cm), which was eluted with 3% ammonium bicarbonate. Fractions containing the product were pooled and lyophilized, and the residue was dried further *in vacuo* at 100° to obtain a colorless solid (0.21 g, 95%), mp 240-241°; hplc: 10% ethanol in 0.1 *M* ammonium acetate, *p*H 7.5, retention time 8.7 minutes; ir (potassium bromide): ν 3380, 3130, 3040, 1700 infl, 1635 cm^{-1}; uv (0.1 *N* hydrochloric acid): λ max 268 nm (ϵ 23,050); (0.1 *N* sodium hydroxide): λ max 235 nm (ϵ 24,200), 273 infl (8,800); ms: found m/e 444; calculated (MH^+), 444.

Anal. Calcd. for $C_{21}H_{25}N_5O_6 \cdot 3.75H_2O$: C, 49.35; H, 6.41; N, 13.70. Found: C, 49.22; H, 5.89; N, 13.82 [28].

2,4-Diamino-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine Dihydrochloride (**12**·2HCl).

A. From 4-Amino-3-cyano-1,2,5,6-tetrahydropyridine (**13**).

A mixture of **13** [27] (8.2 g, 0.066 mole) and guanidine carbonate (12 g, 0.068 mole) in diethyleneglycol dimethyl ether (165 ml) was stirred at 125° for 19 hours. After being cooled to room temperature, the resulting biphasic mixture was treated with an excess of ethereal hydrogen chloride, the upper layer was decanted, and the botton layer was triturated successively with ether and 2-propanol. The 2-propanol extract was concentrated until crystals began to form. Cooling to 0° gave a solid (1.2 g) whose tlc (silica gel, 5:4:1 chloroform-methanol-28% ammonium hydroxide) showed a major spot with R_f 0.5. The original residue remaining after extraction with 2-propanol was redissolved in hot water (60 ml), and 2-propanol (1 *l*) was added. The precipitated solid (1.5 g) was discarded, the mother liquor evaporated to dryness, and the residue crystallized from a mixture of methanol and 2-propanol to obtain another crop (1.1 g) with R_f 0.5 in the above tlc system.

The two solids with R_f 0.5 were combined; total yield 2.3 g (13%). In another experiment, in which the product was purified by repeated column chromatography on silica gel instead of by extraction and crystallization, the yield was increased to 19%. The material obtained by this procedure was indistinguishable from that prepared below.

B. From 1-Benzyl-4-piperidinone (**14**) *via* **15**.

A mixture of **14** (1.9 g, 0.01 mole), cyanoguanidine (0.92 g, 0.011 mole), and dimethyl sulfoxide (3.5 ml) was brought to the reflux temperature over a period of 10 minutes, refluxed for another 10 minutes, and poured into ether (50 ml). The supernatant was decanted, and the residue rewashed with ether. The ether-insoluble oil was dissolved in methanol (35 ml). Cooling to −20° yielded **15** as an orange solid (0.2 g); tlc: R_f 0.77 (silica gel, 28:12:1 chloroform-methanol-28% ammonium hydroxide). The mother liquor was evaporated to dryness and the residue applied onto a silica gel column (2.0 × 28 cm), which was eluted with 85:15:1 chloroform-methanol-28% ammonium hydroxide. The eluate was monitored by tlc, and fractions containing the product with R_f 0.77 were combined and repurified on a similar column by successive elution with 10:10:1 chloroform-acetonitrile-methanol, 10:1 chloroform-methanol, 5:1 chloroform-methanol, and 85:15:1 chloroform-methanol-28% ammonium hydroxide. Fractions containing the product with R_f 0.77 were evaporated and the residue was crystallized from methanol to obtain another 0.04 g of **15**, total yield 0.24 g (8.7%), mp 191-193°; ir (potassium bromide): ν 3400, 3340, 1640, 1575, 1540, cm^{-1}; uv (methanol): λ max 286 nm; (0.1 *N* hydrochloric acid): λ max 263, 268 nm.

Anal. Calcd. for $C_{14}H_{17}N_5 \cdot 1.25H_2O$: C, 60.51; H, 7.07; N, 25.21. Found: C, 60.29; H, 6.77; N, 25.62.

A solution of **15** (0.95 g, 0.0034 mole) in a mixture of 1 *N* hydrochloric acid (12 ml), water (8 ml), and 2-propanol (8 ml) was shaken with 10% palladium-on-carbon (0.1 g) under an atmosphere of hydrogen in a low-pressure Parr apparatus. The progress of the reaction was checked periodically by tlc (silica gel, 28:12:1 chloroform-methanol-28% ammonium hydroxide) to monitor the disappearance of starting material (R_f 0.72) and formation of product (R_f 0.13). When hydrogenolysis was complete, the catalyst was filtered and the filtrate evaporated. Recrystallization of the residue from methanol yielded **12**·2HCl as a colorless solid (0.546 g, 62%), mp >310° dec; ir (potassium bromide): 3350, 3180, 3070, 2930, 2800, 1665, 1530 cm^{-1}; uv (0.1 *N* hydrochloric acid): λ max 271 nm; ^1H nmr (deuterium oxide): δ 3.03 (t, 2H, C_6-CH_2), 3.6 (t, 2H, C_7-CH_2), 4.1 (s, 2H, C_5-CH_2). An analytical sample prepared by recrystallization from a mixture of water and 2-propanol was found to contain both as solvents of crystallization. Elslager and coworkers [18] reported the dihydrochloride *monohydrate* of this compound to have mp 269-271°.

Anal. Calcd. for $C_7H_{11}N_5 \cdot 2HCl \cdot 0.5H_2O \cdot 0.15C_3H_7OH$: C, 34.93; H, 5.98; Cl, 27.68; N, 27.34. Found: C, 34.69; H, 5.74; Cl, 27.37; N, 27.46.

C. From 2-Amino-4-hydrazinopyrido[4,3-*d*]pyrimidine Dihydrochloride (**18**·2HCl) *via* **16** and **17**.

Compound **18**·2HCl (0.32 g, 0.12 mmole), prepared as described below, was dissolved in water (20 ml) and ethanol (40 ml) and the solution was shaken with hydrogen in a low-pressure Parr apparatus in the presence of Davison sponge nickel (1 g) for 22 hours. The catalyst was filtered, dilute hydrochloric acid was added, and the solution concentrated to a volume of 2 ml. An excess of hot 2-propanol was then added and the solution allowed to cool, first to 20°, where crystallization occurred, and then to 0°. The solid was collected and dried *in vacuo* at 80° to obtain a product identical by tlc and ir with the material obtained by Methods A and B; yield 0.29 g (92%).

2-Amino-6-benzyl-4-chloro-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine Hydrochloride (**16**·HCl).

A mixture of **7** [19a,b] (1.8 g, 7.0 mmoles) in phosphorus oxychloride (25 ml) containing 2 drops of *N,N*-dimethylformamide was heated under reflux. Dissolution occurred in a few minutes. After 3.5 hours, the reaction mixture was poured onto ice (300 ml) and the *p*H was adjusted to 8.0 by dropwise addition of 28% ammonia with stirring. After being kept at 0° for 2 hours, the mixture was extracted with chloroform (3 × 250 ml). The chloroform solution was washed with 1 *N* sodium hydroxide, rinsed to neutrality with water, dried over magnesium sulfate, and evaporated. The residue was taken up in benzene (60 ml), ethereal hydrogen chloride was added, and the precipitate was collected and dried *in vacuo* at 60° to obtain a pale-brown powder (1.8 g, 73%), mp 128° dec; tlc: R_f 0.75 (silica gel, 15:5:1 chloroform-methanol-acetic acid); ir (potassium bromide): ν 3420, 2580, 1625, 1595, 1500 cm^{-1}.

Anal. Calcd. for $C_{14}H_{15}ClN_4 \cdot HCl \cdot H_2O$: C, 48.42; H, 5.81; Cl, 20.41; N, 16.13. Found: C, 48.86; H, 5.85; Cl, 20.05; N, 15.89.

2-Amino-6-benzyl-4-hydrazino-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine (**17**).

Anhydrous hydrazine (1.2 g, 38 mmoles) was added to a suspension of **16**·HCl (1.1 g, 3.1 mmoles) in ethanol (25 ml) and water (1 ml), and the mixture was stirred under nitrogen at room temperature. After 17 hours, 1 *N* sodium hydroxide (7 ml) was added and the mixture was evaporated to dryness under reduced pressure. The residue was taken up in 5:1 chloroform-methaol (10 ml) and applied onto a silica gel column (3.0 × 31 cm), which was eluted with 20:1 chloroform-methanol. Fractions were monitored by tlc (silica gel, 28:12:1 chloroform-methanol-28% ammonium hydroxide), and those containing a major product (R_f 0.63) with only minor impurities (R_f 0.77 and 0.92) were combined and evaporated. Crystallization of the residue from 2-propanol afforded tlc-pure **17** as colorless rosettes of needles (0.6 g, 71%); mp 235-238°; ir (potassium bromide): 3440, 1630, 1600, 1580 cm^{-1}.

Anal. Calcd. for $C_{14}H_{18}N_6 \cdot 0.1H_2O$: C, 61.79; H, 6.74; N, 30.89. Found: C, 62.04; H, 6.74; N, 30.60.

2-Amino-4-hydrazino-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine Dihydrochloride (**18**·2HCl).

A solution of **17** (0.54 g, 2.0 mmoles) in ethanol (20 ml) and 1 *N* hydrochloric acid (5 ml) was shaken overnight with hydrogen in a low-pressure Parr apparatus in the presence of 10% palladium on carbon (0.1 g). Completion of the reaction was established by tlc (silica gel, 28:12:1 chloroform-methanol-28% ammonium hydroxide), which showed the disappearance of **17** (R_f 0.62) and formation of a new spot with R_f 0.11. The catalyst was filtered and washed several times with hot water (total 50 ml), and the combined filtrate and washings were evaporated to dryness. The residue was taken up in hot water (7 ml), and hot 2-propanol (50 ml) was added. The solution was left to stand, first at room temperature and then at 0°, and the precipitated solid was collected and dried *in vacuo* at 60°, yield 0.42 g (81%), mp 300° dec; ir (potassium bromide): ν 3400-2800, 1675, 1655, 1605 cm^{-1}. The analytical

sample was prepared by recrystallization from aqueous ethanol.

Anal. Calcd. for $C_7H_{12}N_6 \cdot 2HCl \cdot 0.1C_2H_5OH$: C, 33.55; H, 5.70; N, 32.61. Found: C, 33.32; H, 5.52; N, 32.67.

2,4-Diamino-6-[2-(4-carbethoxyphenyl)ethyl]-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine (**19**).

A stirred mixture of **12**·2HCl (0.45 g, 1.8 mmoles), sodium acetate (0.37 g, 4.6 mmoles), sodium iodide (20 mg, 0.13 mmole), and ethyl 4-(2-bromoethyl)benzoate [20] (0.49 g, 2.0 mmole) in dry dimethyl sulfoxide (35 ml) was kept at 70° (bath) for 50 hours under nitrogen. A clear solution formed after 1 hour. The reaction was monitored by tlc (silica gel, 10:6:1 chloroform-methanol-28% ammonium hydroxide) to follow the disappearance of **12** (R_f 0.46) and formation of **19** (R_f 0.88). After vacuum distillation of the solvent, the residue was taken up in warm 10% acetic acid, and the solution extracted twice with ether, basified to *p*H 9.2 with 28% ammonia, and cooled to 0°. The precipitated solid was filtered, redissolved in a small volume of 5:4:1 chloroform-methanol-28% ammonium hydroxide, and applied onto a silica gel column (1.0 × 22.5 cm), which was eluted with 28:12:1 chloroform-methanol-28% ammonium hydroxide. Fractions containing pure product were pooled and evaporated, and the residue was dried *in vacuo* at 80° to obtain a yellow powder (0.16 g, 26%); mp 210-213° dec; ir (potassium bromide): ν 3450, 1690 (ester C=O), 1630 cm^{-1}.

Anal. Calcd. for $C_{18}H_{23}N_5O_2 \cdot 0.25H_2O$: C, 62.49; H, 6.84; N, 20.24. Found: C, 62.53; H, 6.67; N, 20.09.

2,4-Diamino-6-[2-(4-carboxyphenyl)ethyl]-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine (**6**).

Compound **19** (0.64 g, 1.9 mmoles) was dissolved in a mixture of methanol (70 ml) and 2.5 *N* sodium hydroxide (15 ml), and the solution was kept at room temperature for 6 hours. After being acidified to *p*H 5.0 with 10% acetic acid, the hydrolysis mixture was concentrated under reduced pressure, dilutes with water, and cooled to 0°. The precipitated yellow solid was collected and dried *in vacuo* at 100°, yield 0.57 g (93%), mp 290° dec; ir (potassium bromide): ν 3350, 3200, 1660 cm^{-1}.

Anal. Calcd. for $C_{16}H_{19}N_5O_2 \cdot H_2O$: C, 57.99; H, 6.39; N, 21.14. Found: C, 58.18; H, 6.16; N, 21.01.

Dimethyl *N*-[4-[2-(2,4-Diaminopyrido[4,3-*d*]pyrimidin-6-yl)ethyl]-benzoyl]-L-glutamate (**20**).

Diethyl phosphorocyanidate (29 mg, 0.18 mmole) was added at room temperature to a stirred suspension of **6** (50 mg, 0.15 mmole) in *N,N*-dimethylformamide (2 ml) in which had been dissolved dimethyl L-glutamate hydrochloride (38 mg, 0.18 mmole) and triethylamine (36 mg, 0.36 mmole), after 25 minutes, *N*-methylpyrrolidin-2-one (2 ml) was added. A clear solution formed after 3 hours. Stirring was continued for 26 hours, at which time tlc (silica gel, 10:6:1 chloroform-methanol-28% ammonium hydroxide) indicated formation of **20** (R_f 0.79), with some **6** (R_f 0.5) remaining unchanged. The solvent was distilled under reduced pressure, the residue was taken up in chloroform, and the solution was washed with sodium bicarbonate and dried over magnesium sulfate. Evaporation gave a solid, which was redissolved in 28:12:1 chloroform-methanol-28% ammonium hydroxide (1 ml) and applied onto a silica gel column (1 × 16 cm). Fractions eluted with the same solvent were appropriately combined and evaporated to give a solid, which was dried *in vacuo* at 100°; yield 33 mg (41%); mp 227-228°; ir (potassium bromide): ν 3400, 1740 (ester C=O), 1670-1610 cm^{-1}; uv (0.1 *N* hydrochloric acid): λ max 240 (infl), 270 (infl) nm.

Anal. Calcd. for $C_{23}H_{30}N_6O_5 \cdot 0.5CHCl_3$: C, 53.23; H, 5.80; N, 15.85. Found: C, 53.63; H, 6.11; N, 15.74.

N-[4-[2-(2,4-Diaminopyrido[4,3-*d*]pyrimidin-6-yl)ethyl]benzoyl]-L-glutamic Acid (**4**).

A solution of **20** (25 mg, 0.047 mmole) in methanol (10 ml) and 1 *N* sodium hydroxide (0.3 ml) was kept at room temperature for 5.5 hours, at which time tlc showed hydrolysis to be essentially complete. The solution was acidified to *p*H 6.4 with 6 *N* hydrochloric acid, the solvent was evaporated, and the residue was applied onto a DEAE-cellulose column (10 g, 1.0 × 22 cm), which was eluted first with a large volume of water to remove salts and then with 3% ammonium bicarbonate. Fractions showing a single tlc spot (R_f 0.58, cellulose, *p*H 7.4 buffer; R_f 0.67, silica gel, 10:6:1 chloroform-methanol-28% ammonium hydroxide) were pooled and freeze-dried to a colorless solid, which was dried *in vacuo* at 100°, yield 23 mg (100%), mp 220° dec; hplc: 10% ethanol in 0.1 *M* ammonium acetate, *p*H 7.5, retention time 9.7 minutes; ir (potassium bromide): ν 3400, 3200, 1655, 1635 cm^{-1}; uv (0.1 *N* hydrochloric acid): λ max 226 nm (ϵ 29,000), 269 (7,500); (0.1 *N* sodium hydroxide): λ max 265 nm (ϵ 26,900), 280 (8,850); ms: found m/e 443; calculated (MH^+), 443.

Anal. Calcd. for $C_{21}H_{26}N_6O_5 \cdot 3.5H_2O$: C, 49.89; H, 6.48; N, 16.62. Found: C, 50.15; H, 6.08; N, 16.33.

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