fact, as Unger[23] has pointed out, it is QSAR-gained insight into drug action and design that is of the greatest importance.

Experimental Section

Chemistry. The syntheses of the triazines used in this study have been previously reported[3] except for congener **43**.

3-Nitrobenzyl Phenyl Sulfide. A suspension of thiophenol (4.4 g, 40 mmol), 3-nitrobenzyl chloride (6.8 g, 40 mmol), and potassium carbonate (5.6 g, 40 mmol) in acetone was refluxed for 48 h. The potassium chloride was filtered off, and the filtrate was concentrated under reduced pressure. Distillation in vacuo yielded 8.0 g (85%) of a yellow viscous oil, bp 150–160 °C (2.8 mm). Anal. ($C_{13}H_{11}O_2NS$) C, H.

3-[(Phenylthio)methyl]aniline Hydrochloride. 3-Nitrobenzyl phenyl sulfide (7.5 g, 30 mmol), acetic acid (0.2 mL), and iron powder (40 g, 720 mmol) were stirred in 150 mL of water at 85–95 °C for 10 h. The slurry was alkanized with sodium carbonate, filtered, and washed with hot benzene. The filtrate was collected and extracted with benzene, and the combined extracts were concentrated on a rotary evaporator. The resulting syrupy liquid was dissolved in ether, and HCl gas was passed through the solution until a yellow flocculent solid was formed. The hydrochloride was collected and recrystallized from acetonitrile to yield 6.6 g (87%) of a white solid, mp 152–153.5 °C. Anal. ($C_{13}H_{14}ClNS$) C, H.

Congener 43. 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-[3-[(phenylthio)methyl]phenyl]-*s*-triazine Hydrochloride. A suspension of 3-[(phenylthio)methyl]aniline hydrochloride (2.5 g, 10 mmol) and cyanoguanidine (0.85 g, 10 mmol) in reagent grade acetone was refluxed for 20 h. The pale yellow solid that precipitated was collected by filtration and recrystallized from acetonitrile–ethanol to yield 3.6 g (95%) of a white solid, mp 165–167 °C. Anal. ($C_{18}H_{22}N_5SCl$) C, H.

Methotrexate was supplied by the Division of Cancer Treatment of the National Cancer Institute.

Biology. The original L5178Y/R cells were kindly provided by Dr. J. Bertino, Department of Pharmacology, Yale University School of Medicine, New Haven, CT. For routine passage and during dose–response experiments, L5178Y/R murine leukemia cells were maintained in asynchronous logarithmic growth at 37 °C in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin–streptomycin. The population doubling time was 15–18 h.[21] Twice a week, cells in the mid to late logarithmic stage of growth were diluted (v/v) 1:10- to 1:20-fold with fresh medium and serum in order to keep a portion of the cell stocks in the logarithmic stage of growth at all times. The stock solutions of the triazines were made with unsupplemented medium.

Cell cultures were seeded at 4.0–6.0 × 10^4 cells/mL in duplicate for each drug concentration in a plastic microtiter plate (0.2 mL/well). The triazines that were added to the cell cultures in 1:10 dilution to achieve the desired drug concentration were tested at a minimum of eight different concentrations. After 48 h of continuous drug exposure in a humidified incubator supplied with 95% air and 5% carbon dioxide, the cells were harvested and counted using a Coulter Counter, Model B (Coulter Electronics, Hialeah, FL). A control untreated set of cultures and four duplicate sets of MTX-treated cells were included for each separate dose–response experiment. Duplicate counts were taken on each well and were usually in agreement with each other (±10%).

From the data obtained, a dose–response curve was drawn and the ID_{50} was calculated as in our previous studies.[4] The ID_{50} is defined as the concentration of inhibitor that halves the growth rate, i.e., doubles the generation time. The confidence limits on log $1/ID_{50}$ and π_0 were calculated by utilizing the jackknife procedure.[22]

Substituent Constants. The values for the substituent constants in Table I were taken from our recent compilation.[7]

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Synthesis of Pseudo Cofactor Analogues as Potential Inhibitors of the Folate Enzymes

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Reaction of 5,6,7,8-tetrahydrofolic acid (THF, **7**) with phosgene, thiophosgene, and cyanogen bromide gave the bridged derivatives, 5,10-(CO)-THF (**8**), 5,10-(CS)-THF (**9**), and 5,10-(C=NH)-THF (**11**), respectively. Catalytic hydrogenation of 10-(chloroacetyl)folic acid (**2**) gave 5,10-(CH_2CO)-THF (**12**). A similar reaction with 10-(3-chloropropionyl)folic acid (**3**) gave 10-($ClCH_2CH_2CO$)-THF (**14**) rather than 5,10-(CH_2CH_2CO)-THF (**13**). In the catalytic hydrogenation of 10-ethoxalylfolic acid (**5**), the initial product 10-(EtO_2CCO)-THF (**22**) rearranged readily to give 5-(EtO_2CCO)-THF (**21**). Acylation of THF with chloroacetyl chloride gave a N^5,N^{10}-diacylated product (**18** or **19**), which could not be converted to 5,10-($COCH_2$)-THF (**17**). Reductive alkylation of THF with glyoxylic acid and 5-hydroxypentanal, respectively, gave 5-(HO_2CCH_2)-THF (**24**) and 5-[$HO(CH_2)_5$]-THF (**25**). Reductive dialkylation of THF with formaldehyde gave 5,10-$(CH_3)_2$-THF (**27**), whereas glyoxal gave 5,10-(CH_2CH_2)-THF (**10**). Also, both folic acid and 5-(CHO)-THF were reductively alkylated with formaldehyde to give 10-methylfolic acid (**6**) and 5-(CHO)-10-(CH_3)-THF (**28**), respectively. These compounds were tested as inhibitors of the enzymes involved in folate metabolism and for activity against lymphocytic leukemia P388 in mice.

The six biologically active cofactor forms of 5,6,7,8-tetrahydrofolic acid (THF) (**7**) are substrates for at least 15 enzymes. Key enzymes are GAR and AICAR transformylase (EC 2.1.2.2 and EC 2.1.2.3), serine transhydroxymethylase (EC 2.1.2.1), and thymidylate synthetase (EC 2.1.1.b). The transformylases provide carbon-8 and carbon-2 of inosinic acid, and the methylase and synthetase enzymes catalyze transformations that provide the 5-methyl group of thymidylic acid. Inhibitors of these enzymes will result in the blockage of purine and pyrimidine synthesis, followed by the arrest of DNA synthesis.[1]

In the search for inhibitors of the folate enzymes, the preparation of 5- and 10-substituted, 5,10-disubstituted,

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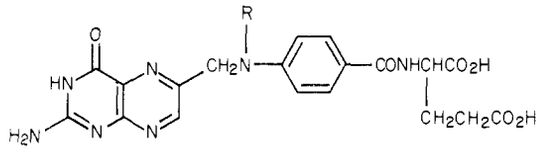
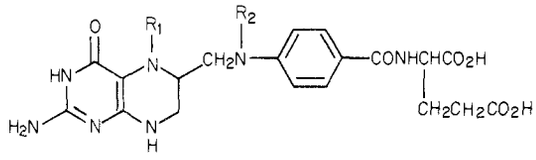


1, R = H
2, R = $ClCH_2CO$
3, R = $Cl(CH_2)_2CO$
4, R = CH_3CO
5, R = EtO_2CCO
6, R = CH_3



7, $R_1 = R_2 = H$
8, $R_1R_2 = CO$
9, $R_1R_2 = CS$
10, $R_1R_2 = CH_2CH_2$
11, $R_1R_2 = C{=}NH$
12, $R_1R_2 = CH_2CO$
13, $R_1R_2 = (CH_2)_2CO$
14, $R_1 = H$; $R_2 = Cl(CH_2)_2CO$
15, $R_1 = H$; $R_2 = CH_3CO$
16, $R_1R_2 = CH_3C^+$
17, $R_1R_2 = COCH_2$
18, $R_1 = ClCH_2CO$; $R_2 = HOCH_2CO$
19, $R_1 = HOCH_2CO$; $R_2 = ClCH_2CO$
20, $R_1R_2 = COCO$
21, $R_1 = EtO_2CCO$; $R_2 = H$
22, $R_1 = H$; $R_2 = EtO_2CCO$
23, $R_1R_2 = EtO_2CC^+$
24, $R_1 = HO_2CCH_2$; $R_2 = H$
25, $R_1 = HO(CH_2)_5$; $R_2 = H$
26, $R_1 = CH_3$; $R_2 = H$
27, $R_1 = R_2 = CH_3$
28, $R_1 = CHO$; $R_2 = CH_3$

and 5,10-bridged substituted derivatives of THF were investigated. Previously, 5,10-(CO)-THF (**8**) and 5,10-(CS)-THF (**9**) were reported to be inactive as inhibitors of thymidylate synthetase from various sources,[2] whereas 5,10-(CH_2CH_2)-THF (**10**) was competitive with 5,10-(CH_2)-THF as an inhibitor of the enzyme.[3] No experimental data on the preparation of these compounds has appeared, and our initial goal was the preparation and evaluation of these and related THF derivatives.

Folic acid (**1**) was reduced with $NaBH_4$, and the THF formed was isolated in the presence of ascorbic acid as previously described.[4] Reaction of a suspension of THF in deoxygenated H_2O with phosgene provided 5,10-(CO)-THF (**8**). Similarly, a solution of THF in aqueous NaOAc was treated with thiophosgene to give 5,10-(CS)-THF (**9**). For the preparation of 5,10-(C=NH)-THF (**11**), folic acid was reduced with $NaBH_4$, and the resulting THF was reacted in situ with cyanogen bromide.

Several approaches were attempted in the preparation of the 5,10-(CH_2CO)-THF (**12**), 5,10-(CH_2CH_2CO)-THF (**13**), 5,10-($COCH_2$)-THF (**17**), and 5,10-(COCO)-THF (**20**). Acylation of folic acid with chloroacetyl chloride gave 10-(chloroacetyl)folic acid (**2**), which was catalytically hydrogenated in CF_3CO_2H to give a mixture of [5,10-(CH)-THF]$^+$, 10-(CH_3CO)-THF (**15**), and 5,10-(CH_2CO)-THF (**12**). The former was identified by TLC data and must be generated by carbon–carbon bond cleavage of the chloroacetyl moiety, presumably via the methenyl analogue, [5,10-($ClCH_2C$)-THF]$^+$. The presence of 10-(CH_3CO)-THF (**15**) in the mixture was supported by the ^1H NMR spectrum of the mixture. The target compound, 5,10-(CH_2CO)-THF (**12**), was separated from the other components by column chromatography on cellulose.

The preparation of 5,10-(CH_2CH_2CO)-THF (**13**) was attempted by a similar procedure. Acylation of folic acid with 3-chloropropionyl chloride gave 10-(chloropropionyl)folic acid (**3**), which was hydrogenated as described above for **2**. Purification of the resulting product by cellulose chromatography gave a low recovery of 10-($ClCH_2CH_2CO$)-THF (**14**) and none of the bridged compound 5,10-(CH_2CH_2CO)-THF (**13**). Apparently, 5,10-(CH_2CH_2CO)-THF (**13**) is not formed readily, and the low recovery of **14** is attributed to the unstable nature of 10-(acyl)-THFs toward air and light. Catalytic hydrogenation of 10-acetylfolic acid (**4**) gave 10-(CH_3CO)-THF (**15**). UV studies showed that neither **15** nor **14** was dehydrated under acidic conditions to give significant amounts of the bridged substituted derivatives of [5,10-(CH)-THF]$^+$ such as **16**. X-ray crystallography studies have shown that the bridge in [5,10-(CH)-THF]$^+$ is nearly planar,[5] and the resulting extended conjugation between the pyrimidine and benzene rings is reflected in the UV absorption maximum [λ_{max} (1 N HCl), 347 nm]. The UV spectrum of **15** [λ_{max} (1 N HCl), 263, 300 sh] indicated the absence of a significant amount of **16**, which is attributed to steric interaction between the 4-oxo atom of the pyrimidine ring and the methyl group of the bridge.

The reaction of THF with chloroacetyl chloride gave a diacylated product rather than the expected 5,10-($COCH_2$)-THF (**17**). Elemental analysis indicated that one of the two chloro groups was hydrolyzed during the basic workup of the reaction to give either **18** or **19**. The 5,10-diacyl structure was supported by the similarity of the UV spectrum with that of 5,10-$(CHO)_2$-THF[6] and by the phenylene proton shift observed in the ^1H NMR spectrum (see below). Removal of one of the acyl groups, followed by formation of a N-5, N-10 bridged compound, was unsuccessful under a variety of conditions. Similarly, the preparation of 5,10-(COCO)-THF (**20**) was unsuccessful in that treatment of THF with oxalyl chloride gave mixtures containing polyacylated products. The preparation of this target compound was also attempted by hydrogenation of 10-ethoxalylfolic acid (**5**). After a basic workup, the product was shown to be a 7:3 mixture (^1H NMR) of 5- and 10-(EtO_2CCO)-THF (**21** and **22**). Apparently, 10-(EtO_2CCO)-THF (**22**) rearranged via [5,10-(EtO_2CC)-THF]$^+$ (**23**), but this intermediate was not observed in the UV spectrum of the product under acidic conditions.

The reductive alkylation of THF with aldehydes has been demonstrated,[7] and treatment of THF with glyoxylic acid and 5-hydroxypentanal in the presence of borohydride gave 5-(HO_2CCH_2)-THF (**24**) and 5-[$HO(CH_2)_5$]-THF (**25**), respectively. The formation of 5-(CH_3)-THF (**26**)[8] from THF, formaldehyde, and $NaBH_4$ under basic conditions gave a sample that was contaminated with a minor amount of a second compound, which was not present in the sample resulting from $NaBH_3CN$ reduction of [5,10-(CH)-THF]$^+Cl^-$.[9] This impurity, identified as 5,10-$(CH_3)_2$-THF (**27**), was the major product when THF was treated under acidic conditions with a 5-fold excess of formaldehyde in the presence of $NaBH_3CN$. Apparently, 5,10-$(CH_3)_2$-THF

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Table I. Inhibition Studies with Bridged THF Derivatives

	inhibition,[b] %				
enzyme[a]	5,10-(CO)-THF	5,10-(CS)-THF	5,10-(C=NH)-THF	5,10-(CH_2CH_2)-THF	[5,10-(CH)-THF]$^+$
I	16	30	0	13	15(10^{-6})
II	25	8	0	16	0
III	40	0	30	10	10
IV	41	55	0	20	40
V	2	5	4	10	22
VI	0	11	0	0	0
VII	0 (10^{-5})	37 (10^{-5})	13	33	19 (10^{-5})
VIIIA	48 (10^{-7})	27 (10^{-5})	24	3	9
VIIIB	51 (10^{-5})	12	24		
IXA	24 (10^{-6})	24 (10^{-6})	61	0	0
IXB	48 (10^{-6})	50 (10^{-6})	43 (10^{-6})		
XA	15	16	21	0	0
XB	16	13	12		

[a] I, dihydrofolate reductase (bovine liver); II, thymidylate synthetase (calf thymus); III, serine transhydroxymethylase (porcine liver); IV, 5,10-methylenetetrahydrofolate reductase (porcine kidney); V, methionine synthetase (porcine kidney); VI, glycinamide ribotide transformylase (pigeon liver); VII, 5-amino-4-imidazolecarboxamide ribotide transformylase (pigeon liver); VIII, 5,10-methylenetetrahydrofolate dehydrogenase (A, porcine liver; B, L1210 tumor tissue); IX, 5,10-methenyltetrahydrofolate cyclohydrolase (A, porcine liver; B, L1210 tumor tissue); X, formyl tetrahydrofolate synthetase (A, porcine liver; B, L1210 tumor tissue). [b] Percent inhibition observed at 10^{-4} M inhibitor concentration; exceptions contain the molar inhibitor concentration in parentheses following the percent inhibition.

is formed by reduction of the hydroxymethyl groups of 5,10-($HOCH_2$)$_2$-THF. A similar reaction sequence has been reported for the preparation of methyl 5,6,7,8-tetrahydro-5-10-dimethylpteroate.[10] In a related reaction, reductive alkylation at both N-5 and N-10 was observed in the treatment of an aqueous solution of THF with 40% aqueous glyoxal solution and $NaBH_3CN$ to give 5,10-(CH_2CH_2)-THF (**10**), which was purified by cellulose column chromatography. Reductive alkylations at N-10 of both folic acid and 5-(CHO)-THF were effected with formaldehyde and $NaBH_3CN$ to give 10-methylfolic acid (**6**)[11] and 5-(CHO)-10-(CH_3)-THF (**28**),[12] respectively. In contrast, the reductive alkylation of folic acid with propanal, chloroacetaldehyde, and ethyl glyoxylate under similar conditions was unsuccessful.

The positions of substitution in these compounds were determined by a combination of methods. Unlike THF, THF derivatives (e.g., 5-CHO-THF) substituted in the pyrazine ring are more stable toward oxidation,[13] and an indication of stability can be obtained by the determination of the UV spectra and HPLC chromatograms. In addition, examination of the ^{1}H NMR spectra of 10-acylfolic acids (δ 7.4–7.7, 7.6–8.1) either in Me_2SO-d_6 or D_2O showed that one pair of the phenylene protons were considerably deshielded relative to one pair of the phenylene protons of folic acid (δ 6.6, 7.7) and 5-substituted THF derivatives (δ 6.5–7.0, 7.5–7.9).[14] The UV spectra and HPLC chromatograms showed that 5,10-(C=X)-THF, where X = O, S, and NH (**8**, **9**, and **11**), were reasonably stable compounds, and the ^{1}H NMR spectra showed the downfield shift of one pair of the phenylene protons as described above. Similar phenylene proton shifts were observed for 10-acylfolic acids, 10-(acyl)-THFs, and the bridged compound 5,10-(CH_2CO)-THF (**12**). This shift is attributed to the electron-withdrawing, unsaturated moiety substituted at N-10. Of interest is that both folic acid and 5-substituted THF derivatives in CF_3CO_2D showed the phenylene proton shift, which is attributed to protonation at N-10. The structures of 5,10-(Me)$_2$-THF (**27**) and 5,10-(CH_2CH_2)-THF (**10**) were confirmed by treatment with aqueous nitrous acid. These experiments gave an immediate positive starch–iodide test, indicating that both N-5 and N-10 were substituted.[12]

Biological Evaluation. The bridged compounds, 5,10-(C=X)-THF (X = O, S, and NH) (**8**, **9**, and **11**) were evaluated as inhibitors of ten folate-dependent enzymes (Table I). Essentially no inhibition of the majority of these enzymes was observed. However, both 5,10-methylene-THF dehydrogenase (EC 1.5.1.5) and 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9) were inhibited significantly by **8** and **9** at micromolar concentrations of these folate analogues. In fact, of over 100 potential antifolates examined,[15] the carbonyl and the thiocarbonyl derivatives proved to be the most effective inhibitors found for folate-dependent enzymes other than dihydrofolate reductase.

These studies would suggest that the trifunctional protein [formyl–methenylmethylenetetrahydrofolate synthetase (combined)][16] has at least two folate binding sites: a common site shared by the dehydrogenase and cyclohydrolase and a second site, which was uneffected by **8** and **9**, associated with the synthetase activity. The possibility exists that 5,10-bridged compounds might be designed and synthesized to contain reactive substituents that would enable them to be used as selective affinity or photoaffinity probes of the folate binding site of the dehydrogenase and cyclohydrolase. To determine the in vivo activity of 5,10-(CO)-THF (**8**), this compound (400, 200, and 100 mg/kg dose) plus methotrexate (3 mg/kg dose) were tested against lymphocytic leukemia P388/vincristine in mice on a chronic schedule.[17] Combination therapy, however, gave a lower increase in life span than methotrexate alone.

Although 5,10-(CH_2CH_2)-THF (**10**) was reported as an inhibitor of thymidylate synthetase,[3] this compound

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showed insignificant activity against the enzyme from either calf thymus or from blast cells of patients with acute myelocytic leukemia[18] [16 and 12% inhibition ($\sim 10^{-4}$ M), respectively]. Of interest, 5,10-$(CH_3)_2$-THF (**27**) was also inactive against thymidylate synthetase, but both 5,10-$(CH_3)_2$-THF (**27**) and 5,10-(CH_2CH_2)-THF (**10**) showed 33–39% inhibition (10^{-4} M) against AICAR transformylase. The chloroacetyl derivatives, **2** and either **18** or **19**, were studied as inhibitors of thymidylate synthetase from L1210 cells,[19] but no significant inhibition was observed.[20] Against dihydrofolate reductase (bovine liver), **2** gave an ID_{50} of 5.7×10^{-5} M (methotrexate, $ID_{50} = 4.8 \times 10^{-9}$ M).[20]

The folic acid and THF derivatives, with the exception of **2** ($ED_{50} \approx 8$ μM), showed no significant cytotoxicity in the HEP-2 cell culture screen.[21] Also, the same compounds showed no activity against L1210 leukemia cells implanted ip in mice on either a single-dose or chronic schedule.[22]

In summary, **8** and **9** were inhibitors of both 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase, and these agents should be useful in enzymatic studies with these enzymes.

Experimental Section

Melting points were determined on a Kofler Heizbank unless otherwise indicated. Absence of melting point data indicates an indefinite melting point. The ultraviolet absorption spectra were determined with a Cary Model 17 spectrophotometer. Each compound was dissolved in the solvent indicated in parentheses and diluted with the appropriate solvent. The ^{1}H NMR spectra were determined with a Varian XL-100-15 spectrometer operating at 100 MHz or a T-60A operating at 60 MHz (internal Me_4Si or DSS). The relative peak areas are given to the nearest whole number, and chemical shifts quoted in the case of multiplets are measured from the approximate center. Mass spectral data were taken with a Varian MAT 311A instrument equipped with a combination E1/F1/FD ion source. High-pressure liquid chromatography (HPLC) was carried out on an ALC-242 liquid chromatograph equipped with a UV detector (254 nm), an M-6000 pump, and a 30 cm × 4 mm (i.d.) column of μ-Bondapak C_{18} (Waters Associates, Inc.).

10-(Chloroacetyl)folic Acid (2). To a suspension of $1{\cdot}2H_2O$ (23.9 g, 50.0 mmol) in anhydrous DMAC (250 mL) was added dropwise with stirring a solution of chloroacetyl chloride (22.6 g, 200 mmol) in anhydrous DMAC (100 mL). After 20 h at room temperature, the resulting solution was evaporated to dryness in vacuo, and the residue was suspended in H_2O (400 mL) and adjusted to pH 3.4 with dilute NaOH. The insoluble material was collected by filtration, washed with H_2O, and then dissolved in boiling H_2O (1.8 L). After filtration through a steam-heated funnel, the filtrate was cooled to deposit the product as a tan solid: yield 17.8 g (66%); mp >260 °C with charring; UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N NaOH) at pH 7, 236 (24.3), 272 (19.3), 347 nm (6.23); ^{1}H NMR (Me_2SO-d_6, 6% w/v), δ 4.22 (s, CH_2Cl), 5.10 (s, 9-CH_2), 7.60 and 7.94 (2 d, C_6H_4), 8.67 (s, 7-CH). Anal. ($C_{21}H_{20}ClN_7O_7{\cdot}H_2O$) C, H, N.

10-(3-Chloropropionyl)folic Acid (3). A solution of 3-chloropropionyl chloride (22.2 g, 175 mmol) in anhydrous DMAC (100 mL) was added dropwise to a mechanically stirred suspension of $1{\cdot}2H_2O$ (23.9 g, 50.0 mmol) in DMAC (300 mL). After 18 h at room temperature, the dark red solution was evaporated to dryness in vacuo (50 °C), and the resulting gum was washed with Et_2O and dried in vacuo over P_2O_5. The semisolid residue was dissolved in H_2O (400 mL), and the solution was adjusted to pH 3.5 with NaOH to deposit the product (25.0 g), which was recrystallized from H_2O (2 L): yield 21.5 g (81%); mp indefinite with decomposition above 220 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N NaOH) in 0.1 N NaOH, 256 (37.3), 364 nm (7.72); ^{1}H NMR (Me_2SO-d_6, 7.5%, w/v) δ 7.52 and 7.92 (2 d, C_6H_4), 8.68 (s, 7-CH). Anal. ($C_{22}H_{22}ClN_7O_7$) C, H, N; Cl: calcd, 6.66; found, 6.23.

10-Acetylfolic Acid (4). A solution of acetyl chloride (12.2 g, 155 mmol) in anhydrous DMAC (100 mL) was added dropwise to a mechanically stirred suspension of $1{\cdot}2H_2O$ (23.9 g, 50.0 mmol) in DMAC (250 mL). The reaction was slightly exothermic and gave an orange-colored solution within 30 min after the addition was completed. The solution was allowed to stand for 18 h at room temperature and evaporated to dryness in vacuo (40 °C), and the resulting gum was dried in vacuo over P_2O_5. The gum was suspended in H_2O (1.5 L), adjusted to pH 3.6 with NaOH, and heated to boiling. The resulting solution was filtered and allowed to cool slowly to give a stiff gel, which could not be collected by centrifugation. The combined materials in a total volume of about 2.2 L was dissolved by the addition of solid $Ca(OH)_2$. The solution (pH 7.1) was filtered and diluted with EtOH (2.5 L). The cream-colored solid that precipitated was collected by filtration, washed with EtOH, and dried in vacuo over P_2O_5: yield 20.6 g (69%); mp >360 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N NaOH, 255 (35.6), 365 nm (7.83); ^{1}H NMR (D_2O, 3% w/v), δ 7.39 and 7.86 (2 d, C_6H_4), 8.54 (s, 7-CH). Anal. ($C_{21}H_{19}N_7O_7{\cdot}1.1Ca{\cdot}0.3C_2H_6O{\cdot}3H_2O$) C, H, Ca, N.

10-Ethoxalylfolic Acid (5). To a suspension of $1{\cdot}2H_2O$ (4.77 g, 10.0 mmol) in DMAC (100 mL) was added dropwise with stirring ethyl oxalyl chloride (4.44 g, 32.5 mmol). The resulting dark red solution was stirred at room temperature for 18 h and evaporated to dryness in vacuo. The gum was washed with Et_2O (2 × 200 mL) and suspended in H_2O (200 mL), and the resulting slurry was dissolved by the addition of 50% NaOH to pH 4.7 and then reprecipitated by the addition of HCl to pH 2.8: yield 3.65 g. This crude sample was suspended in H_2O (100 mL) and adjusted to pH 7.1 with $Ca(OH)_2$. After filtration, the filtrate was diluted with EtOH (20 mL) and the solid that precipitated was removed by filtration. The filtrate was diluted with an additional 3 volumes of EtOH to precipitate the calcium salt of the product: yield 1.89 g (30%); mp >350 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N NaOH, 255 (35.6), 364 nm (7.99); ^{1}H NMR (CF_3CO_2D, 7.5% w/v), δ 7.64 and 8.05 (2 d, C_6H_4), 9.03 (s, 7-CH). Anal. ($C_{23}H_{21}N_7O_9{\cdot}Ca{\cdot}0.2C_2H_6O{\cdot}2.4H_2O$) C, H, Ca, N.

10-Methylfolic Acid (6).[11] Folic acid (1) dihydrate (500 mg, 1.05 mmol) was dissolved in H_2O (25 mL) by adjusting the pH to 6.4 with 1 N NaOH. This solution under N_2 was treated with 38% HCHO (414 mg, 5.25 mmol) and $NaBH_3CN$ (100 mg, 1.59 mmol) and maintained at pH 6.5 for 45 min by occassional addition of 1 N HCl. The solution was stirred for 20 h and adjusted to pH 2.8 with 6 N HCl, and the yellow precipitate of **6** was collected by filtration, washed with H_2O, and dried at 100 °C in vacuo (P_2O_5): yield 318 mg (44%); UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N NaOH) in 0.1 N HCl, 220 (23.1), 250 (sh) (12.9), 307 nm (24.1); at pH 7, 217 (24.4), 280 (sh) (24.9), 303 (27.1), 365 nm (sh) (6.63); in 0.1 N NaOH, 222 (20.6), 255 (24.9), 303 (26.6), 366 nm (9.26); ^{1}H NMR (Me_2SO-d_6, 6.3% w/v), δ 2.02 (m, $CH_2CH_2CO_2H$), 2.33 (t, CH_2CO_2H), 3.20 (s, CH_3), 4.36 (m, NHC*H*), 4.81 (s, CH_2N), 6.81 and 7.75 (2 d, C_6H_4), 7.06 (s, NH_2), 8.17 (d, NH), 8.50 (s, 7 H). Anal. ($C_{20}H_{21}N_7O_6$) C, H, N.

5,6,7,8-Tetrahydro-N^5,N^{10}-carbonylfolic Acid (8). Phosgene was bubbled through a suspension of **7** (2.12 g, 3.41 mmol) in O_2-free H_2O (230 mL) at a rate of approximately two bubbles per second for 1 h. The solution under N_2 was adjusted to pH 2.8 with 50% NaOH and cooled in an ice bath. The resulting yellow precipitate was collected by filtration under N_2, washed with cold H_2O at pH 2.8, and dried in vacuo (P_2O_5): yield 1.16 g (67%); UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N HCl, 283 nm (22.9); at pH 7, 290 nm (22.2); in 0.1 N NaOH, 276 nm (21.9); ^{1}H NMR (Me_2SO-d_6, 6.3% w/v), δ 7.72 and 7.93 (2 d, C_6H_4); ^{1}H NMR (CF_3CO_2D, 6.3% w/v), δ 7.73 and 7.97 (2 d, C_6H_4). Anal. ($C_{20}H_{21}N_7O_7{\cdot}H_2O{\cdot}0.6HCl$) C, H, N.

5,6,7,8-Tetrahydro-N^5,N^{10}-(thiocarbonyl)folic Acid (9). A stirred solution of **7** (2.28 g, 4.49 mmol) and $NaOAc{\cdot}3H_2O$ (2.24

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g, 16.5 mmol) in oxygen-free H_2O (60 mL) was treated dropwise with thiophosgene (0.431 mL, 5.66 mmol) and stirred vigorously for 90 min. The reaction mixture was adjusted to pH 3 with 6 N HCl and stirred for 20 min. The precipitate was collected by filtration, washed with H_2O, and redissolved in oxygen-free H_2O (120 mL) by the dropwise addition of 1 N NaOH to give a pH of 7.5. The solution was treated with charcoal, filtered, and adjusted to pH 2.7 with 1 N HCl. The tan precipitate was collected by centrifugation, washed with H_2O at pH 2.7, and dried in vacuo (P_2O_5): yield 1.81 g (80%); UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N NaOH) in 0.1 N HCl, 281 nm (23.4); at pH 7, 217 (35.1), 285 nm (20.9); in 0.1 N NaOH, 276 (17.9), 295 nm (sh) (17.0); ^{1}H NMR (Me_2SO-d_6, 8.5% w/v) δ 7.92 (s, C_6H_4). Anal. ($C_{20}H_{21}N_7O_6S \cdot H_2O$) C, H, N, S.

N^5,N^{10}-Ethylene-5,6,7,8-tetrahydrofolic Acid (10). A solution of freshly prepared crude **7** (31 g, 50 mmol) in water (900 mL) at pH 6 under N_2 was treated with 40% aqueous glyoxal solution (100 mL) followed by a solution of $NaBH_3CN$ (30 g) in H_2O (75 mL). After 30 min, the solution was diluted with a solution of $CaCl_2$ (60 g) in H_2O (150 mL), adjusted to pH 7.5 with dilute NaOH, and diluted with 5 volumes of EtOH. The resulting precipitate (57.9 g) was dissolved under N_2 in H_2O (400 mL) containing ascorbic acid (5 g), and the solution was adjusted to pH 3.7 with 6 N HCl to give crude **10**: yield 17.4 g. This sample was dissolved in 0.1 M 2-mercaptoethanol (150 mL) by the addition of concentrated NH_4OH and adjusted to pH 5 with HOAc. The resulting red solution was applied to a cellulose column (1 kg, Whatman CF-1) and eluted with 0.1 M NH_4OAc (pH 5) that was 0.1 M in 2-mercaptoethanol. The product traveled on the column as a reddish-brown band. The fractions containing product (TLC) were pooled and concentrated in vacuo at 45 °C to about 0.1 volume, followed by treatment under N_2 with HCl to pH 3 to precipitate **10**: yield 12 g (~48%); FDMS, m/e 472 [$(M + 1)^+$], 453 [$(M - H_2O)^+$]; UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N NaOH) in 0.1 N HCl, 217 (28.9), 269 (21.6), 290 nm (sh) (19.3); at pH 7, 216 (29.5), 287 nm (26.8); in 0.1 N NaOH, 285 nm (23.9); ^{1}H NMR (Me_2SO-d_6, 8% w/v), δ 6.96 and 7.77 (2 d, C_6H_4); ^{1}H NMR (CF_3CO_2D, 6% w/v) δ 3.6–4.7 (br m, CH_2), 7.36 and 7.98 (2 d, C_6H_4). Anal. ($C_{21}H_{25}N_7O_6 \cdot 1.58H_2O$) C, H, N.

A portion (3.0 g) of the above sample was suspended in deaerated H_2O (60 mL) and adjusted to pH 9.5 with solid $Ca(OH)_2$. After filtration under N_2, the filtrate was adjusted to pH 7.5 and diluted with EtOH to precipitate the calcium salt: yield 0.88 g. Anal. ($C_{21}H_{23}N_7O_6 \cdot Ca \cdot C_2H_6O \cdot 2H_2O$) C, H, Ca, N, ash.

5,6,7,8-Tetrahydro-N^5,N^{10}-(imidocarbonyl)folic Acid (11). A suspension of $1 \cdot 2H_2O$ (955 mg, 2.0 mmol) in H_2O (25 mL) was adjusted to pH 7.8 with 1 N NaOH, and the resulting solution under nitrogen was treated with $NaBH_4$ (1.00 g, 26.4 mmol) in small portions over a period of 30 min while occassionally adjusting the pH to 8.0 with glacial acetic acid. The solution was stirred for 15 min and acidified with acetic acid to pH 6.5 to destroy excess $NaBH_4$. The solution was treated with cyanogen bromide (318 mg, 3.00 mmol), stirred for 2 h under nitrogen, treated with $CaCl_2$ (278 mg, 2.50 mmol), and diluted with EtOH (125 mL). The light orange precipitate of crude calcium salt was collected in a refrigerated centrifuge, washed with EtOH, and dried in vacuo: yield 1.24 g. A mixture of 900 mg of the crude product in H_2O (5 mL) was taken to pH 5.8 with 6 N HCl and stirred for 1 h. The mixture was filtered, and the precipitate was washed with H_2O (2 mL). The filtrate and wash were applied to a column (16 mm × 120 cm) of Sephadex G-10 (95 g) in H_2O. The column was developed with H_2O at a flow rate of 2 mL/h, and the fractions were monitored by TLC (Avicel, 0.1 M PO_4^{-3}, pH 7). The fraction containing the product was concentrated to 5 mL in vacuo and diluted with EtOH (50 mL). The precipitate of calcium salt was collected in a refrigerated centrifuge, washed with EtOH, and dried in vacuo (P_2O_5): yield 208 mg (25%); mp >260 °C (Kofler Heizbank); UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N HCl, 220 (sh) (23.1), 280 nm (23.4); at pH 7, 222 (32.4), 283 nm (21.8); in 0.1 N NaOH, 284 nm (26.5); ^{1}H NMR (Me_2SO-d_6, 5% w/v) δ 1.18 (s, CH_3 of EtOH), 7.57 and 7.97 (2 d, C_6H_4). Anal. ($C_{20}H_{19}N_8O_6Ca \cdot 0.3C_2H_5OH \cdot 3.6H_2O$) C, H, N.

5,6,7,8-Tetrahydro-N^5,N^{10}-(2-oxoethylene)folic Acid (12). A solution of **2** (3.00 g, 5.60 mmol) in CF_3CO_2H (75 mL) containing prereduced Pt (from 300 mg PtO_2 in 25 mL of CF_3CO_2H) was hydrogenated at room temperature and atmospheric pressure. Two molar equivalents of H_2 was taken up in 3.5 h. The catalyst was removed by filtration (Celite), and the filtrate was evaporated to dryness in vacuo. TLC showed that the reaction product was a mixture in which one component was [5,10-CH-THF]$^+$. The ^{1}H NMR showed that the mixture contained an acetyl moiety, presumably 10-CH_3CO-THF (**15**). The residue was suspended in deaerated H_2O (60 mL) and adjusted to pH 11 with solid $Ca(OH)_2$. The dark solution was filtered to remove resinous material, and the filtrate was adjusted to pH 7.2 with HCl and diluted successively with 1 and 4 volumes of EtOH. The first precipitate (0.26 g) was discarded, and the second precipitate (1.06 g) was eluted from a cellulose (400 g, Whatman CC31) column with pH 6.5, 0.1 M NH_4OAc that was 0.01 M in 2-mercaptoethanol at a rate of 2 mL/min. The faster-moving UV-absorbing band appeared in 5 h, and 24 10-mL fractions were collected. Based on TLC results, fractions 14–24 were combined, evaporated to dryness, and dissolved in deaerated H_2O by the addition of solid $Ca(OH)_2$ to pH 7.1. This sample was diluted with EtOH to give a trace amount of a precipitate, which was removed by filtration. The filtrate was then diluted with a large volume of EtOH to precipitate the product (0.39 g). This material was dissolved in H_2O, and the solution was acidified to pH 4.5. The flocculent solid was removed by filtration, and the filtrate was adjusted to pH 3.6. The solid that precipitated was reconverted to the calcium salt as described above: yield 0.18 g (5%); mp >350 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N HCl (unstable), 255 (br; 17.6), 275 (sh; 16.2); at pH 7, 245 (sh; 18.2), 270 (sh; 15.4); in 0.1 N NaOH, 247 (17.9), 265 nm (sh; 15.9); ^{1}H NMR (D_2O, 5% w/v) δ 3.56 and 3.86 (m and br s, CH_2), 7.46 and 7.93 (2 d, C_6H_4). Anal. ($C_{21}H_{21}N_7O_7 \cdot Ca \cdot 0.6C_2H_6O \cdot 3H_2O$) C, H, Ca, N.

10-(3-Chloropropionyl)-5,6,7,8-tetrahydrofolic Acid (14). A solution of **3** (3.00 g, 5.64 mmol) in CF_3CO_2H (125 mL) containing platinum oxide (0.2 g) was hydrogenated at room temperature and atmospheric pressure. The hydrogen uptake corresponded to 112% of theory and occurred in 70 min. After filtration under N_2, the filtrate was evaporated to dryness in vacuo, and the residue was dissolved in deaerated H_2O (50 mL). This solution was filtered to remove resinous material, and the filtrate was adjusted to pH 3.5 with HCl. The yellow solid that precipitated was collected by filtration, washed with cold deaerated HCl (pH 3.5), and dried in vacuo over P_2O_5: yield 1.96 g. A portion (1.4 g) of this sample was eluted from a cellulose (350 g, Whatman CC31) column with 0.1 M KH_2PO_4 buffer (pH 6) that was 0.02 M in 2-mercaptoethanol. The major UV-absorbing band was collected and acidified to pH 3.6, and the solid that precipitated was collected by filtration, washed with deaerated HCl (pH 4), and dried in vacuo over P_2O_5: yield 0.21 g; mp charring >230 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N HCl) in 0.1 N HCl (unstable), 252 (br; 18.9), 316 nm (br; 3.66); ^{1}H NMR (Me_2SO-d_6, 7.5% w/v), δ 7.50 and 7.92 (2 d, C_6H_4). Anal. ($C_{22}H_{26}ClN_7O_7 \cdot H_2O$) C, H, Cl, N.

10-Acetyl-5,6,7,8-tetrahydrofolic Acid (15). A solution of the calcium salt of **4** (2.97 g, 5.00 mmol) in CF_3CO_2H (100 mL) containing platinum oxide (0.25 g) was hydrogenated at room temperature and atmospheric pressure. The theoretical amount of H_2 was taken up in 25 min. After filtration under N_2, the filtrate was evaporated to dryness, and the residue was dissolved in deaerated H_2O (60 mL). Under N_2 this solution was filtered, adjusted to pH 8.5 with $Ca(OH)_2$, refiltered, and adjusted to pH 7.1 with HCl. The yellow solution was diluted with 1 volume of EtOH, and the solid that precipitated was removed by filtration. The filtrate was diluted with 2 additional volumes of EtOH to give the product: yield 1.92 g (66%); mp >350 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N HCl (10% 2-mercaptoethanol), 260 (17.7), 305 nm (sh; 2.92); ^{1}H NMR (D_2O, 8% w/v) δ 1.95 (s, CH_3CO), 7.46 and 7.92 (2 d, C_6H_4). The phenylene peaks showed shoulders, which suggested that **15** was undergoing oxidation. Also, HPLC chromatograms indicated that solutions of **15** in pH 3.6 buffer were undergoing conversion to 10-acetyl-7,8-dihydrofolic acid. Anal. ($C_{21}H_{23}N_7O_7 \cdot Ca \cdot 0.77C_2H_6O \cdot H_2O$) C, H, N.

Acylation of THF with Chloroacetyl Chloride. To a solution of **7** (10.1 g, ~20 mmol) in anhydrous DMAC (150 mL) was added dropwise with stirring a solution of chloroacetyl chloride (7.91 g, 70.0 mmol) in anhydrous DMAC (50 mL). After 1 h the solution was evaporated to dryness, and the residue was dried in vacuo over P_2O_5 and then dissolved in H_2O (150 mL) by the addition of 50% NaOH to pH 8. Adjustment of the solution to

pH 3.3 with dilute HCl gave a sand-colored precipitate: yield 8.4 g. A suspension of this sample in deaerated H_2O (145 mL) was adjusted to pH 11 by the portionwise addition of solid $Ca(OH)_2$. After filtration the filtrate was adjusted to pH 7.5 with dilute HCl and the yellow solid that deposited was removed by filtration. The filtrate was diluted with EtOH (~70 mL) until a permanent precipitate was formed, which was removed by filtration after cooling at 5 °C for 1 h. The filtrate was diluted with 5 volumes of EtOH to give a flocculent white precipitate of the calcium salt, either **18** or **19**: yield 6.10 g (~49%); mp >260 °C; UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N NaOH, 267 (19.3), 303 nm (sh; 10.5); ^{1}H NMR (D_2O, 7% w/v), δ 2.22, 3.46, and 4.04 (m, br m, and br s, CH_2), 7.49 and 7.89 (2 d, C_6H_4). Anal. ($C_{23}H_{24}ClN_7O_9 \cdot Ca \cdot 0.2C_2H_6O \cdot 0.7H_2O$) C, H, Ca, Cl, N.

Mixture of 5- and 10-Ethoxalyl-5,6,7,8-tetrahydrofolic Acid (21 and 22). A solution of **5** (632 mg, 1.00 mmol) in CF_3CO_2H (50 mL) containing platinum oxide (50 mg) was hydrogenated at room temperature and atmospheric pressure. The hydrogen uptake corresponded to 117% of theory. After filtration the filtrate was evaporated to dryness in vacuo, the residue was dissolved in deaerated H_2O (50 mL), and the solution was adjusted to pH 7.5 with $Ca(OH)_2$. The filtered solution was diluted with 1 volume of EtOH, and the precipitate was removed by filtration. The filtrate was diluted with 5 volumes of EtOH to precipitate the calcium salt of the product, which was collected by filtration and reprecipitated using the same conditions: yield 236 mg (38%); UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N HCl, 283 nm (24.3); at pH 7, 219 (31.5), 283 nm (25.5); ^{1}H NMR (D_2O, 7.5% w/v), δ 6.67 and 7.64 (2 d, C_6H_4) (**21**, ~70%), 7.46 and 7.92 (2 d, C_6H_4) (**22**, ~30%); ^{1}H NMR in CF_3CO_2D showed three sets of phenylene peaks, which suggested the presence of the 5,10-methenyl analogue (**23**) under these conditions. Anal. ($C_{23}H_{25}N_7O_9 \cdot 1.07Ca \cdot 0.25C_2H_6O \cdot H_2O$) C, H, Ca, N.

5-(Carboxymethyl)-5,6,7,8-tetrahydrofolic Acid (24). A solution of $1 \cdot 2H_2O$ (10.0 g, 20.9 mmol) in H_2O (200 mL, pH ~7) was cooled in an ice-bath and reduced to THF by the addition, with stirring over 10 min, of a solution of $NaBH_4$ (10 g) in H_2O (66 mL). After an additional 30 min, excess $NaBH_4$ was destroyed with glacial HOAc. Glyoxylic acid (50% in H_2O, 3.7 g, ~25 mmol) was added to give a precipitate, which was dissolved after 30 min by the addition of 50% NaOH to pH 7.5. The clear solution of the adduct was reduced with a solution of $NaBH_4$ (5 g) in H_2O (34 mL). One hour after the addition was complete, the solution was adjusted to pH 3.8 with HOAc and refrigerated for 18 h. The inorganic precipitate was removed by filtration, and the filtrate was evaporated to dryness in vacuo at 40 °C. The resulting thick oil was triturated and washed with ethanol to give the sodium salt of **24**, which was collected by filtration and washed with additional ethanol: yield 12.1 g. A solution of this salt in deaerated H_2O (200 mL) was adjusted to pH 12 with solid $Ca(OH)_2$, and the turbid mixture was filtered (Celite) to remove boron salts. The filtrate was adjusted with HCl to pH 7.5 and diluted with a solution of $CaCl_2$ (5 g) in H_2O (10 mL) to give a precipitate of the calcium salt: yield 7.71 g. An additional amount (3.83 g) of the calcium salt was obtained by dilution of the filtrate with 2.5 volumes of ethanol. A solution of the combined salts (11.5 g) in deaerated H_2O (300 mL) was adjusted to pH 12 with 50% NaOH, solid $Ca(OH)_2$ (150 mg) was added, and the resulting slurry was filtered under N_2 pressure. The filtrate was adjusted with HCl to pH 3.8, and the precipitate of **24** was collected by filtration, washed with deaerated HCl (pH 3.7, 2 × 150 mL), and dried in vacuo over P_2O_5: yield 4.87 g (43%). This material is photosensitive: FDMS, m/e 504 [$(M + 1)^+$]; UV λ_{max} ($\epsilon \times 10^{-3}$) (1% 2-mercaptoethanol in 0.1 N NaOH) in 0.1 N HCl, 275 (sh; 15.4), 292 nm (sh; 17.4); at pH 7, 292 nm (27.8); in 0.1 N NaOH, 293 nm (27.8); ^{1}H NMR (CF_3CO_2D, 5% w/v) δ 3.2–4.8 (m, CH, CH_2), 7.68 and 8.07 (2 d, C_6H_4). Anal. ($C_{21}H_{25}N_7O_8 \cdot 1.8H_2O$) C, H, N.

5-(5-Hydroxypentyl)-5,6,7,8-tetrahydrofolic Acid (25). A solution of $1 \cdot 2H_2O$ (10.0 g, 20.9 mmol) in H_2O (200 mL, pH ~7) was reduced to THF by the addition, with stirring over 15 min, of a solution of $NaBH_4$ (10 g) in H_2O (50 mL). After an additional 30 min, excess $NaBH_4$ was destroyed with HCl (pH 6), followed by adjustment of the pH to 6.8 with NaOH. This solution was treated with 5-hydroxypentanal (2.56 g, 25.1 mmol) and, after 1 h, with a solution of $NaBH_3CN$ (5 g) in H_2O (50 mL). The solution was maintained between pH 6.1 and 6.6 by the addition of HCl over a period of 1 h. The solution was cooled in an ice bath and adjusted with HCl to pH 3.8 to give a small amount of yellow-orange precipitate. This mixture was treated under N_2 with solid $Ca(OH)_2$ (pH 11.5), resulting in the separation of a granular precipitate of boron salts (34 g). Adjustment of the filtrate with HCl to pH 9.5 gave a second precipitate, which was removed by filtration. The filtrate was adjusted with HCl to pH 7.5 and diluted with 1 volume of ethanol. The resulting precipitate (~1 g) was removed by filtration, and the filtrate was diluted with an additional 4 volumes of ethanol to give practically pure **25**: yield 6.46 g. Analysis showed the presence of boron (0.15%). Further purification was effected by repeated reprecipitation of this material from an aqueous solution with ethanol, the 1-volume precipitate being discarded as described above: yield 3.30 g (26%); UV λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) in 0.1 N HCl, 216 (28.2), 268 (18.6), 290 nm (16.6); at pH 7, 214 (27.6), 288 nm (25.9); in 0.1 N NaOH, 287 nm (24.9); ^{1}H NMR (D_2O, 6% w/v), δ 6.69 and 7.64 (2 d, C_6H_4). Anal. ($C_{24}H_{31}N_7O_7 \cdot Ca \cdot 0.15C_2H_6O \cdot 1.2H_2O$) C, H, Ca, N.

5,6,7,8-Tetrahydro-5,10-dimethylfolic Acid (27). With external ice-bath cooling and protection from air by a stream of N_2, a suspension of $1 \cdot 2H_2O$ (10.0 g, 20.9 mmol) in H_2O (200 mL) was dissolved by the dropwise addition of 50% NaOH (2.3 mL). A solution of $NaBH_4$ (10.0 g) in H_2O (50 mL) was added with stirring over 10 min, and after an additional 30 min, the reaction mixture was adjusted to pH 5 with 9 N HCl (~40 mL). This solution of THF was treated with 37% formaldehyde (8.50 mL, ~105 mmol) and then after 30 min with a solution of $NaBH_3CN$ (6.60 g, 105 mmol) in H_2O (50 mL) over 30 min while maintaining the pH (5.0–5.6) by addition of 9 N HCl. After an additional 30 min, the reaction mixture was acidified to pH 3.8 with 9 N HCl, followed by the addition of 2-mercaptoethanol (2 mL). The resulting frothy mixture was stirred for 1 h while HCN was evolved. The crude product was collected by filtration under N_2 and washed with ice-cold, deaerated, dilute HCl (pH 3.7): yield 2.92 g (28%). A TLC of the product was similar to that of the purified sample of **27** described below.

The filtrate was treated first with $CaCl_2$ (5 g) and then with solid $Ca(OH)_2$ to pH 11 to produce a voluminous white solid (25.8 g), which was removed by filtration under N_2. The filtrate was adjusted to pH 7.5 with dilute HCl and diluted with 3 volumes of EtOH to give the calcium salt of crude **27**: yield 5.72 g. After a second reprecipitation with EtOH, the sample (3.10 g) was dissolved in deaerated H_2O (40 mL) and acidified to pH 3.8 with dilute HCl to give the product: yield 1.28 g (12%); mp, gradual sintering with charring from 190 °C and decomposition at 215–220 °C; FDMS, m/e 474 [$(M + 1)^+$]; UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N NaOH) in 0.1 N HCl, 215 (22.0), 270 (sh; 13.0), 301 nm (20.0); at pH 7, 217 (sh; 23.8), 300 nm (25.1); in 0.1 N NaOH, 292 (sh; 22.0), 307 nm (24.6); ^{1}H NMR (Me_2SO-d_6, 5% w/v) δ 2.46 and 2.94 (2 CH_3), 6.68 and 7.74 (2 d, C_6H_4); ^{1}H NMR (CF_3CO_2D, 10% w/v) δ 2.95 and 3.50 (2 CH_3), 7.80 and 8.20 (2 d, C_6H_4). Anal. ($C_{21}H_{27}N_7O_6 \cdot H_2O$) C, H, N.

5-Formyl-10-methyl-5,6,7,8-tetrahydrofolic Acid (28).[12] A stirred solution of 5-(CHO)-THF (1.00 g, 1.56 mmol) in oxygen-free water (20 mL) was treated with 38% HCHO (616 mg, 7.80 mmol) and $NaBH_3CN$ (147 mg, 2.34 mmol). The solution was maintained at pH 6 by the gradual addition of 1 N HCl for 30 min, stirred for 18 h, and acidified to pH 3 with 6 N HCl. The precipitated **28** was collected by filtration under N_2, washed with H_2O, and dried in vacuo (P_2O_5): yield 629 mg (80%); UV λ_{max} ($\epsilon \times 10^{-3}$) (0.01 N NaOH) in 0.1 N HCl, 289 nm (27.4); at pH 7, 221 (31.8), 292 nm (28.1); in 0.1 N NaOH, 287 (26.2), 307 nm (sh; 25.1); ^{1}H NMR (Me_2SO-d_6, 6% w/v) δ 2.02 (m, $CH_2CH_2CO_2H$), 2.34 (t, CH_2CO_2H), 2.93 (s, CH_3), 4.35 (m, $CHNH$), 4.92 (m, 6 H), 6.32 (s, NH_2), 6.72 and 7.74 (2 d, C_6H_4), 7.03 (t, 8 H), 8.10 (d, $CHNH$), 8.76 (s, CHO). Anal. ($C_{21}H_{25}N_7O_7 \cdot H_2O$) C, H, N.

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