plement. **Materials.**—Sheep red blood cells (RBC) suspended in Alsever's soln, guinea pig hemolysin (antibody), and lyophilized guinea pig complement were purchased from Grand Island Biologicals, Oakland, Calif., or Hyland Laboratories, Costa Mesa, Calif. RBC were standardized to 10^9 cells/ml of buffer A as previously described.[4] Hemolysin was diluted 1:800 with buffer A and was stable at 3° indefinitely; as used in the assay below, this gives 5 times the amt needed for max velocity.

Solutions.—Buffer A was 5 m*M* Tris hydrochloride containing 0.5 m*M* $MgCl_2$–0.15 m*M* $CaCl_2$–0.15 *M* NaCl–0.1% gelatin. Buffer B was pH 5.4 K phosphate buffer of 0.02 ionic strength. Citrate–saline was a 1:4 mixture of 0.075 *M* Na citrate and 0.15 *M* NaCl.

Separation of C'1.—The lyophilized complement was dissolved in restoring soln at 0°, then 10 ml was dialyzed at 3° against 3 l. of buffer B by continuous flow for 10–15 hr. The dialyzed suspension was centrifuged at 3° at 20,000 rpm in a No. 40 head of a Spinco L centrifuge. The pellet contained C'1 and the supernatant, the remainder of the components of complement,[14,15] and is called the R fraction.

The supernatant R fraction was adjusted to pH 7 with 0.1 *N* NaOH, then brought to 0.15 ionic strength by addition of the appropriate amt of 1.71 *M* NaCl. The soln was kept frozen at −20° in 1-ml aliquots.

The pellet of C'1 was rinsed twice with 2-ml portions of buffer B. The solid was resuspended in 30 ml of 0.15 *M* NaCl and stirred 1 hr at 0°. This mixture was then dialyzed and centrifuged as described above. The supernatant was discarded and the pellet of C'1 stirred 1 hr at 0° with 30 ml of 0.15 *M* NaCl. The soln was clarified by centrifugation and the supernatant containing C'1 was frozen in 1-ml aliquots.

Recombination Assay and Standardization.—The soln of C'1 and R were dild 1:2 with buffer A. In a centrifuge tube was placed 0.25 ml of RBC (10^9/ml) and 0.25 of hemolysin (1:800) in a bath at 37°. After 15 min the contents were treated with 0.30 ml of buffer A and 100 μl of C'1; after 7 min, 100 μl of R was added. After 14 min the lysis reaction was stopped by addition of 2.75 ml of ice-cold citrate–saline. The tube was centrifuged for 3 min in a clinical centrifuge, then the OD of the hemoglobin in the supernatant was read at 541 mμ in a glass cuvette.

When either the C'1 or the R fraction is replaced by buffer, there should be no lysis above a control where both the C'1 and R fractions are replaced by buffer; no lysis shows that separation of C'1 and R was sufficient for the recombination assay. The R soln is further dild to a concn 1:3 and 1:4. If the rate is the same as 1:2, then the 1:3 or 1:4 diln is used with buffer A.

The amount of C'1 is then decreased by further diln with buffer A until the above conditions given an OD reading of 0.30–0.42; the C'1 is usually dild in the range of 1:5 to 1:7. This assay is then rate limited by the concn of C'1.

Irreversible Inhibition of C'1a.—In 2 tubes are placed 323 μl of C'1 at the proper diln; these are then placed in a bath at 37° for 10 min to activate C'1 to C'1a.[15] To one tube is added 10 μl of inhibitor in MeOEtOH, and to the other tube is added 10 μl of MeOEtOH containing no inhibitor. Meanwhile two tubes containing 0.25 ml of RBC (10^9/ml) and 0.25 ml of 1:800 hemolysin was fixed for 15 min at 37°. To each RBC tube was added 0.30 ml of buffer A and 100 μl of C'1a incubation mixture plus or minus inhibitor. After 7 min, 100 μl of R fraction was added, and the RBC were allowed to lyse a predetermined time (7–15 min), then quenched by addition of 2.75 ml of ice-cold citrate–saline. The OD for hemoglobin was read after centrifugation as described in the previous paragraph; the per cent irreversible inhibition was determined by comparing the OD of the control tube *vs.* the OD of the inhibitor tube.

In practice 5–6 tubes were run simultaneously, one control tube, 3–4 tubes contg an inhibitor, and one lysis control where the C'1 and R fractions of the control tube were replaced by buffer. The amount of lysis of RBC caused by the compd after 1:10 diln in this assay has usually already been determined in the whole complement assay of the compd.

Folic Acid Analogs. Modifications in the Benzene-Ring Region. 1. 2'- and 3'-Azafolic Acids[1]

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2'- and 3'-azafolic acids were synthesized as part of a program to design and obtain folic acid analogs that might have antineoplastic activity because of their altered ability to function as one-carbon transfer agents. The reductive condensation of 2-acetamido-6-formylpteridin-4(3*H*)-one (**2**) with diethyl *N*-(5-aminopicolinoyl)-glutamate (**7b**) followed by blocking group hydrolysis afforded 2'-azafolic acid (**10**). Alternatively, **2** was condensed with **7b** nonreductively, and the product anil was isolated and subsequently converted into **10**. Acetylation of the hydrolyzed reductive condensation product of **2** and ethyl 6-aminonicotinate (**1b**) afforded N^2,N^{10}-diacetyl-3'-azapteroic acid (**3c**). Coupling of **3c** with diethyl glutamate gave diethyl N^2,N^{10}-diacetyl-3'-azafolate (**5b**); acetylation of the reductive condensation product of **2** and diethyl *N*-(6-aminonicotinoyl)-glutamate (**4**) also yielded **5b**. Blocking group hydrolysis of **5b** then afforded 3'-azafolic acid (**6**). Compounds **4**, **6**, **7b**, and **10** were found to be inactive in initial tests against leukemia L1210 in mice; they were also found to be noncytotoxic when tested *vs.* HEp-2 cells in culture. Analogs **6** and **10** were observed to be growth supporting when tested *vs. Streptococcus faecalis*.

N^{10}-Formyltetrahydrofolate, N^5,N^{10}-methenyltetrahydrofolate, and N^5,N^{10}-methylenetetrahydrofolate are formed enzymatically from tetrahydrofolate and formate or formaldehyde donors. These tetrahydrofolate derivatives function as agents in the transfer of one-carbon units.[2] Structural alterations between the pteridine and the glutamate portions of folic acid type molecules could alter the capacity of such structures to accept or to donate one-carbon units. For example, the potential of folic acid type molecules for forming one-carbon transfer agents may be lessened by reducing electron availability at position 10 (N^{10}). Reduction of electron availability might be effected either by introducing electron-withdrawing substituents on the benzene ring or by replacing the benzene ring with certain heterocyclic rings. Conversely, the presence of electron-donating groups on the benzene ring may, by increasing electron availability at the N^{10} position, stabilize one-carbon transfer agents and thereby render more difficult the transfer of a one-carbon unit from the folic acid type cofactor to the substrate. These struc-

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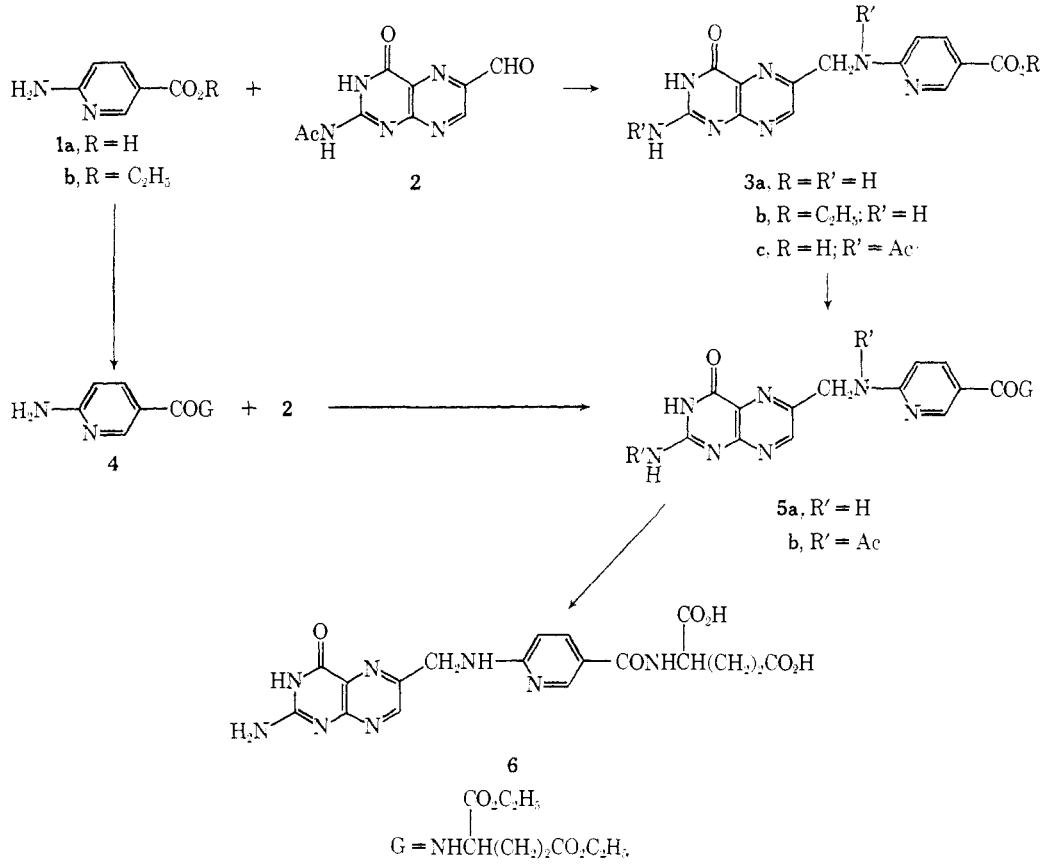
(1) This investigation was supported by Chemotherapy, National Cancer Institute, National Institutes of Health, Contract PH43-64-51, and by the Charles F. Kettering Foundation.

(2) M. Friedkin, *Annu. Rev. Biochem.*, **32**, 185 (1963); F. M. Huennekens in "Biological Oxidations," T. P. Singer, Ed., Wiley, New York, N. Y., 1968, pp 439–513.

SCHEME I



tural changes may affect folic acid metabolism at sites other than, or in addition to, the dihydrofolate reductase stage, which is considered[3] to be the primary site of action of the well-known antagonists methotrexate and aminopterin. Two folic acid analogs in which the benzene ring is replaced by the pyridine ring and in which electron density at N^{10} may be expected to be reduced have been synthesized as part of a program on the synthesis of folic acid analogs of the type described above.

Chemistry.—The synthetic route chosen for the preparation of 3′-azafolic acid (**6**) and 2′-azafolic acid (**10**) is based on the method of Sletzinger, *et al.*,[4] in which folic acid had been obtained *via* the reductive condensation of 2-acetamido-6-formylpteridin-4(3*H*)-one (**2**) with *p*-aminobenzoylglutamic acid in the presence of *p*-toluenethiol. This method appeared to offer a short route to **6** and **10**, and a general route to folic acid analogs in which the benzoylglutamic acid group may be replaced by other moieties. Intermediate **3c** (Scheme I) was initially obtained by acetylation of the crude product (**3a**) of reductive condensation of **2** with **1a**, but it was found that the use of **1b** in the condensation, followed by hydrolysis of the resultant **3b** to **3a** and acetylation, afforded better yields of **3c**. Coupling of the blocked acid **3c** with diethyl glutamate afforded **5b**. Alternatively, **5b** was obtained by performing the diethyl glutamate coupling (**1a** to **4**) prior to the steps of reductive condensation (of **2** with **4**) and acetylation (**5a** to **5b**). Although the overall yields of **5b** by either route were comparable, the latter route (utilizing **4**) was preferred since less of the laboriously obtained[4] **2** was consumed.

Loss of the N^2-acetyl group during the preparation of reductive condensation products **3a**, **3b**, and **5a** (and **8a**, Scheme II) was confirmed by their uv spectral maxima (0.1 *N* NaOH) at 365 nm.[5] Complete and selective hydrolysis of the N^2- and N^{10}-acetamide and glutamate ester functions of **5b** afforded the desired 3′-azafolic acid (**6**).

Similarly, reductive condensation of **2** with **7b**, obtained by hydrogenation of **7a**, afforded **8a**, as shown in Scheme II. Acetylation of **8a** gave **8b**, and selective hydrolysis of the N^2- and N^{10}-acetamide functions was attempted; hydrolysis of the picolinamide function appeared to compete with cleavage of the N^{10}-acetamide function, and the use of **8b** was abandoned. Direct hydrolysis of **8a** afforded the desired 2′-azafolic acid (**10**).

The nonreductive condensation of **2** with **7b** afforded the anil **9**, and **9** was converted into **10** by a process of $NaBH_4$ reduction, anaerobic alkaline hydrolysis, and reoxidation. The use of $NaBH_4$ represents a new method for reduction of anils of this type, although borohydride has been used for the reduction of a variety of other anil types[6] as well as for the reduction of the pyrazine ring of folic acid[7] and related pteridines.[8] A similar anil, derived from **2** and *N*-(*p*-aminobenzoyl)-

(3) W. C. Werkheiser, *Cancer Res.*, **23**, 1277 (1963); J. R. Bertino, *ibid.*, **23**, 1286 (1963).

(4) M. Sletzinger, D. Reinhold, J. Grier, M. Beachem, and M. Tishler, *J. Amer. Chem. Soc.*, **77**, 6365 (1955).

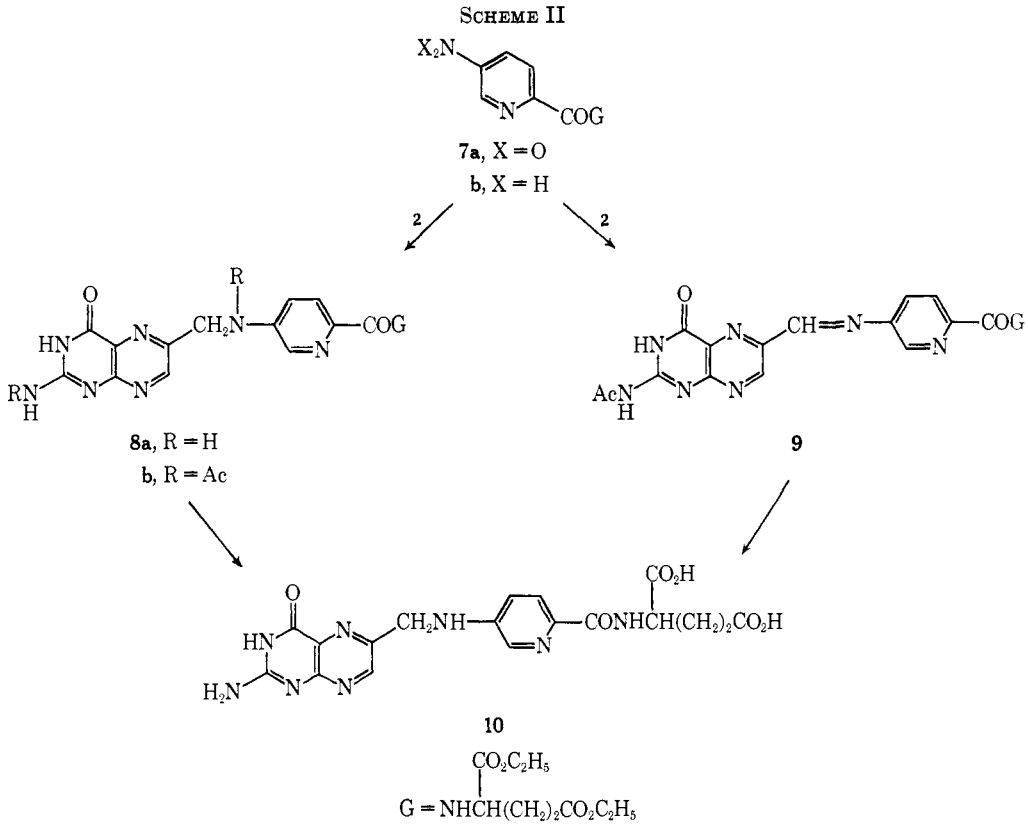
(5) This absorbtion maximum was routinely observed for structures possessing the 2-amino-3,4-dihydro-4-oxo-6-pteridinyl moiety; an absorption maximum of 350 nm (0.1 *N* NaOH) was observed to be characteristic of the corresponding 2-acetamido compounds.

(6) B. R. Baker and C. E. Morreal, *J. Pharm. Sci.*, **52** (9), 840 (1963).

(7) (a) V. S. Gupta and F. M. Huennekens, *Arch. Biochem. Biophys.*, **120**, 712 (1967). (b) K. G. Scrimgeour and K. S. Vitols, *Biochemistry*, **5**, 1438 (1966).

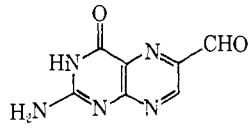
(8) T. Shiota, M. N. Disraely, and M. P. McCann, *J. Biol. Chem.*, **239**, 2260 (1964).

SCHEME II



glutamic acid, had been previously converted to folic acid[9] by catalytic hydrogenation and by formic acid and its Et_3N^+ salt.

Although hydrated samples of **6** and **10** having satisfactory elemental analyses could be obtained after purification by DEAE-cellulose column chromatography,[7b,10] examination of the samples by tlc revealed the presence of a blue-fluorescent impurity common to both products. Its identity was established as 2-amino-6-formylpteridin-4(3*H*)-one (**11**) by tlc comparisons with an authentic sample in several chromatographic systems. The reduction of the level of occurrence of this impurity from approximately 5% to less than 1% in **10** by the use of mercaptoethanol in the chromatographic purification solutions indicated that it had arisen by air oxidation of **10** during chromatography. A similar observation[11] of air oxidation has been made previously. The level of occurrence of this impurity in **6** was also less than 1%.



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Biological Evaluation.[12]—Compounds **4** and **7b**, when tested against leukemia L1210[13] in mice at a dose level of 400 mg/kg (day 1 only), produced no evidence of toxicity and no increase in life span. The analogs **6** and **10**, when tested against leukemia L1210 in mice at the highest tolerated[14] single doses (75 and 150 mg/kg, respectively, day 1 only), did not significantly[13] increase life span. Compounds **6** (at 75 mg/kg) and **10** (at 48 mg/kg, and also at several lower dose levels) were also tested against leukemia L1210 on a chronic dose schedule (qd 1–9), but neither compound produced a significant increase in life span.

Compounds **4**, **7b**, **6**, and **10** exhibited no cytotoxicity when tested *vs.* HEp-2 cells in culture.

Analogs **6** and **10** were found to be growth supporting[16] when tested *vs. Streptococcus faecalis* ATCC 8043 in

(9) M. Sletzinger and M. Tishler, U. S. Patent 2,740,784 (1956); *Chem. Abstr.*, **50**, 15601e (1956).

(10) Appreciation is expressed to Drs. Charles Baugh and Carlos Krumdieck, Department of Nutrition, Alabama Medical College, Birmingham, Ala., for helpful discussions.

(11) J. M. Whiteley, J. Drais, J. Kirchner, and F. M. Huennekens, *Arth. Biochem. Biophys.*, **126**, 955 (1968).

(12) Biological testing was performed by the Chemotherapy Department of Southern Research Institute under contract with Chemotherapy, National Cancer Institute, National Institutes of Health; *in vivo* testing was carried out under the supervision of Drs. F. M. Schabel, Jr., and W. R. Laster, Jr.; cell culture tests were under the supervision of Dr. G. J. Dixon, and bacteriological testing was performed under the supervision of Dr. R. F. Pittillo.

(13) Mice were implanted ip with 10^5 L1210 cells on day 0. "Day 1 only" means that a single dose was injected about 24 hr after L1210 cell implantation; a chronic dose schedule means that daily injections were initiated 1 day after implantation and continued through day 9. A significant increase in life span in the treated animals is considered to be a survival time at least 25% longer than the control animals, *i.e.*, $T/C \geq 125\%$.

(14) Compound **6** was tested and found toxic at dose levels above 75 mg/kg; **10** was not tested above 150 mg/kg, but this dose was presumed to be approaching the toxic level because of the degree of weight loss observed. Other data pertaining to the toxic dose level of **10** were obtained in a one-mouse toxicity test. Four healthy mice were given a single injection of **10** on day 0 at individual dose levels of 400, 200, 100, and 50 mg/kg, and toxicity was measured in terms of death before day 30. On this basis, **10** was acutely toxic at 400 and 200 mg/kg, and chronically toxic at 100 mg/kg; this is consistent with the former results. It is of interest to note that folic acid itself has been observed[15] to be acutely toxic at a single dose level of 300 mg/kg administered on day 3 following leukemia L1210 cell implantation in mice. Dose levels of 150 and 75 mg/kg were nontoxic.

(15) J. A. R. Mead, A. Goldin, R. L. Kisliuk, M. Friedkin, L. Plante, E. J. Crawford, and G. Kwok, *Cancer Res.*, **26**, 2374 (1966).

(16) It has been previously reported[17] that a radioactive material which supported the growth of *S. faecalis* R and *L. citrovorum* 8081 was obtained by paper chromatography of a cell-free extract of *Enterococcus stei* grown in the presence of 6-aminonicotinic acid ([^{14}C]COOH). Evidence indicated this material was analogous to folic acid, and it was, therefore, presumably 3'-azafolic acid or a metabolic product thereof.

(17) A. Wacker, E. Lochmann, and L. Träger in "Pteridine Chemistry," W. Pfleiderer and E. C. Taylor, Ed., MacMillan Co., New York, N. Y., 1964, p 409.

Flynn's Broth in the presence of that concentration (0.0004 μmole/ml) of folic acid which gives half-maximum growth.

Experimental Section

Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of theoretical values. Melting points were determined with a Kofler Heizbank (gradiently heated bar) apparatus. Pmr data were determined with a Varian A-60A spectrometer and are given in ppm downfield from Me_4Si. Chemical shifts of pmr multiplets are reported as the apparent center of the multiplet. Uv spectra were determined with a Cary Model 14 recording spectrophotometer (wavelengths in nm). Linde Type 4A molecular sieves were used to dry solvents designated anhyd, and in certain reaction procedures where indicated. Na_2SO_4 was used to dry soln in organic solvents, and solvent evapns were done *in vacuo*. The DEAE-cellulose used was Mannex (Mann Research Laboratories) of the capacity noted. Before chromatographic use, the DEAE-cellulose was converted into the phosphate form by washing with a large excess of 0.5 *M* potassium phosphate buffer (pH 7.0), then with H_2O, and with H_2O contg mercaptoethanol. Soln of NaCl used in elution were maintained at pH 7.0 by use of 0.005 *M* potassium phosphate buffer. Isolations and washings by centrifugation were done at 1800–2000 rpm; washing by centrifugation of **6** and **10** was done with dil HCl: 3–4 drops of 1 *N* HCl per 50 ml of H_2O. Analytical samples reported as hydrated were handled in a dry bag.

Tlc was run on Avicel cellulose-coated plates (Cell-A), Bakerflex cellulose sheets (Cell-B), Bakerflex DEAE-cellulose sheets (Cell-DEAE), and Brinkmann's silica gel H, according to Stahl (SGH). SGH was also used for column chromatography. Solvent systems used were A: *n*-BuOH–AcOH–H_2O (5:2:3); B: *n*-BuOH–AcOH–H_2O (4:1:5, upper layer); C: 5% aq Na_2HPO_4; D: 5% NH_4OH–3% aq NH_4Cl; E: *i*-PrOH–2 *N* aq HCl (65:35); F: 0.5 *M* NaCl–0.2 *M* mercaptoethanol–0.005 *M* potassium phosphate buffer of pH 7.0; G: $CHCl_3$–MeOH–AcOH (87:10:3); H: $CHCl_3$–MeOH (97:3); I: $CHCl_3$–MeOH (96:4); J: $CHCl_3$–MeOH (95:5); K: $CHCl_3$–MeOH (9:1); L: $CHCl_3$–EtOAc (95:5).

Diethyl *N*-(5-Nitropicolinoyl)glutamate (7a).—Diethyl glutamate·HCl (23.97 g, 0.1 mole) was added to a soln of 16.8 g (0.1 mole) of 5-nitropicolinic acid[18] and 20.6 g (0.1 mole) of DCI in 1700 ml of anhyd pyridine, and the reaction mixture was stirred at room temp in a closed flask for 5 days. The pptd dicyclohexylurea was removed by filtration and washed with $CHCl_3$. The filtrate was evapd, and the residue was triturated with $CHCl_3$ to remove further amounts of dicyclohexylurea. The $CHCl_3$ soln was washed with 1 *N* HCl, satd aq $NaHCO_3$, and H_2O. An insol cryst material contained in these washes was identified as 5-nitropicolinic acid (2.02 g). The $CHCl_3$ soln was dried and evapd, and the residue was chromatographed on a column of SGH (300 g) by development with $CHCl_3$. Fractions of low purity were rechromatographed, and finally all tlc-homogeneous (SGH, solvent L) fractions were combined. Evapn yielded a slowly crystg syrup: 14.11 g (78%, based on unrecovered 5-nitropicolinic acid); pmr (CCl_4) δ 1.28 (m, 4 lines, 6 H, CH_3), 2.30 (m, 4 H, CH_2CH_2), 4.07 (quartet, 2 H, OCH_2), 4.25 (quartet, 2 H, OCH_2), 6.42 (m, 1 H, NCH), 8.35 (d, 1 H, C_3-H), 8.42 (m, 1 H, NH), 8.65 (d of doublets, 1 H, C_4-H), 9.42 (d, 1 H, C_6-H); uv max (EtOH) 245 (ε 9120), 278 (8510). *Anal.* ($C_{15}H_{19}N_3O_7$) C, H, N.

Diethyl *N*-(5-aminopicolinoyl)glutamate (7b), was obtained by atmospheric hydrogenation (EtOH) of **7a** (14.1 g, 0.04 mole) over Raney Ni catalyst. Removal of the catalyst by filtration and solvent evap yielded a cryst solid. Trituration of this material with PhH afforded crystals: 11.18 g (87%); mp 104°; uv max (EtOH) 283 (ε 16,400), 300 (sh); pmr (DMSO-d_6) δ 1.15 (t, 3 H, CH_3), 1.18 (t, 3 H, CH_3), 2.20 (m, 4 H, CH_2CH_2), 4.01 (m, 4H, OCH_2), 4.50 (m, 1 H, NCH), 5.92 (s, 2 H, NH_2), 7.00 (d of doublets, 1 H, C_4-H), 7.72 (d, 1 H, C_3-H), 7.98 (d, 1 H, C_6-H), 8.37 (d, 1 H, CONH). *Anal.* ($C_{15}H_{21}N_3O_5$) C, H, N.

Diethyl *N*-(5-{[(2-Acetamido-3,4-dihydro-4-oxo-6-pteridinyl)-methylene]amino}picolinoyl)glutamate (9).—2-Acetamido-6-formylpteridin-4(3*H*)-one[4] (**2**) (490 mg, 2.1 mmoles) was dissolved in 8 ml of anhyd DMSO, and 680 mg (2.1 mmoles) of **7b** was added along with some molecular sieves. The closed flask was stirred at room temp for 4 days before the reaction soln was filtered into 300 ml of anhyd Et_2O containing 10 ml of anhyd Me_2CO. The yellow solid (735 mg) was isolated by filtration, and the filtrate was evapd almost to dryness. The residue was dissolved in 2 ml of Me_2CO and added dropwise to 200 ml of Et_2O contg 5 ml of Me_2CO. A second yellow solid (310 mg) was thus obtained after filtration. By pmr analysis, the first crop was found to contain about 12% DMSO and the second crop none; the total amount of product was therefore 967 mg (85%). This material was used in the next step without further purification. An analytical sample was obtained by recryst from Me_2CO: uv max (DMF) 333 (shoulder, ε 20,200), 367 (23,600); pmr (DMSO-d_6) δ 2.27 (s, 3 H, $COCH_3$), 8.09 (m, 2 H, C_5'-H, C_6'-H), 8.68 (m, 1 H, C_3'-H), 8.92 (s, 1 H, CH=N), 8.92 (d, 1 H, CONH), 9.53 (s, 1 H, C_7-H), 12.23 (m, 2 H, CONH). Other pmr absorptions were as expected. *Anal.* ($C_{24}H_{26}N_8O_7$) C, H, N.

***N*-(5-{[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]-amino}picolinoyl)glutamic Acid (2'-Azafolic Acid, 10). A. From Isolated Schiff Base.**—Compd **9** (1.38 g, 2.56 mmoles) was dissolved in 200 ml of anhyd DMF and 292 mg (7.7 mmoles) of $NaBH_4$ was added. After the initial vigorous reaction, the flask was closed, and the reaction was allowed to proceed overnight. The DMF was evapd, the residue was dissolved in 500 ml of deaerated 0.1 *N* NaOH, and hydrolysis was carried out at room temp under N_2 for 4 hr. The aq soln was acidified with HCl to destroy excess $NaBH_4$, and after several minutes the soln pH was adjusted to 7.5 before the addition of 20 ml of 30% H_2O_2. After 30 min, the soln was acidified to pH 3.5. The gelatinous ppt was collected and washed with dil HCl by centrifugation. An aq suspension of this crude product was treated with enough NH_4OH to effect soln (pH <7.0), and the soln was made 0.1 *M* in mercaptoethanol. This soln was applied to a DEAE-cellulose column (6 g, std capacity, phosphate form). The column was eluted with a linear gradient of NaCl (500 ml of 0.5 *M* NaCl in the reservoir and 500 ml of 0.0 *M* NaCl in the mixing bottle; both soln were 0.1 *M* in mercaptoethanol). Elution of the fractions was monitored by uv absorption, and the desired fractions were combined and diluted 1:10 with H_2O containing mercaptoethanol. This soln of the folic acid analog was passed through a short DEAE-cellulose column (1 g, high capacity, basic form), and the desired product was collected thereon. This column was eluted with 50 ml of 1 *M* NaCl contg mercaptoethanol, and the yellow eluate acidified to pH 3.5. The ppt was redissolved in 60 ml of dil NaOH and repptd by acidification to pH 3.5. This material was collected and washed 3 times with dil HCl by centrifugation. The orange mass was dried *in vacuo* (P_2O_5), and the product was pulverized before final drying (P_2O_5) for 24 hr at 0.35 mm and room temp: 60 mg, 5% yield; uv max (0.1 *N* HCl) 227 (ε 25,900), 291 (18,600), 333 (16,700); uv max (pH 7) 281 (26,300), 305 (sh); uv max (0.1 *N* NaOH) 255 (24,900), 283 (22,300), 304 (sh), 363 (9250); pmr (CF_3CO_2D) δ 2.62 (m, 4 H, CH_2CH_2), 5.05 (s, 2 H, NCH_2), 5.05 (m, 1 H, NCH), 8.00 (d of doublets, 1 H, C_5'-H), 8.50 (d, 1 H, C_6'-H), 8.63 (d, 1 H, C_3'-H), 9.05 (s, 1 H, C_7-H). *Anal.* ($C_{18}H_{18}N_8O_6 \cdot 1.25\ H_2O$) C, H, N.[19] A comparable reaction had previously afforded a yield of 11%.

B. *Via* Reductive Condensation Procedure.[4]—A soln of **7b** (5.33 g, 16.5 mmoles) and *p*-toluenethiol (13.0 g, 105 mmoles) in

(18) J. Schmidt-Thomé and H. Goebel, *Hoppe-Seyler's Z. Physiol. Chem.*, **288**, 237–243 (1951). The acid was obtained in average yields of 90% from the carboxamide as reported. We were never able to obtain the reported 44% yield in the conversion of 2-bromo-5-nitropyridine to 5-nitropicolinamide; careful control of the (fusion) reaction temp between 208 and 212° gave the best yields (19% and 26%).

(19) The characterization of **6** and **10** as hydrates corresponds to the reporting of other analogs[20] of folic acid and folic acid[21] itself as being hydrated. Before the hygroscopic nature of **6** and **10** was determined, a wide range of analytical results was obtained on the same sample of **6** (and **10**); all of the results were easily accommodated by the assumption of hydration, varying from 2.0 to 4.5 moles of H_2O per mole of compd. After the use of a dry bag for handling dried samples of **6** and **10** became routine, it was found that both compd could be dried and analyzed as hemihydrates. The drying conditions and degrees of hydration reported herein were the most easily reproduced.

(20) T. L. Loo, *J. Med. Chem.*, **8**, 139 (1965); C. W. Mosher, E. M. Acton, O. P. Crews, and L. Goodman, *J. Org. Chem.*, **32**, 1456 (1967); J. I. DeGraw, J. P. Marsh, Jr., E. M. Acton, O. P. Crews, C. W. Mosher, A. N. Fujiwara, and L. Goodman, *ibid.*, **30**, 3408 (1965).

(21) S. Uyeo, S. Mizukami, T. Kuhota, and S. Takagi, *J. Amer. Chem. Soc.*, **72**, 5339 (1950).

375 ml of anhyd methoxyethanol was heated to vigorous reflux in a flask fitted with an N_2 inlet and a reflux condenser atop a condensate trap filled with molecular sieves. The flask was flushed with N_2, and **2** (3.5 g, 15 mmoles) was added. The reaction was allowed to proceed at reflux under N_2 atmosphere for 6 hr, after which the dark, cooled soln was added with stirring to 1500 ml of Et_2O. After refrigeration, the gelatinous ppt was isolated by filtration, washed with Et_2O, and dried (5.15 g, 69% crude). The combined filtrates were evapd to an oily residue which yielded more crude product (1.90 g, 25% crude) on treatment with Et_2O.

The combined solids were hydrolyzed by dissolving in 2250 ml of deaerated 0.1 *N* NaOH and allowing the soln to stir at room temp under N_2. At the end of 4 hr, the soln was acidified with HCl to pH 6, filtered, and acidified further to pH 3.5. The gelatinous ppt was isolated and washed once with dil HCl by centrifugation. The wet mass was dried (P_2O_5) *in vacuo* and yielded 6.22 g (88% crude) of a brown powder. This was dissolved in 60 ml of 1 *N* NaOH, and this soln was diluted with H_2O to 3000 ml and made 0.1 *M* in mercaptoethanol; the soln (pH 6) was applied to a DEAE-cellulose column (50 g, high capacity, basic form). The column was washed with H_2O containing mercaptoethanol and then was eluted with 1 *M* NaCl (0.1 *M* in mercaptoethanol). Elution was ended when the eluate became colorless and yielded no ppt on acidification. Acidification of the total eluate to pH 3.5 yielded an orange, gelatinous ppt, which was isolated and washed twice with dil HCl by centrifugation. The wet mass was dried (P_2O_5) *in vacuo*, and yielded 3.55 g (50% crude) of an orange powder. Tlc on Cell-B (solvent A) revealed two blue-fluorescent impurities, although uv spectral data compared well with the final analytical specimen.

The orange powder was dissolved in 300 ml of H_2O by the addition of 17 ml of 1 *N* NH_4OH, and this soln was diluted to 3500 ml with H_2O containing 15 ml of mercaptoethanol before application to a DEAE-cellulose column (75 g, std capacity, phosphate form). The column was washed with 2000 ml of 0.2 *M* mercaptoethanol and then was eluted with a linear gradient of NaCl (2000 ml of 0.6 *M* NaCl in the reservoir and 2000 ml of 0.0 *M* NaCl in the mixing bottle; both soln were 0.2 *M* in mercaptoethanol). Elution of the fractions was monitored by uv absorption, and the desired fractions were combined and acidified to pH 3.5 with HCl. The ppt was collected and washed, by centrifugation, with dil HCl containing mercaptoethanol, and was then redissolved in H_2O containing mercaptoethanol by the addition of a minimum amount of NaOH. Acidification with HCl to pH 3.5 yielded a ppt which was collected and washed 4 times with dil HCl by centrifugation. The wet mass was dried (P_2O_5) *in vacuo*, and the product was pulverized before final drying (P_2O_5) for 24 hr at 0.35 mm and room temp; 1.18 g (17% yield). The uv and pmr spectra obtained for this sample were equiv to those given for **10**. *Anal.* ($C_{18}H_{18}N_8O_6 \cdot 0.75H_2O$) C, H, N.[19] A comparable reaction had previously afforded a 14% yield.

Both products described above were identical by tlc comparison and were observed to contain a single uv-absorbing (principal) component and a single uv-fluorescent (minor) component on Cell-A (solvents A and B), Cell-B (solvents A and B), Cell-DEAE (solvent E), and SGH (solvent A). The minor component was identified as 11 by tlc comparison with an authentic sample in the systems listed above. When the two products were analyzed by side-by-side tlc comparisons with soln of folic acid containing 1,3, 5, 7, and 9% of 11, the amount of 11 in these products was estimated to be less than 1% (Cell-A, solvent A; folic acid and **10** had identical R_f values in this tlc system).

Diethyl *N*-(6-Aminonicotinoyl)glutamate (4). **A.**—6-Aminonicotinic acid[22] (6.9 g, 0.05 mole) was dissolved in warm, anhyd DMF, and the soln was filtered and cooled in an ice bath before the addition of Et_3N (15.2 g, 0.15 mole) and ethyl chloroformate (5.4 g, 0.05 mole). After 1 hr, diethyl glutamate·HCl (12.0 g, 0.05 mole) was added, and the reaction mixture was stirred at room temp for 3 days. The ppt was removed by filtration and washed with DMF, and the filtrate was evapd. An EtOAc soln of the residue was washed with aq Na_2CO_3 and H_2O, dried, and evapd to a solid (13.4 g). Application of this material in $CHCl_3$ to a chromatographic column (SGH, 200 g) and development with solvent I afforded **4**: 4.0 g, 24% yield; mp 130°; tlc homogeneous (SGH, solvent J); uv max (EtOH) 270 (ε 19,400); pmr (DMSO-d_6) δ 1.17 (t, 3 H, CH_3), 1.20 (t, 3 H, CH_3), 2.08 (m, 4 H, CH_2CH_2), 4.05 (quartet, 2 H, OCH_2), 4.13 (quartet, 2 H, OCH_2), 4.45 (m, 1 H, NCH), 6.48 (s, 2 H, NH_2), 6.48 (d, 1 H, C_5-H), 7.87 (d of doublets, 1 H, C_4-H), 8.33 (d, 1 H, CONH), 8.54 (d, 1 H, C_2-H). *Anal.* ($C_{15}H_{21}N_3O_5$) C, H, N.

B.—6-Aminonicotinic acid[22] (5.5 g, 0.04 mole) was suspended in 700 ml of anhyd pyridine and allowed to react with 8.3 g (0.04 mole) of DCI and 9.6 g (0.04 mole) of diethyl glutamate·HCl. After 5 days, the ppt was removed by filtration and the filtrate was evapd. The residue was triturated with $CHCl_3$, and the soln was applied to a chromatographic column (SGH, 200 g) and developed as before. Combination of the tlc-homogeneous (SGH, solvent J) fractions afforded **4**: 3.6 g, 28% yield, mp 130°.

6-{[(2-Acetamido-3,4-dihydro-4-oxo-6-pteridinyl)methyl]-acetylamino}nicotinic Acid (N^2,N^{10}-Diacetyl-3'-azapteroic Acid, 3c). **A.**—A suspension of 6-aminonicotinic acid[22] (1.8 g, 0.013 mole), *p*-toluenethiol (6.2 g, 0.05 mole), and 5 ml of pyridine in 150 ml of anhyd methoxyethanol was heated to vigorous reflux in a flask fitted with a N_2 inlet and a reflux condenser atop a condensate trap filled with molecular sieves. The flask was flushed with N_2, and **2** (2.33 g, 0.01 mole) was added. The reaction was allowed to proceed at reflux under N_2 for 5 hr. The cooled reaction mixture was filtered, and the filter cake was washed with Me_2CO. The filtrate yielded more ppt, and the combined, air-dried solids were repptd from warm, dil NaOH by acidification (to pH 4.6) with HCl after charcoal decolorization. The brown solid was washed with H_2O and Me_2CO and dried *in vacuo* (2.3 g, 74% crude).

The product was heated in refluxing Ac_2O for 5 hr under N_2. After partial cooling the insol were removed by filtration and washed with Ac_2O. Partial evapn yielded further insol which were similarly removed. Addition of Et_2O to the residue obtd on complete evapn yielded a yellow solid. This material was suspended in 40 ml of H_2O, and concd aq NH_3 was added until soln was complete (pH 9.5). The pH was lowered to 2.6 with 1 *N* HCl, and a ppt was removed and discarded. The filtrate was refrigerated and yielded a yellow cryst solid that was washed with H_2O and Me_2CO and dried *in vacuo:* 307 mg, 8% overall yield; tlc showed a trace impurity (SGH, solvent G); uv max (0.1 *N* NaOH) 256 (ε 29,500), 351 (7350). *Anal.* ($C_{17}H_{15}N_7O_5$) C, H, N. A comparable reaction later afforded a 14% overall yield.

B.—The reaction conditions described above were utilized in a condensation of **2** (3.88 g, 16.7 mmoles) with **1b** (3.32 g, 20 mmoles) in 200 ml of methoxyethanol containing 10.5 g (85 mmoles) of *p*-toluenethiol. The cooled reaction mixt was filtered, and the solid on the filter was washed with Me_2CO. Evapn of the filtrate and treatment of the residue with Et_2O afforded more solid. The combined solids were dissolved in 0.1 *N* NaOH and decolorized with charcoal, and the soln was allowed to stir under N_2 for 16 hr. Acidification to pH 4.1 yielded solid that was filtered and washed with H_2O. After drying *in vacuo*, the solid was acetylated, and the product was isolated as in the preceding procedure. A yellow cryst solid resulted: 1.15 g, 17% yield; tlc was equiv to that of the analytical sample; uv max (0.1 *N* HCl) 280 (ε 22,100), 333 (8260); uv max (pH 7) 257 (19,700), 274 (18,600), 338 (7850); uv max (0.1 *N* NaOH) 256 (29,300), 350 (7250); pmr (DMSO-d_6) δ 2.25 (s, 3 H, $COCH_3$), 2.32 (s, 3 H, $COCH_3$) 5.42 (s, 2 H, NCH_2), 7.87 (d, 1 H, C_5'-H), 8.33 (d of doublets, 1 H, C_6'-H), 8.95 (d, 1 H, C_2'-H), 8.97 (s, 1 H, C_7-H), 12.00 (m, 3 H, CONH, CO_2H).

Diethyl *N*-(6-{[(2-Acetamido-3,4-dihydro-4-oxo-6-pteridinyl)-methyl]acetylamino}nicotinoyl)glutamate (8b). **A. Coupling of 3c with Diethyl Glutamate.**—Compd **3c** (978 mg, 2.46 mmoles) was dissolved in 100 ml of warm, anhyd DMF, and the soln was protected from moisture and cooled in an ice bath. Anhyd Et_3N (249 mg, 2.46 mmoles) was added along with isobutyl chloroformate (336 mg, 2.46 mmoles), and the soln was stirred at 0° for 1 hr. Et_3N (249 mg, 2.46 mmoles) was added with diethyl glutamate·HCl (580 mg, 2.46 mmoles), and the reaction was allowed to proceed at room temp for 20 hr. The solvent was evapd using a warm H_2O bath, and the residue was treated with 100 ml of EtOAc and filtered. The filter cake was washed with EtOAc, and the combined filtrates were washed twice with 20 ml of 0.5 *N* HCl, once with 20 ml of satd aq $NaHCO_3$, and twice with 20 ml of H_2O. The $NaHCO_3$ and following H_2O washes were combined and extd with EtOAc before acidifying to pH 4. All EtOAc soln were combined and dried before complete evapn. The residue was treated with Et_2O, and the solid obtd weighed 395 mg (28%).

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The acidified aq soln was extd with EtOAc. These extracts were washed with H_2O and dried before evapn. The residue was treated with Et_2O, and the solid obtd weighed 210 mg (15%). Analysis by tlc (SGH, solvent K) indicated the two solids were of nearly identical compn and of excellent purity. Their reppth from Et_2O–EtOAc did not yield material having satisfactory elemental analyses, and the remaining product was subjected to column chromatography (SGH, 20 g; developed with solvent K). The best fractions were combined: 339 mg, 24% yield; tlc (SGH, solvent K) indicated slight impurity; uv max (0.1 *N* NaOH) 256 (ϵ 33,900), 351 (7440). *Anal.* ($C_{26}H_{30}N_8O_8$) C, H, N.

B. Acetylation of the Product of Reductive Condensation of 2 and 4.—Compd **4** (3.55 g, 11 mmoles), **2** (2.33 g, 10 mmoles), and *p*-toluenethiol (8.7 g, 70 mmoles) in 300 ml of anhyd methoxyethanol were utilized in the reductive condensation procedure previously described (B, synthesis of **10**). Celite (10 g) was added to the product suspended in Et_2O, and the total filter cake was air-dried before acetylation as previously described (A, synthesis of **3c**). The solid phase was removed by filtration and washed with Ac_2O and Me_2CO. The filtrates were combined and evapd, and the residue was triturated with Et_2O. The insol fraction was extd with warm EtOAc several times, and the combined exts were washed with H_2O and dried. Evapn yielded 3.3 g of solid (57% crude yield).

This solid was chromatographed on a column of SGH (150 g) by development with solvent K. Product-containing fractions were rechromatographed (SGH, 75 g, solvent K). The best fractions were combined and evapd, and the residue obtd was triturated with Et_2O. The product was isolated by filtration: 740 mg, 13% yield; tlc was identical with that of the analytical sample; uv max (0.1 *N* NaOH) 256 (ϵ 33,700), 351 (7680); pmr (DMSO-d_6) δ 2.22 (s, 3 H, $COCH_3$), 2.24 (s, 3 H, $COCH_3$), 5.36 (s, 2 H, NCH_2), 7.87 (d, 1 H, C_5'-H), 8.32 (d of doublets, 1 H, C_6'-H), 8.88 (d, 1 H, CONH of glutamic acid), 8.89 (d, 1 H, C_2'-H), 8.95 (s, 1 H, C_7-H), 12.10 (m, 2 H, CONH). Other pmr spectral data obtd for this sample were equiv to those given for **4**. *Anal.* ($C_{26}H_{30}N_8O_8$) H; C: calcd, 53.60; found, 52.27; N: calcd, 19.24; found, 18.76.

***N*-(6-{[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino}nicotinoyl)glutamic Acid (3'-Azafolic Acid).**—Compd **8b** (759 mg, 1.3 mmoles) was dissolved in 390 ml of deaerated 0.1 *N* NaOH, and the soln was allowed to stir at room temp under N_2. At the end of 16 hr, the soln was acidified to pH 3.5, and the ppt was isolated and washed with dil HCl by centrifugation. The gelatinous mass was dried (P_2O_5) *in vacuo*, and the solid obtd was dissolved in H_2O contg 2 equiv of NH_4OH. This soln was diluted to 1 l. and was made 0.1 *M* in mercaptoethanol; the resulting soln (pH <7) was applied to a DEAE-cellulose column (22 g, std capacity, phosphate form). The column was eluted with a linear NaCl gradient (1000 ml of 0.7 *M* NaCl in the reservoir and 1000 ml of 0.0 *M* NaCl in the mixing bottle; both soln were 0.1 *M* in mercaptoethanol). Elution of the fractions was monitored by uv absorption, and the desired fractions were combined and acidified to pH 3.5 with HCl.

The ppt was collected and washed with dil HCl by centrifugation. The gelatinous mass was dried (P_2O_5) *in vacuo*, and the product was pulverized before final drying (P_2O_5) for 24 hr at room temp and 0.5 mm. The yellow solid weighed 384 mg, 63% yield (8% yield from **2**); a comparable reaction had previously afforded 53% yield (13% from **2**); tlc: **6** appeared homogeneous (uv detection) on SGH (solvent A), Cell-A (solvent A), Cell-B (solvents C, D, E). On Cell-B (solvent A), **6** produced a pattern of 2 overlapping spots; repetition of this same pattern from each of the original 2 spots on 2-dimensional tlc indicated the pattern was a chromatographic artifact. On Cell-DEAE (solvent F), **6** appeared to contain two blue-fluorescent impurities. Side-by-side comparison of **6** with **10** in this tlc system indicated that the amount of **11** present in **6** was much less than 1%; the amount of the second impurity appeared comparable; uv max (0.1 *N* HCl) 261 (ϵ 24,800), 316 (15,500); uv max (pH 7) 277 (32,000), 347 (8020); uv max (0.1 *N* NaOH) 258 (28,200), 275 (29,000), 365 (8900); pmr (CF_3CO_2D) δ 2.62 (m, 4 H, CH_2CH_2), 5.13 (m, 1 H, NCH), 5.18 (s, 2 H, NCH_2), 7.42 (d, 1 H, C_5'-H), 8.58 (d of doublets, 1 H, C_6'-H), 8.84 (d, 1 H, C_2'-H), 9.05 (s, 1 H, C_7-H). *Anal.* ($C_{15}H_{18}N_8O_6 \cdot 1.5H_2O$) C, H, N.[19]

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Choline Acetyltransferase Inhibitors. Styrylpyridine Analogs with Nitrogen-Atom Modifications

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Among steric and electronic features of styrylpyridine analogs previously associated with their activity as inhibitors of choline acetylase were molecular coplanarity and the π-electron-deficient cationic pyridinium moiety. Azomethines, capable of assuming, but presumably not preferring, molecular coplanarity, are represented by both active and inactive trans isomer types. The active inhibitor is less potent than the styrylpyridine analog. An aminomethylstilbene had no inhibitory activity; the cationic and steric features are believed favorable, but the absence of a π-electron-deficient moiety is not. 2-Pyridoneimines were intermediate in potency and ionization between corresponding pyridine and pyridinium analogs. These observations are consistent with previously reported structure–activity relationships and proposed enzyme receptor interactions.

In previous articles,[2,3] a variety of steric and electronic parameters of analogs of styrylpyridines were assessed in relation to influences on potency and specificity as inhibitors of choline acetylase (ChA) (choline acetyltransferase). A simple, potent prototype is the *trans*-*N*-methyl-4-(1-naphthylvinyl)pyridinium salt, I. There are now described analogs that include azomethines, in which the α and β carbon atoms are selectively replaced by N, 2-pyridoneimine (1,2-dihydro-2-iminopyridine) species, and *trans*-4-aminomethylstilbene. For the reasons next outlined, these have been designed

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