spectrometry. Analysis of the crude product by mass spectrometry at 70 eV showed m/e 277, 291, 305, and 319, which are molecular ions for 4 and mono-, di-, and trimethylated 4. A spectrum at 20 eV showed the ratio of molecular ions to be 1.7:8.2:10.0:2.8, respectively. The spectrum of trimethoprim (13) has prominent peaks at m/e 290 (M^+), 275 ($M^+ - CH_3$), 259 ($M^+ - OCH_3$), and 123. A high-resolution mass spectrum showed m/e 123 to have the compositon $C_5H_7N_4$, corresponding to the pyrimidomethylene portion of the molecule. The use of defocused metastable ions in the first field-free region showed that the m/e 290, 275, and 259 all yielded m/e 123. This information can be used to analyze how much of 4 is monomethylated in the benzene vs. pyrimidine rings, since m/e 291 should yield m/e 124 or m/e 138, respectively. A DADI metastable spectrum of m/e 291 produced a peak from the transition m/e 291 → 138, but none for m/e 291 → 124. The accurate mass of m/e 291 and 138 was that expected for $C_{14}H_{17}N_3O$ and $C_6H_8N_3O$, indicating that, within the limits of detection, monomethylation occurs on the pyrimidine portion of the molecule.

Biological Assays. Antibacterial assays were carried out using methods described in ref 3. Assays of dihydrofolate reductase inhibition were carried out using partially purified enzyme from rat liver[33] and affinity-column-purified enzyme from *E. coli*.[34] The assay method was that of Baccanari et al.,[34] in phosphate buffer. Compounds were incubated for 5 min with enzyme and buffer at 37 °C, followed by the addition of NADPH and then FH_2 to initiate the reactions. The inhibitory potencies are reported as the concentration required to reduce the reaction rate by 50% (IC_{50}), as calculated from titrations of three to six concentrations of compound, or as the percent inhibition observed at the highest concentration tested. Examination of data collected over a period of 16 years by several operators showed the 90% tolerance limits for values reported to be ±52.5%.

Acknowledgment. The authors are indebted to Dr. George H. Hitchings for his continued encouragement during the course of this investigation. Dr. Morton Harfenist contributed valuable suggestions concerning the use of the Mannich route. Microanalyses were performed by Drs. Samuel Blackman and Stuart Hurlbert and their staffs. Dr. David Brent and Doris Rouse are responsible for the mass spectral data on methylation products of 4 and the interpretation. The antibacterial assays were carried out under the supervision of Dr. S. R. M. Bushby; Robert Ferone and staff performed the DHFR assays.

(33) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, 1, 126 (1965).

(34) D. Baccanari, A. Phillips, S. Smith, D. Sinski, and J. J. Burchall, *Biochemistry*, 14, 5267 (1975).

2,4-Diamino-5-benzylpyrimidines and Analogues as Antibacterial Agents. 3. C-Benzylation of Aminopyridines with Phenolic Mannich Bases. Synthesis of 1- and 3-Deaza Analogues of Trimethoprim

Barbara S. Rauckman and Barbara Roth*

The Wellcome Research Laboratories, Burroughs Wellcome Company, Research Triangle Park, North Carolina 27709.
Received September 10, 1979

Electrophilic substitution of 2,4-diaminopyridine by 2,6-disubstituted-4-[(*N*,*N*-dimethylamino)methyl]phenols and by halogens (bromine and fluorine) produces 3-benzyl and 3-halo derivatives, plus a small amount of disubstitution at the 3,5 positions. Treatment of a 2,4-diamino-3-halopyridine with phenolic Mannich bases gives 5- and N-benzylation. 2,4-Diamino-3-bromo-5-(4-hydroxy-3,5-dimethoxybenzyl)pyridine was methylated on the phenolic group in good yield and dehalogenated to produce 3-deazatrimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyridine]. This compound is about 300-fold less active as an inhibitor of *Escherichia coli* dihydrofolate reductase than is trimethoprim. 2,6-Diaminopyridine is very readily dibenzylated at the 3,5 positions, as well as on an amino group, by a phenolic Mannich base; use of a fourfold excess of the pyridine provided a 3-benzylated 2,6-diaminopyridine in 50% yield; this was inactive as an inhibitor of dihydrofolate reductase at 10^{-4} M. 2-Amino- and 4-aminopyridines do not produce C-benzylated products under the conditions reported here.

The significance of trimethoprim (1)[1] as a broad-spectrum antibacterial agent[2] made it of importance to investigate related heterocycles. This paper describes the synthesis and biological activities of the 3-deaza- (**31d**) and 1-deaza-4′-demethyl (**4a**) analogues of trimethoprim and some derivatives.

Chemistry. One synthetic route to trimethoprim involves the condensation of a phenolic Mannich base with 2,4-diaminopyrimidine to produce a 5-(substituted-benzyl)pyrimidine.[3] It seemed plausible that such a condensation might succeed with aminopyridines, although a variety of products were possible. Since 2,6-diaminopyridine (**2**) and a dicarbomethoxy intermediate to 2,4-diaminopyridine (**17**) are commercially available, investigation of this route seemed particularly desirable.

Scheme I illustrates the reactions of 2,6-disubstituted pyridines with the Mannich bases from two phenols. When heated in a solvent such as ethylene glycol to about 120–150 °C, such Mannich bases lose dimethylamine to produce an intermediate methene quinone, which is polarized positively at the methene carbon. This then can attack an electron-rich center, as is expected to be present at the 3 and 5 positions of **2**. Use of molar equivalents of **2** and **3a** actually produced 3,5-disubstitution chiefly; introduction of one benzylic moiety apparently aids materially in facilitating 5-substitution. The desired monosubstituted benzyl derivative, **4a**, was produced by using an excess of the pyridine. Small amounts of an N-benzylated product, **6a**, were also obtained.

Somewhat surprisingly, it was found that the Mannich base from a 2,6-dialkylphenol (**3b**) reacted about as successfully with 2,6-diaminopyridine as did the 2,6-dimethoxy analogue. This is in marked contrast to the pyrimidine series, where this was not the case.[3] In fact, by using equivalent amounts of **2** and **3b**, a reasonable yield of **4b**

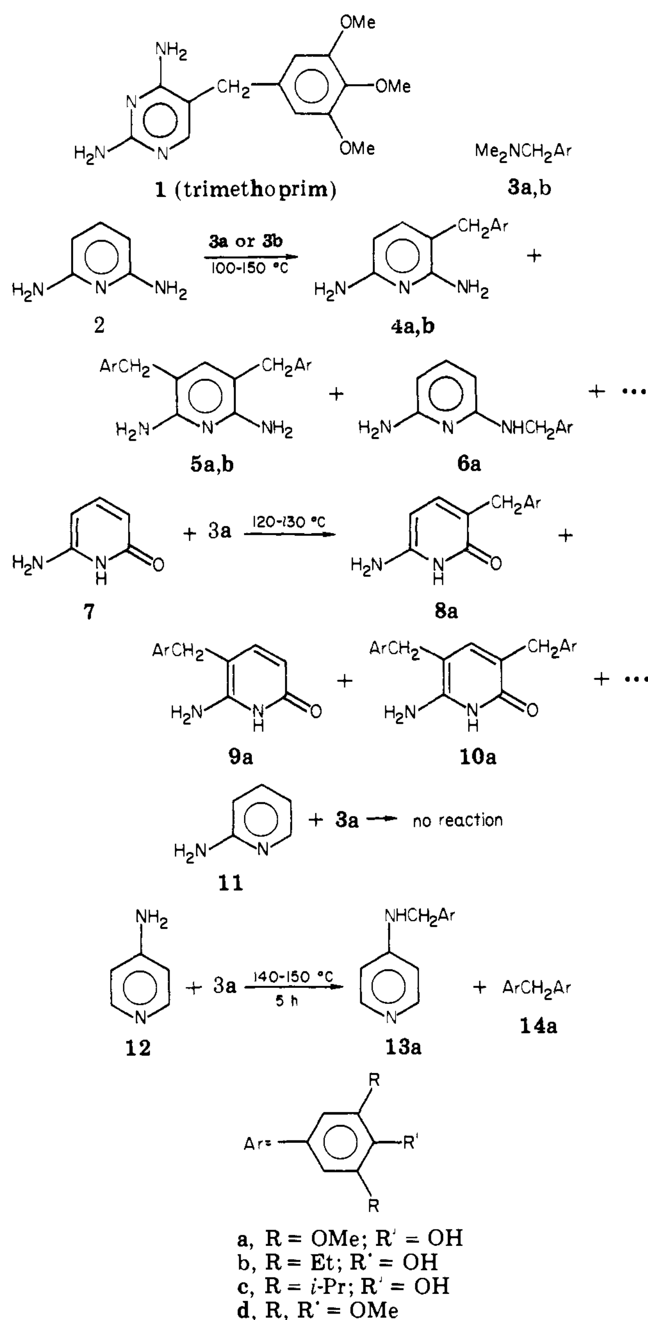
(1) B. Roth, E. A. Falco, G. H. Hitchings, and S. R. M. Bushby, *J. Med. Pharm. Chem.*, 5, 1103 (1962).

(2) (a) "Evaluations on New Drugs: Trimethoprim–Sulfamethoxazole", *Drugs*, 1, 7 (1971). (b) "Symposium on Trimethoprim–Sulfamethoxazole", *J. Infect. Dis.*, 128, supplement (Nov. 1973).

(3) For Paper 2 of this series, see: B. Roth, J. Z. Strelitz, and B. S. Rauckman, *J. Med. Chem.*, 23, preceding paper in this issue (1980).

0022-2623/80/1823-0384$01.00/0 © 1980 American Chemical Society

Scheme I



was obtained, in addition to the disubstituted product.

By analogy with earlier studies of electrophilic substitution on 2(1*H*)-pyridinone and 2-aminopyridine,[4,5] which produced ortho substitution of the former and para substitution of the latter, it was thought that 6-amino-2-(1*H*)-pyridinone (**7**) might produce **8a** exclusively as a C-monobenzylated product. Actually, both 3- and 5-monosubstitution (**8a** and **9a**) occurred when an excess of the pyridinone was used; **10a** was the predominant C-benzylated product isolated using equivalent amounts of the two reagents, as was the case with **2**.

Several attempts were made to benzylate 2-aminopyridine (**11**) with Mannich base **3a**, but this substance proved unreactive to either N- or C-substitution under the conditions described here. 4-Aminopyridine (**12**) did produce an N-substituted product, **13a**, in fairly low yield, along with **14a**, which is formed by heating **3a** in the absence of an electrophile.[3] No C-substituted product was isolated.

2,4-Diaminopyridine (**17**), although described at an early date from the readily available 2,4-bis(methoxycarbonyl)pyridine by Curtius rearrangement,[6] has not been studied extensively, possibly because the synthetic route is rather poor. Azo derivatives have been described in the patent literature,[7] without assignment of substitution position. The 3-nitro and 3-amino derivatives of **17** were prepared by another route.[8] We prepared **17** by the original Curtius route,[6] but at each step isolated byproducts which were a consequence of incomplete reaction on both the 2 and 4 positions. Column chromatography was normally required to separate the products. The synthesis and byproducts are described below.

Scheme II illustrates the reactions of **17** with Mannich bases and with halogens. Initial electrophilic attack was found to occur exclusively at the 3 position rather than at 5 position with both types of reagent. Subsequent reaction occurred to give 3,5-disubstitution. Thus, **17** produced **18a** and **19a** with Mannich base **3a**; no 5-monosubstituted derivative was found among the reaction products. Likewise, bromine produced **20** and **21** exclusively. On the other hand, 2,4-bis[(ethoxycarbonyl)amino]pyridine (**15**) was brominated in the 5 position only (**16**).

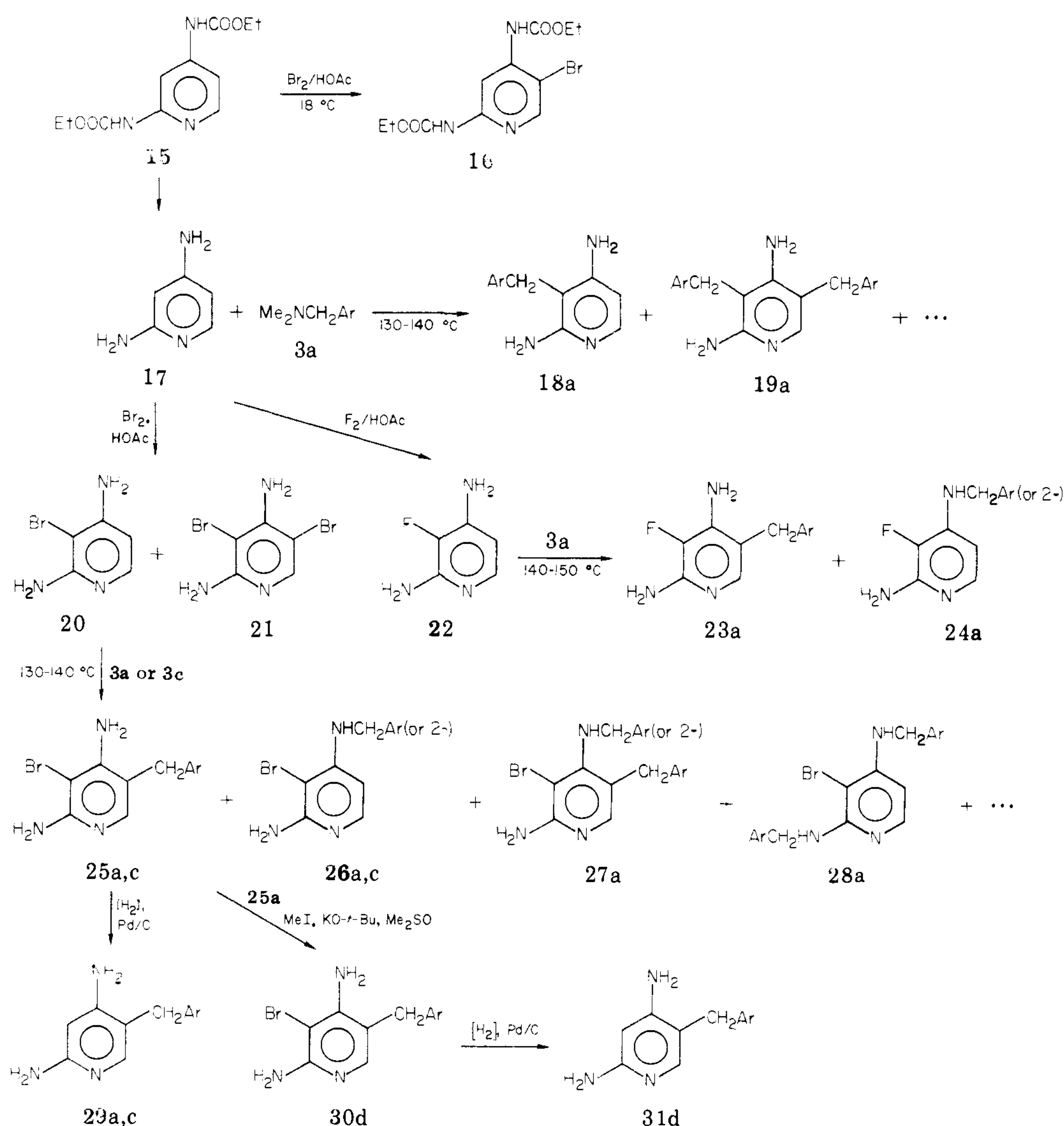
Advantage was taken of the ready formation of **20** in seeking a possible route to **31d**. Treatment of **20** with **3a** did produce 5-benzylation. Unfortunately, this was not a straightforward reaction, as might have been expected by analogy with **19a** and **21**. Not only did mono-N-benzylation occur to produce **26a** in addition to **25a**, but dibenzylation occurred as well to give **27a** and **28a**. The mono- and dibenzylated products could be separated by chromatography. The resultant monobenzylated mixture was then readily separated by fractional crystallization; the final yield of **25a** was about 16%.

Methylation of **25a** on the phenolic group, rather than on the pyridine ring, was accomplished in the presence of a strong base in Me_2SO. The resultant bromopyridine was readily debrominated catalytically to give **31d**, the 3-deaza analogue of trimethoprim, which was the main object of this study. Concerning the methylation, it should be pointed out that **25a**, rather than **29a**, provided a more straightforward target for this reaction, since in the latter case the more basic pyridine ring would be expected to compete with the phenol for methylation.

Compound **31d** is a stronger base than is trimethoprim by about 2 pH units (Table I). It was believed that the inductive effect of a fluorine atom at the 3 position of the pyridine ring should lower the pK_a, hopefully by 2 units, without producing undesirable bulk. Therefore, a study was made of the direct fluorination of 2,4-diaminopyridine.[9] This was accomplished to give the desired product, **22**, in 35% yield.[9] Mannich substitution gave the desired 2,4-diamino-3-fluoro-5-(4-hydroxy-3,5-dimethoxybenzyl)pyridine, **23a**, in 12% yield. The dissociation

(4) B. A. Fox and T. L. Threlfull, "Organic Syntheses", Collect. Vol V, Wiley, New York, 1973, 346.
(5) C. A. Salemink and G. M. van der Want, *Recl. Trav. Chim. Pays-Bas*, **68**, 1013 (1949); see also L. A. Paquette, "Principles of Modern Heterocyclic Chemistry", W. A. Benjamin, New York, 1968, pp 232–234.
(6) H. Meyer and H. Tropsch, *Monatsh. Chem.*, **35**, 189 (1914).
(7) U.S. Patent 2068353 (1937); *Chem. Abstr.*, **31**, 1959 (1937).
(8) (a) Swiss Patent 260573 (1949); *Chem. Abstr.*, **44**, 2040 (1950). (b) F. Kögl, G. M. Van der Want, and C. A. Salemink, *Recl. Trav. Chim. Pays-Bas*, **67**, 129 (1948).
(9) This study and the synthesis of **18** were carried out by Ed Tyczkowski, Armageddon Chemical Co., 431 Salem, Durham, N.C.

Scheme II[a]



[a] For Ar, see Scheme I.

Table I. Dissociation Constants of Substituted Pyridines at 20 °C

no.	pyridine substituents[a] 2	3	4	5	6	dissociation constants +	
11	NH_2					6.82[b]	
12			NH_2			9.25[c]	
17	NH_2		NH_2			9.25 ± 0.01	
15	NHCOOEt		NHCOOEt			5.04 ± 0.01	
36	$CONH_2$		NHCOOEt			3.32 ± 0.02	
20	NH_2	Br	NH_2			7.44 ± 0.02	
21	NH_2	Br	NH_2	Br		5.50 ± 0.01	
31d	NH_2		NH_2	W		9.03 ± 0.02	
25a	NH_2	Br	NH_2	X		7.20 ± 0.02	10.18 ± 0.03
23a	NH_2	F	NH_2	X		7.15 ± 0.02	~10.2
26a	NH_2[d]	Br	NHX[d]			7.33 ± 0.01	10.18 ± 0.03
24a	NH_2[d]	F	NHX[d]			7.26 ± 0.02	~10.2
2	NH_2				NH_2	6.73 ± 0.02	
6a	NH_2				NHX	6.51 ± 0.01	10.14 ± 0.03
4a	NH_2	X			NH_2	6.60 ± 0.02	10.29 ± 0.04
4b	NH_2	Y			NH_2	6.59 ± 0.01	10.92 ± 0.04
5a	NH_2	X		X	NH_2	6.69 ± 0.04	10.54 ± 0.05
5b	NH_2	Y		Y	NH_2	6.63 ± 0.04	11.74 ± 0.05
8a	=O	X			NH_2	1.31 ± 0.02	*e*
10a	=O	X		X	NH_2	1.51 ± 0.03	*e*

[a] Benzylic substituents are designated as follows: W = 3,4,5-trimethoxybenzyl; X = 4-hydroxy-3,5-dimethoxybenzyl; Y = 4-hydroxy-3,5-diethylbenzyl; Z = 4-hydroxy-3,5-diisopropylbenzyl. [b] A. Albert, R. Goldacre, and J. Phillips, *J. Chem. Soc.*, 2240 (1948). [c] R. G. Bates and H. B. Hetzer, *J. Res. Natl. Bur. Stand., Sect. A*, 64, 427 (1960). [d] 2- and 4-substituents may be reversed. [e] Not determined; overlapping pK_a values in 10-12 region.

Table II. Inhibitory Activities of Aminopyridine Derivatives vs. Dihydrofolate Reductases

	pyridine substituents[a]					$I_{50} \times 10^6$, M	
no.	2	3	4	5	6	*E. coli*	rat liver
31d	NH_2		NH_2	W		140	7%; ~8 800
29a	NH_2		NH_2	X		37	37%; ~10 000
23a	NH_2	F	NH_2	X		510	32%; ~35 000
25a	NH_2	Br	NH_2	X		11%; ~42 000	21%; ~42 000
29c	NH_2		NH_2	Z		114,[b] 96	36%; ~27 000
25c	NH_2	Br	NH_2	Z		3 300	0%; ~23 700
18a	NH_2	X	NH_2			26%; ~40 000	41 000
4a	NH_2	X			NH_2	0%; ~10 000	34%; ~10 000
4b	NH_2	Y			NH_2	12 000	26%; ~40 000
5b	NH_2	Y		Y	NH_2	31%; ~1 000,[b] 65%; ~1 700[c]	19%; ~1 000
5a	NH_2	X		X	NH_2	29%; ~40 000	24 000
6a	NH_2				NHX	29%; ~2 000,[b] 25%; ~1 000[c]	0%; ~2 000
8a	=O	X			NH_2	0%; ~1 030	2.4%; ~1 000
10a	=O	X		X	NH_2	13 000	14 000

[a] Benzylic substituents are designated as follows: W = 3,4,5-trimethoxybenzyl; X = 4-hydroxy-3,5-dimethoxybenzyl; Y = 4-hydroxy-3,5-diethylbenzyl; Z = 4-hydroxy-3,5-diisopropylbenzyl. [b] Two independent assays. [c] Compound very insoluble; difficult assay.

constant of this product was almost identical with that of trimethoprim.[10]

Table I presents the dissociation constants of the various new pyridines, compared to some literature values. It will be noted that the addition of a second amino group to **11** or **12** (ortho to the ring N in each case) to give **2** or **17** does not increase the basicity. This effect is largely inductive.[11] The 4-amino substituent has a much larger resonance component (cf. **11** and **12**). Similar effects have been noted in the pyrimidine series.[10] Halogenation of the aminopyridines at the 3 or 5 position likewise produces a large inductive component in the substituent effect; note that 3-bromination of **17** lowers the dissociation constant by 1.8 units and that introduction of a second halogen at the 5 position lowers the pK_a value by an additional increment of 1.9.

Biological Results

Considerable evidence exists that 2,4-diaminopyrimidine derivatives act as inhibitors of the enzyme dihydrofolate reductase [5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase (EC 1.5.1.3)] in their protonated form.[10,12-15] This is also believed to be true of methotrexate, based on X-ray crystallography of the inhibitor in association with the crystalline enzyme.[16,17] It is a plausible assumption that the simple monocyclic pyrimidines interact with the enzyme at the same site as does the pteridine moiety of methotrexate, i.e., with the charged atom (N-1 in methotrexate) directed toward Asp-27 (*E. coli* numbering), the only anionic residue in the cleft,[16,17] although orientations might be expected to vary.

Protonation of trimethoprim and other diaminopyrimidines also occurs at N-1.[10,18,19] It would then be expected that a 2,4-diaminopyridine would bear a closer relationship to trimethoprim in its interaction with dihydrofolate reductase than would a 2,6-diaminopyridine, since the protonation site relative to the three substituents would remain constant in the former case.[15]

Furthermore, it would be expected that the increased basicity of a 3-deazatrimethoprim would not be deleterious to binding to dihydrofolate reductase, based on the argument that trimethoprim binds as its cation (although cell permeability might be lowered).

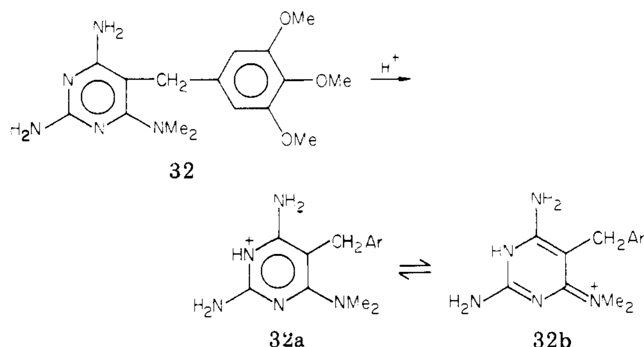
The inhibitory activities of the pyridines described here are shown in Table II. 3-Deazatrimethoprim, **31d**, is roughly 300 times less active as an inhibitor of *E. coli* dihydrofolate reductase than is trimethoprim; however, its 4′-demethyl analogue, **29a**, is about four times more active than is **31d**. Addition of a 3-F (**23a**) drops the activity about 14-fold. The remaining compounds have low activity. Compound **4a**, 1-deaza-4′-demethyltrimethoprim, showed no inhibitory activity at 10^{-4} molar.

The considerable loss in activity effected by replacement of the N-3 atom of trimethoprim by carbon is somewhat surprising. The effect would seem not to be related to the increased basicity of **31d**; based on the above arguments, it might, in fact, increase the binding. Furthermore, restoration of the dissociation constant to 7 by introduction of a fluorine atom (**23a**) had a marked deleterious effect.

The fact that **29a** appears more active than **31d**, whereas the 4′-demethyl analogue of trimethoprim is only about one-half as active,[3] suggests that the pyridine analogue may have a somewhat different orientation in interaction with the enzyme than does trimethoprim.[20] The charge-density patterns would be expected to be different in the two heterocycles; furthermore, the loss of potential hydrogen bonding of N-3 to the enzyme (possibly to the backbone carbonyl at Ile-5, as with methotrexate) could well result in a change in orientation and actual repulsion between C-3 and the backbone. This then could change the spatial

(10) B. Roth and J. Z. Strelitz, *J. Org. Chem.*, **34**, 821 (1969).
(11) M. Charton, *J. Am. Chem. Soc.*, **86**, 2033 (1964).
(12) B. Roth, *J. Med. Chem.*, 12, 227 (1969).
(13) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, New York, 1967.
(14) (a) A. M. Perault and B. Pullman, *Biochim. Biophys. Acta*, **52**, 266 (1961). (b) R. Collin and B. Pullman, *ibid.*, **89**, 232 (1964).
(15) J. A. Montgomery, R. D. Elliott, S. L. Straight, and C. Temple, *Ann. N.Y. Acad. Sci.*, **186**, 227 (1971).
(16) D. A. Matthews, R. A. Alden, J. T. Bolin, S. T. Freer, R. Hamlin, N. Xuong, J. Kraut, M. Poe, M. Williams, and K. Hoogsteen, *Science*, **197**, 452 (1977).
(17) D. A. Matthews, R. A. Alden, J. T. Bolin, D. J. Filman, S. T. Freer, R. Hamlin, W. G. J. Hol, R. L. Kisliuk, E. J. Pastore, L. T. Plante, N. Xuong, and J. Kraut, *J. Biol. Chem.*, **253**, 6946 (1978).
(18) B. Roth, B. S. Hurlbert, J. Z. Strelitz, and G. H. Hitchings, "Abstracts of Papers", 152nd Meeting of the American Chemical Society, New York, 1966, American Chemical Society, Washington, D.C., 1966; unpublished low temperature NMR data.
(19) D. J. Brown, W. Hoerger, and S. F. Mason, *J. Chem. Soc.*, 4035 (1955).
(20) G. H. Hitchings and B. Roth, Symposium Proceedings, Enzyme Inhibitors as Drugs, London, 1979, Macmillan, London, in press.

Scheme III



relationship between the 4′-methoxy and hydrophobic side chains on the enzyme; possible crowding by the methyl could be alleviated to some extent with a 4′-hydroxyl substituent in place of the methoxy group.

It should be noted that the 3-deaza analogue of methotrexate[15] had about one-fifth of the activity of its parent against pigeon liver dihydrofolate reductase and one-fifteenth the activity against *S. faecalis* dihydrofolate reductase. However, this large molecule has more potential binding sites, so that a one-atom change may be less important. The 1-deaza analogue had $^1/_{400}$ the activity of methotrexate; however, its pK_a value was only 4.5, as compared with 7.4 for the 3-deazapteridine.

Compound **4a** was originally prepared for testing on an R-plasmid-resistant dihydrofolate reductase from *E. coli*.[21] Most diaminopyrimidines had been found to be virtually inactive as inhibitors of this enzyme; however, the 6-(dimethylamino) derivative of trimethoprim (**32**)[22] was unique in that it was 40–80 times more active against the plasmid enzyme than against the chromosomal enzyme.[21] A possible explanation relates to the protonation site of **32**. This compound would assume a different orientation from trimethoprim in interaction with the enzyme if it were protonated at N-3 (**32a**), rather than at N-1 (see Scheme III). This is a plausible assumption on both steric and electronic grounds,[23] provided that the 5-substituent does not interfere with the formation of the charge-delocalized species **32b**, with trigonal N. Paper 4 of this series characterizes **32** and its physical properties.[22] Assuming 3-protonation for the present argument, the 6-(dimethylamino) function should then assume the 4-amino locus of trimethoprim in interaction with DHFR. However, this bulky group might prohibit normal binding, as it does in fact with the chromosomal reductase.[22] It would be expected that pyridine 4a, which lacks the dimethylamino function, would assume the orientation of **32**, provided that there were an ionic attraction to the enzyme as described above. Thus, it could possibly have increased inhibitory activity toward the R-plasmid enzyme. In fact, the compound showed only about 26% inhibition at 10^{-4} M against R483 R-plasmid enzyme. It is not improbable that the mutation involves the anionic site (Asp-27).

Experimental Section

Melting points were determined with either a Thomas-Hoover or a Uni-Melt melting apparatus (the latter for melting points greater than 250 °C) and are uncorrected. Proton NMR spectra were carried out on a Varian A-60 or HT-100 spectrophotometer with tetramethylsilane as internal reference. UV spectra were recorded on a Cary 118 UV–visible spectrophotometer. Elemental analyses were carried out by Dr. B. S. Hurlbert and staff using a Carlo Erba Strumentazione elemental analyzer Model 1106 or by the Atlantic Microlab, Inc., Atlanta, Ga. Mass spectra by Dr. D. Brent and staff were run on a Varian MAT 731 or Varian CH-5-DF.

The methods and buffers used for pK_a determinations and UV spectra were those of Roth and Strelitz.[10] A Beckman Research pH meter was used for pH measurements. Buffers were 0.01 N or $^1/_{150}$ M for phosphate.

Column chromatographic separations were carried out using Brinkman silica gel, particle size 0.063–0.2 mm.

Reaction of 2,6-Diaminopyridine (2) with Phenolic Mannich Bases. A. With 2,6-Dimethoxy-4-[(*N*-*N*-dimethylamino)methyl]phenol (3a). (1) **In a 1:1 Ratio.** A mixture of 10.9 g (0.1 mol) of **2**, 21.1 g (0.1 mol) of **3a**, and 0.54 g (0.01 mol) of NaOMe in 130 mL of ethylene glycol was heated under N_2 at 130–150 °C for 3 h. Dimethylamine was evolved and collected in order to observe the progress of the reaction. After 3 h, over 80% of the theoretical amount had been collected. The solution was cooled and neutralized with glacial HOAc to pH 6. After the solution was left standing overnight, a copious cream precipitate formed. Recrystallization from absolute EtOH gave 9.65 g (43.8%) of **2,6-diamino-3,5-bis(4-hydroxy-3,5-dimethoxybenzyl)pyridine (5a)**, which was recrystallized in the presence of HCl to give its hydrochloride: mp 254–257 °C dec; NMR (Me_2SO-d_6) δ 3.62 [s, 4, $(CH_2)_2$], 3.68 [s, 12, $(OMe)_4$], 6.49 (s, 4, Ar), 7.06 [br s, 4, $(NH_2)_2$], 7.30 (s, 1, pyr 4 H), 8.07 [br, 2, (Ar OH)$_2$], 13.4 (br, 1, NH^+); MS *m/e* 441, 410, 288, 275, 274, 167. UV cation (0.01 N HCl) λ_{max} 236 nm (ε 23300), sh 280 (3000), 342 (15400); neutral species (pH 8.4, Tris) sh 232 (22600), 313 (9700); anion (0.1 N NaOH) sh 242 (23700), 308 (13800). Anal. ($C_{23}H_{27}N_3O_6$·HCl) C, H, Cl, N. No monobenzylated pyridine was isolated; however, traces were probably formed.

(2) **In a 4:1 Ratio.** A mixture of 17.5 g (0.16 mol) of **2**, 8.44 g (0.04 mol) of **3a**, and 0.3 g (0.006 mol) of NaOMe in 90 mL of ethylene glycol was heated under N_2 at 100–120 °C for 3.5 h. After the mixture cooled, 4 mL of HOAc was added, and the solvent was removed in vacuo. The residue was slurried with H_2O several times to remove residual solvent; on standing overnight it solidified. The aqueous layer was extracted three times with 80 mL of EtOAc, and the organic solvent was removed. The solid and EtOAc extracts were combined and purified by column chromatography ($CHCl_3$–MeOH, 30:1), which yielded 5.53 g of **2,6-diamino-3-(4-hydroxy-3,5-dimethoxybenzyl)pyridine (4a)** (50.2%) and 63 mg of **5a** (0.7%). Compound **4a** was crystallized twice from EtOAc: mp 152–153.5 °C; NMR (Me_2SO-d_6) δ 3.52 (s, 2, CH_2), 3.70 [s, 6, $(OMe)_2$], 5.13 [br s, 4, $(NH_2)_2$], 5.70 (d, 1, pyr 5 H, *J* = 8 Hz), 6.47 (s, 2, Ar), 6.90 (d, 1 pyr 4 H, *J* = 8 Hz); MS *m/e* 275, 260, 244, 228, 122. UV cation (0.01 N HCl) λ_{max} 240 nm (ε 15700), sh 280 (1700), 336 (15400); neutral species (pH 8.6, Tris) 233 (14400), 308 (8900); anion (0.1 N NaOH) 240 (15400), 303 (10700). Anal. ($C_{24}H_{17}N_3O_3$) C, H, N.

(3) **In a 2:1 Ratio.** A reaction similar to (2) using a 2:1 ratio of the pyridine to the Mannich base produced 34.7% of **4a**, 8.2% of **5a**, and 6.2% of **2-amino-6-(4-hydroxy-3,5-dimethoxybenzylamino)pyridine (6a)**. Compound **6a** was purified on a column (EtOAc), followed by crystallization from EtOAc: mp 170–172 °C; NMR (Me_2SO-d_6) δ 3.74 [s, 6, $(OMe)_2$], 4.26 (d, 2, NHCH_2, *J* = 6 Hz), 5.32 (br s, 2, NH_2), 5.65 (d, 2, pyr 3 H, 5 H, *J* = 8 Hz), 6.22 (t, 1, N*H*CH_2, *J* = 6 Hz), 6.63 (s, 2, Ar), 7.05 (t, 1, pyr 4 H, *J* = 8 Hz), 8.05 (br, 1, ArOH); D_2O exchange, 5.32, 6.22, and 8.05 disappear, 4.26 collapses to br s; MS *m/e* 275, 182, 167, 109. UV cation (0.01 N HCl) λ_{max} 238 nm (ε 13000), sh 280 (1200), 338 (17500); neutral species (pH 8.6, Tris) sh 235 (12000), sh 281 (2600), 311 (9200); anion (0.1 N NaOH) 248 (15300), 310 (10000). Anal. ($C_{14}H_{17}N_3O_3$) C, H, N.

B. With 2,6-Diethyl-4-[(*N*,*N*-dimethylamino)methyl]phenol (3b). A mixture of 10.9 g (0.1 mol) of **2**, 6.91 g (0.033 mol) of **3b**, and 0.3 g (0.006 mol) of NaOMe in 100 mL of ethylene glycol was treated as with experiment A(1). There was thus produced 3.77 g (42%) of **2,6-diamino-3-(3,5-diethyl-4-hydroxybenzyl)pyridine (4b)** and 1.38 g (19%) of **2,6-diamino-3,5-bis(3,5-diethyl-4-hydroxybenzyl)pyridine (5b)**. The former (**4b**) melted at 160–161 °C (EtOAc): NMR (Me_2SO-d_6) δ 1.07 [t, 6, $(CH_2CH_3)_2$], 2.54 [q, 4, $(CH_2CH_3)_2$], 3.46 (s, 2, CH_2), 4.98 (br s, 2, NH_2), 5.10 (br s, 2, NH_2), 5.64 (d, 1, pyr 5 H, *J* = 8 Hz), 6.72

(21) K. H. Pattishall, J. Acar, J. J. Burchall, F. W. Goldstein, and R. J. Harvey, *J. Biol. Chem.*, **252**, 2319 (1977).
(22) B. Roth, E. Aig, K. Lane, and B. Rauckman, *J. Med. Chem.*, submitted (paper 4 of this series).
(23) B. Roth and J. Z. Strelitz, *J. Org. Chem.*, **35**, 2696 (1970).

(s, 2, Ar), 6.80 (d, 1, pyr 4 H, J = 8 Hz), 7.77 (br s, 1, Ar OH); MS m/e 271, 254, 242, 225. UV cation (0.01 N HCl) λ_{max} 242 nm (ϵ 13 300), 279 (1900), 336 (15 300); anion (0.1 N NaOH) 241 (16 100), 304 (12 200). Anal. ($C_{16}H_{21}N_3O$) C, H, N.

Compound **5b** melted at 175–175.5 °C (EtOH): NMR (Me_2SO-d_6) δ 1.07 [t, 12, $CH_2CH_3)_4$], 2.54 [q, 8, ($CH_2CH_3)_4$], 3.46 [br s, 4, $(CH_2)_2$], 4.87 [br s, 4, $(NH_2)_2$], 6.72 (s, 4, Ar), 6.75 (s, 1, pyr 4 H), 7.79 [s, 2, (Ar OH)$_2$]; MS m/e 433, 404, 284, 283, 270, 163. UV cation (0.01 N HCl) λ_{max} 242 nm (ϵ 16 000), 278 (3500), 343 (15 800); anion (0.1 N NaOH) 242 (24 000), 306 (16 800). Anal. ($C_{27}H_{35}N_3O_2$) C, H, N.

This reaction was repeated with a 1:1 molar ratio of pyridine to Mannich base. The products were **4b** (29%) and **5b** (54.6%).

Reaction of 6-Amino-2(1*H*)-pyridinone (7) with 3a. Compound **7** (33.03 g, 0.3 mol), **3a** (21.12 g, 0.1 mol), and NaOMe (0.54 g, 0.01 mol) in 130 mL of β-methoxyethanol were heated under N_2 at 120–130 °C for 5 h and neutralized. The solvent was removed, and water was added to the residue, followed by adjustment to pH 6–7. This mixture was extracted with $CHCl_3$, followed by $CHCl_3$–MeOH (3:1). The organic extracts were combined, the solvent was removed, and the residue was slurried in 400 mL of warm EtOAc–MeOH (12:1) until some of it had dissolved. The insoluble portion (5.82 g) was separated, and the filtrate was purified on a column (EtOAc–MeOH, 12:1), which produced **6-amino-3-(4-hydroxy-3,5-dimethoxybenzyl)-2-(1*H*)-pyridinone (8a)**: yield 2.68 g; mp 224–225.5 °C (EtOH); NMR (Me_2SO-d_6) δ 3.44 (s, 2, CH_2), 3.69 [s, 6, $(OMe)_2$], 5.19 (d, 1, pyr 5 H, J = 7.5 Hz), 5.69 (br s, 2, NH_2), 6.45 (s, 2, Ar), 6.92 (d, 1, pyr 4 H, J = 7.5 Hz), 9.27 (br, 2, NH, OH); MS m/e 276, 261, 245, 123. UV cation (2 N HCl) λ_{max} 235 nm (ϵ 18 800), sh 285 (2500), 322 (12400); neutral species (pH 7, phosphate) 232 (14 400), sh 270 (1900), 330 (14 800); anion (0.1 N NaOH) 245 (16 400), sh 274 (4900), 306 (10 300). Anal. ($C_{14}H_{16}N_2O_4$) C, H, N.

The structure of isomer **8a** was confirmed by conversion of the 2-oxo group to a methanesulfonyloxy function, followed by catalytic hydrogenation (H_2, Pd/C)[24] to give the 2-NH_2-5-CH_2Ar-6-H derivative: NMR (Me_2SO-d_6) δ 3.62 (s, 2, CH_2), 3.71 [s, 6, $(OMe)_2$], 5.66 (br s, 2 NH_2), 6.38 (d, 1, pyr 3 H, J = 8.5 Hz), 6.45 (s, 2, Ar), 7.22 (dd, 1, pyr 4 H, J = 2.5 and 8.5 Hz), 7.80 (br d, 1, pyr 6 H, J = 2.5 Hz), 8.05 (br, 1, ArOH).

The insoluble fraction from the above was recrystallized (EtOH), thus producing **6-amino-5-(4-hydroxy-3,5-dimethoxybenzyl)-2(1*H*)-pyridinone (9a)**: 1.21 g; mp 250–252 °C; NMR (Me_2SO-d_6) δ 3.46 (s, 2, CH_2), 3.70 [s, 6, $(OMe)_2$], 5.45 (d, 1, pyr 3 H, J = 8.5 Hz), 5.65 (br s, 2 NH_2), 6.45 (s, 2, Ar), 7.00 (d, 1, pyr 4 H, J = 8.5 Hz), 9.13 (br, 2, NH, OH); MS m/e 276, 261, 245, 167, 123. UV cation (2 N HCl) λ_{max} 230 nm (ϵ 16 200), sh 285 (2600), 318 (12 400); neutral species (pH 7, phosphate) 234 (16 200), sh 270 (1850), 330 (14 600); anion (0.1 N NaOH) 240 (15 900), 307 (10 800). Anal. ($C_{14}H_{16}N_2O_4$) C, H, N.

The filtrates from this crystallization produced 2.27 g of a mixture of about 85% **8a** and 15% **9a** (NMR analysis). The total yield of the two isomers was 16.9% of **8a** and 5.4% of **9a**. A small amount of dibenzylated product **10a** (see below) was also formed, as indicated by TLC comparisons.

When the above reaction was carried out using equimolar amounts of **7** and **3a** in ethylene glycol, a copious precipitate formed when water was added to the neutralized reaction mixture. This was dissolved in alkali, reprecipitated, and recrystallized from EtOH, which gave **6-amino-3,5-bis(4-hydroxy-3,5-dimethoxybenzyl)-2(1*H*)-pyridinone (10a)**: yield 49%; mp 231–232 °C; NMR (Me_2SO-d_6) δ 3.43 [s, 4, $(CH_2)_2$], 3.65 [s, 12, $(OMe)_4$], 5.54 (br s, 2, NH_2), 6.42 (s, 4, Ar), 6.92 (s, 1, pyr 4 H), 8.60 [br, 3, NH, $(OH)_2$]; MS m/e 442, 411, 289, 276, 275, 257, 167. UV cation (6 N HCl) λ_{max} 232 nm (ϵ 22 500), sh 281 (2600), 324 (10 200); neutral species (pH 7, phosphate) sh 237 (18 000), sh 281 (2500), 338 (12 300); anion (1 N NaOH) 246 (20 400), 297 (9300), 316 (9800). Anal. ($C_{23}H_{26}N_2O_7$) C, H, N.

No monobenzylated product was isolated using this method; however, a small amount could have been present in the filtrates.

4-[(4-Hydroxy-3,5-dimethoxybenzyl)amino]pyridine (13a). 4-Aminopyridine (**12**; 4.70 g, 50 mmol) and **3a** (10.5 g, 50 mmol) in 65 mL of ethylene glycol were heated under N_2 at 140–150 °C for 5 h. After the mixture cooled, the solvent was removed in vacuo, H_2O was added, and the pH was adjusted to 8.5. The product was extracted into $CHCl_3$, the solvent was removed, and the extract was purified on a column ($CHCl_3$–MeOH, 10:1), which gave 3.0 g (23%) of **13a**: mp 196–197 °C (EtOH); NMR (Me_2SO-d_6) δ 3.74 [s, 6, $(OMe)_2$], 3.85 (br d, 2, NHCH_2, J = 6 Hz), 6.52 (dd, 2, pyr 3 H, 5 H, J = 1.5 and 6 Hz), 6.65 (s, 2, Ar), 6.90 (br t, 1, N*H*CH_2, J = 6 Hz), 8.00 (br dd, 2, pyr 2 H, 6 H, J = 1.5 and 6 Hz); MS m/e 260, 167. UV neutral species (pH 7.2, phosphate) λ_{max} 273 nm (ϵ 22 200); anion (0.1 N NaOH) 255 (24 700), sh 290 (5900). Anal. ($C_{14}H_{16}N_2O_3$) C, H, N.

A second product isolated from the column was **bis(4-hydroxy-3,5-dimethoxyphenyl)methane (14a)**:[3] yield 2.18 g (27%); mp 109–110 °C (EtOAc/hexane). This product, formed by self-condensation of the Mannich base, was formed to some extent in all of these reactions but normally to a lesser degree.

2,4-Diaminopyridine (17).[6] This compound was prepared as briefly described by Meyer and Tropsch[6] from 2,4-bis(methoxycarbonyl)pyridine. Since byproducts were isolated, the reactions are described. The starting material was converted to its dihydrazide by the usual procedure, using 3 equiv of $NH_2NH_2 \cdot H_2O$. Although a quantitative reaction occurred, only about 85% of the product was the desired **dihydrazide (33)**: mp 244–247 °C; NMR (Me_2SO-d_6) δ 4.64 [br s, 4, $(NH_2)_2$], 7.89 (dd, 1 pyr 5 H, J = 2 and 6 Hz), 8.33 (d, 1, pyr 3 H, J = 2 Hz), 8.73 (d, 1, pyr 6 H, J = 6 Hz), 9.92 (br s, 1, NH), 10.23 (br s, 1, NH); MS m/e 195, 165, 164, 137, 136. UV neutral species (pH 7, phosphate) sh 215 nm (ϵ 13 600), λ_{max} 271 (5900). Anal. ($C_7H_9N_5O_2$) C, H, N.

The remaining product, separated by crystallization from MeOH, was a monohydrazide, **2-hydrazido-4-(methoxycarbonyl)pyridine (34)**: mp 170.5–172 °C; NMR (Me_2SO-d_6) δ 3.95 (s, 3, Me), 4.65 (br s, 2, NH_2), 8.00 (dd, 1, pyr 5 H, J = 1.5 and 6 Hz), 8.36 (d, 1, pyr 3 H, J = 1.5 Hz), 8.82 (d, 1, pyr 6 H, J = 6 Hz), 10.0 (br, 1, NH); MS m/e 195, 164, 137, 136. UV neutral species (pH 7, phosphate) sh 215 nm (ϵ 14 350), λ_{max} 281 (4500). Anal. ($C_8H_9N_3O_3$) C, H, N.

The mixture of **33** and **34** was normally used in the next reaction to form the mono- and diazides and the mono- and bis[(ethoxycarbonyl)amino]pyridines. This was done by dissolving the mixture in water plus 3 equiv of concentrated HCl and $CHCl_3$ at 0–5 °C and slowly adding 2 equiv of $NaNO_2$ in H_2O. After neutralization of the mixture to pH 6, the mono- and diazides were extracted into $CHCl_3$. Some of the solvent was removed (**note: explosion hazard!**),[6,25] and absolute EtOH was added, followed by refluxing overnight; this produced a copious precipitate of **2,4-bis[(ethoxycarbonyl)amino]pyridine (15)** in admixture with **2-[(ethoxycarbonyl)amino]-4-[methoxy(and ethoxy)carbonyl]pyridine (35)**. Products **15** and **35** were formed in about a 3:1 ratio and could be separated by column chromatography (hexane–$CHCl_3$, 1:4). The **dicarbamate (15)** formed in 76% yield: mp 171–172.5 °C (EtOH); NMR ($CDCl_3$) δ 1.30 [t, 6, $(CH_2CH_3)_2$], 4.27 [q, 4, $(CH_2CH_3)_2$], 7.37 (br, 1 NH), 7.48 (dd, 1, pyr 5 H, J = 2 and 6 Hz), 7.87 (d, 1, pyr 3 H, J = 2 Hz), 8.25 (d, 1, pyr 6 H, J = 6 Hz), 9.94 (br s, 1, NH); MS m/e 253, 181. UV cation (0.1 N HCl) λ_{max} 223 nm (ϵ 41 600), 257 (19 600), 278 (15 200); neutral species (pH 8, Tris) 224 (39 200), sh 238 (20 600), 273 (3900), sh 280 (3300). Anal. ($C_{11}H_{15}N_3O_4$) C, H, N.

Product **35** was present as a 1:1 mixture of methyl and ethyl esters, according to NMR analysis, and melted at 138–140 °C (MeOH): NMR ($CDCl_3$) δ 1.38 [2 t, 4.5, $(CH_2CH_3)_{1.5}$], 3.97 (s, 1.5, $Me_{0.5}$), 4.33 (q, 2, CH_2CH_3), 4.45 [q, 1, $(CH_2CH_3)_{0.5}$], 7.58 (dd, 1, pyr 5 H, J = 1.5 and 6 Hz), 8.51 (d, 1, pyr 6 H, J = 6 Hz), 8.60 (d, 1, pyr 3 H, J = 1.5 Hz), 9.83 (br s, 1, NH); MS m/e 238 (100%), 224 (81.5%).

Other products were isolated from the column purification, including **2-carbamoyl-4-[(ethoxycarbonyl)amino]pyridine (36)**: mp 202–205 °C (EtOH); NMR (Me_2SO-d_6) δ 1.25 (t, 3, CH_2CH_3), 4.15 (q, 2, CH_2CH_3), 7.52 (br s, 1, NH), 7.63 (dd, 1, pyr 5 H, J = 2 and 6 Hz), 8.02 (br, 1, NH), 8.12 (d, 1, pyr 3 H, J = 2 Hz), 8.42 (d, 1, pyr 6 H, J = 6 Hz), 10.25 (br s, 1, N*H*COOEt); MS m/e 209, 166. UV cation (1 N HCl) λ_{max} 221 nm (ϵ 21 000),

(24) M. Nussim and Y. Weinik, British Patent 1 542 804 (1979).

(25) P. A. S. Smith, *Org. React.*, **3**, 337 (1946).

270 (15 500); neutral species (pH 7, phosphate) 218 (29 000), 241 (12 500), sh 273 (3100). Anal. ($C_9H_{11}N_3O_3$) C, H, N.

Hydrolysis of the dicarbamate **15** was carried out using 5 equiv of 5 N KOH in absolute EtOH at reflux for 6 h. The solvent was removed, H_2O was added, and the product was extracted with EtOAc, giving **2,4-diaminopyridine (17)**: yield 70%; mp 103–106 °C; NMR (Me_2SO-d_6) δ 5.19 (br s, 2, NH_2), 5.44 (br s, 2, NH_2), 5.56 (d, 1, pyr 3 H, J = 2 Hz), 5.78 (dd, 1, pyr 5 H, J = 2 and 5.5 Hz), 7.40 (d, 1, pyr 6 H, J = 5.5 Hz); MS m/e 109, 82, 81. UV cation (0.01 N HCl) λ_{max} 261 nm (ε 8900); neutral species (0.01 N NaOH) sh 241 (7000), 275 (2000). Anal. ($C_5H_7N_3$) C, H, N.

It was not necessary to separate **15** and **35** before hydrolysis; the separation was easier afterwards. This was accomplished by removal of the solvent after hydrolysis, addition of water, and adjustment to pH 1 and then to 7; a copious precipitate of **2-amino-4-carboxypyridine (37)** separated: mp 345 °C; NMR (TFA-d) δ 7.55 (dd, 1, pyr 5 H, J = 1.5 and 7 Hz), 7.90 (d, 1, pyr 3 H, J = 1.5 Hz), 8.03 (d, 1, pyr 6 H, J = 7 Hz); MS m/e 138, 109, 94, 82, 81, 69. Compound **17** could then be separated by adjusting the pH to about 13 and extracting with EtOAc as before. A typical reaction carried out on 10.1 g of a 3:1 mixture gave 3.12 g of **17** (70.1%) and 1.12 g of **37** (19.8%).

Carbon-13 NMR data was used to determine which isomers were correct for compounds **36** and **37** based on calculations using model compounds (Table III; see paragraph at the end of this paper concerning Supplementary Material). Since not all substituents have been calculated for their effects at all positions on pyridine, the substituent effects were approximated using CHO or $COCH_3$ for COOH, NH_2 for NH_2 and NHCOOEt, and $COCH_3$ for $CONH_2$.[26] The pyridine carbon at 161.79 ppm for C-2 of **37** confirms that it is **2-amino-4-carboxypyridine**, and the lack of a pyridine carbon near 162 ppm for C-2 of **36** rules out the 4-carbamoyl isomer and establishes it to be **2-carbamoyl-4-[(ethoxycarbonyl)amino]pyridine**. The peak with the lowest signal intensity is assigned as C-2 rather than C-4 for all three compounds, since this position has very few proton dipoles near enough to contribute to an efficient mechanism for relaxation, which causes the signal intensity to be lower. The correct isomers of **34** and **35** can then be deduced from the assignment of isomer **37** and from the reaction sequence.

Reaction of 2,4-Diaminopyridine (17) with Phenolic Mannich Base 3a. A mixture of 4.53 g (42 mmol) of **17**, 8.76 g (42 mmol) of **3a**, and 70 mL of ethylene glycol was heated under N_2 at 130–140 °C for 4 h. The reaction was then treated in the manner of **4a** and purified by column chromatography ($CHCl_3$–MeOH, 6:1, 4:1) to give 4.04 g of **2,4-diamino-3-(4-hydroxy-3,5-dimethoxybenzyl)pyridine (18a**; 35.4%) and 1.86 g of **2,4-diamino-3,5-bis(4-hydroxy-3,5-dimethoxybenzyl)pyridine (19a**; 20.3%). Compound **18a** melted at 235–238 °C dec (EtOH); NMR (Me_2SO-d_6) δ 3.62 (s, 2, CH_2), 3.68 [s, 6, $(OMe)_2$], 5.05 (br s, 2, NH_2), 5.38 (br s, 2, NH_2), 5.97 (d, 1, pyr 5 H, J = 6 Hz), 6.54 (s, 2, Ar), 7.42 (d, 1, pyr 6 H, J = 6 Hz); MS m/e 275, 260, 229. UV cation (0.1 N HCl) sh 219 nm (ε 41 000), λ_{max} 272 (9500); anion (0.1 N NaOH) sh 240 (14 300), 278 (6700). Anal. ($C_{14}H_{17}N_3O_3$) C, H, N.

Compound **19a** melted at 237–242 °C dec (EtOH); NMR (Me_2SO-d_6) δ 3.61 [s, 4, $(CH_2)_2$], 3.64 [s, 12, $(OMe)_4$], 4.99 [br s, 4, $(NH_2)_2$], 6.43 (s, 2, Ar), 6.46 (s, 2, Ar), 7.32 (s, 1, pyr 6 H), 7.9 [br, 2, (Ar $OH)_2$]; MS m/e 441, 426, 410. UV cation (0.1 N HCl) sh 221 nm (ε 49 500), λ_{max} 273 (9900), sh 281 (9400); anion (0.1 N NaOH) sh 245 (22 400), 282 (11 400). Anal. ($C_{23}H_{27}N_3O_6$·$0.75H_2O$) C, H, N.

Bromination of 2,4-Diaminopyridine. 2,4-Diaminopyridine (**17**; 2.0 g, 18 mmol) was brominated in 20 mL of glacial HOAc at 18 °C using 0.95 mL (2.78 g, 17 mmol) of bromine. (Slightly less than 1 equiv of bromine was used, since the compound tends to dibrominate easily.) The solvent was removed after 3 h, water was added, and the solution was adjusted to pH 6 with HOAc. The aqueous layer was extracted with $CHCl_3$, from which was isolated 0.33 g of **2,4-diamino-3,5-dibromopyridine (21**; 6.8%). The aqueous layer was adjusted to pH 12 and extracted again with $CHCl_3$; this extract yielded 2.99 g of **2,4-diamino-3-bromopyridine (20**; 86.7%). Compound **20** was pure after extraction: mp 118–122 °C; NMR ($CDCl_3$) δ 4.57 (br s, 2, NH_2), 4.87 (br s, 2, NH_2), 6.05 (d, 1, pyr 5 H, J = 6 Hz), 7.64 (d, 1, pyr 6 H, J = 6 Hz); MS m/e 189, 187, 108. UV cation (0.01 N HCl) λ_{max} 221 nm (ε 34 900), sh 266 (7300), 277 (8000); neutral species (0.01 N NaOH) 217 (37 900), sh 237 (9200), 279 (2200). Anal. ($C_5H_6BrN_3$) C, H, Br, N.

Compound **21** required column purification (EtOAc): mp 174–179 °C; NMR ($CDCl_3$) δ 4.9 [br, 4, $(NH_2)_2$], 7.85 (s, 1, pyr 6 H); MS m/e 269, 267, 265, 188, 186. UV cation (0.1 N HCl) λ_{max} 226.5 nm (ε 42 100), 274 (5800), sh 285 (5500); neutral species (0.01 N NaOH) 221.5 (39 800), 290 (2100). Anal. ($C_5H_5Br_2N_3$·$0.33H_2O$) C, H, Br, N.

5-Bromo-2,4-bis[(ethoxycarbonyl)amino]pyridine (16). 2,4-Bis[(ethoxycarbonyl)amino]pyridine (**15**; 0.77 g, 3 mmol) was brominated in 6 mL of HOAc at 18 °C with 0.17 mL (0.56 g, 3 mmol) of bromine. After 1 h, the white precipitate was isolated and slurried with base: yield 0.28 g (27.4%) of **16**; mp 212–213 °C; NMR ($CDCl_3$) δ 1.35 [t, 6, $(CH_2CH_3)_2$, J = 7 Hz], 4.28 [2 q, 4, $(CH_2CH_3)_2$, J = 7 Hz], 7.26 (br s, 1, NH), 8.27 (s, 1, pyr 3 H), 8.71 (br s, 1, NH), 8.97 (s, 1, pyr 6 H); MS m/e 333, 331, 252, 224, 189, 187. UV cation (1 N HCl) λ_{max} 236 nm (ε 40 300), 264 (15 100), 291 (9000). Anal. ($C_{11}H_{14}BrN_3O_4$) C, H, Br, N.

The filtrate from above was purified on a column ($CHCl_3$–MeOH, 20:1), which gave 0.19 g of **15** and 0.18 g of **16**. Correcting for the recovered starting material, **15**, the total yield of **16** was 60.5%.

Reaction of 2,4-Diamino-3-bromopyridine (20) with Phenolic Mannich Bases. A. With 3a. Equimolar quantities of **20** and **3a** (35 mmol) were treated as described above for **12** and **3a**. Column chromatography ($CHCl_3$–MeOH, 50:1) gave 3.66 g (29.5%) of a 2.4:1 mixture of monobenzylated products and 2.69 g (29.5%) of a 2:1 mixture of dibenzylated products, plus a recovery of 2.18 g of **20** (33%). Recrystallization of the monobenzylated fraction from 95% EtOH separated the products; **2,4-diamino-3-bromo-5-(4-hydroxy-3,5-dimethoxybenzyl)pyridine (25a)** crystallized first: yield 1.96 g; mp 232–233.5 °C dec; NMR (Me_2SO-d_6) δ 3.64 (s, 2, CH_2), 3.70 [s, 6, $(OMe)_2$], 5.46 [br s, 4, $(NH_2)_2$], 6.50 (s, 2, Ar), 7.37 (s, 1, pyr 6 H), 8.07 (br, 1, Ar OH); MS m/e 355, 353, 324, 322, 274, 189, 187, 167. UV cation (0.1 N HCl) λ_{max} 205 nm (ε 47 800), 222 (48 400), 277 (9550); anion (0.1 N NaOH) sh 250 (15 800), sh 283 (7500). Anal. ($C_{14}H_{16}BrN_3O_3$) C, H, Br, N.

Concentration of the filtrate from **25a** yielded 0.8 g, which was crystallized as the HCl salt from HCl in EtOH/Et_2O. This gave **2(or 4)-amino-3-bromo-4(or 2)-[(4-hydroxy-3,5-dimethoxybenzyl)amino]pyridine hydrochloride (26a·HCl)**: mp 230–231 °C dec; NMR (Me_2SO-d_6) δ 3.73 [s, 6, $(OMe)_2$], 4.44 (br d, 2, $NHCH_2$, J = 6 Hz), 6.35 (d, 1, pyr 5 H, J = 7.5 Hz), 6.62 (s, 2, Ar), 7.42 (br, s, 2, NH_2), 7.68 (d, 1, pyr 6 H, J = 7.5 Hz), 7.84 (br t, 1, $NHCH_2$, J = 6 Hz), 8.20 (br, 1, ArOH), 13.0 (br, 1, NH^+); MS m/e 355, 353, 274, 189, 187, 167. UV cation (0.01 N HCl) λ_{max} 204 nm (ε 44 700), 224 (36 700), 279 (13 600); anion (0.1 N NaOH) 257 (17 900), sh 280 (9100). Anal. ($C_{14}H_{16}BrN_3O_3$·HCl) C, H, Br, Cl, N.

The dibenzylated pyridine fraction isolated from the column was recrystallized twice, mp 152–157 °C (EtOH/H_2O). NMR analysis showed that it was a mixture of two isomers in a 2:1 ratio: **5-(4-hydroxy-3,5-dimethoxybenzyl)-2(or 4)-[(4-hydroxy-3,5-dimethoxybenzyl)amino]pyridine (27a)** and **2,4-bis[(4-hydroxy-3,5-dimethoxybenzyl)amino]pyridine (28a)**; MS m/e 521, 519, 438, 355, 353, 182, 167. Anal. ($C_{23}H_{26}BrN_3O_6$) C, H, Br, N.

B. With 3c. Equimolar amounts of **20** and **3c** were treated as in the previous reaction with **3a**, on a 41.8-mmol scale. There were obtained 6.30 g (39.9%) of monobenzylated products, 1.0 g (8.4%) of dibenzylated products, and 2.72 g (34.6%) of recovered **20**. Recrystallization of the monobenzylated fraction from EtOH–H_2O (2:1) gave 2.68 g of a mixture of **2,4-diamino-3-bromo-5-(4-hydroxy-3,5-diisopropylbenzyl)pyridine (25c)** and **2(or 4)-amino-3-bromo-4(or 2)-[(4-hydroxy-3,5-diisopropylbenzyl)amino]pyridine (26c)** in a 10:3 ratio (NMR). A second crystallization from 85% EtOH gave 1.76 g of **25c** in 94% isomeric purity; a third crystallization from absolute EtOH gave 1.11 g of **25c** with greater than 99% isomeric purity: mp 192–193.5 °C; NMR (Me_2SO-d_6) δ 1.10 [d, 12, $(CHMe_2)_2$, J = 7 Hz], 3.24 [septet,

(26) F. W. Wehrli and T. Wirthlin, "Interpretation of Carbon-13 NMR Spectra", Heyden & Son, Ltd., London, 1976.

2,(C*H*Me$_2$)$_2$, J = 7 Hz], 3.61 (s, 2, CH$_2$), 5.46 [br s, 4, (NH$_2$)$_2$], 6.83 (s, 2, Ar), 7.30 (s, 1, pyr 6 H), 7.80 (s, 1, Ar OH); MS *m/e* 379, 377, 364, 362, 336, 334. UV cation (0.01 N HCl) λ_{max} 222 nm (ϵ 46 100), 277 (9400); approximate neutral species (pH 8.7, phosphate) 218 (44 000), 280 (4000); anion (0.1 N NaOH) sh 240 (19 400), 290 (6000). Anal. ($C_{18}H_{24}BrN_3O$) C, H, N.

2,4-Diamino-5-(4-hydroxy-3,5-dimethoxybenzyl)pyridine Hydrobromide (29a). Compound **25a** (0.5 g, 1.4 mmol) was hydrogenated in a Parr shaker with H_2/5% Pd on C in MeOH with 1 equiv of concentrated HCl. This produced 0.38 g (72%) of **29a**·HBr: mp 225–228 °C dec (H_2O); NMR (Me$_2$SO-d_6) δ 3.54 (s, 2, CH$_2$), 3.70 [s, 6, (OMe)$_2$], 5.83 (s, 1, pyr 3 H), 6.51 (s, 2, Ar), 6.84 (s, 2, NH$_2$), 7.01 (br s, 2, NH$_2$), 7.16 (s, 1, pyr 6 H), 8.13 (s, 1, Ar OH), 10.43 (br, 1, NH$^+$); MS *m/e* 275, 260, 244, 122. UV cation (0.01 N HCl) λ_{max} 220 nm (ϵ 52 400), 263 (10 000); unstable in base. Anal. ($C_{14}H_{17}N_3O_3$·HBr·H_2O) C, H, N.

2,4-Diamino-5-(4-hydroxy-3,5-diisopropylbenzyl)pyridine Hydrobromide (29c). Compound **25c** was hydrogenated as for **29a** to produce 0.54 g (71%) of **29c**·HBr: mp 124–126 °C (H_2O); NMR (Me$_2$SO-d_6) δ 1.11 [d, 12, (CH*Me*$_2$)$_2$, J = 7 Hz], 3.27 [septet, 2, (C*H*Me$_2$)$_2$, J = 7 Hz], 3.56 (s, 2, CH$_2$), 5.85 (s, 1, pyr 3 H), 6.85 (s, 2, Ar), 6.90 (br s, 2, NH$_2$), 7.09 (br s, 2, NH$_2$), 7.16 (s, 1, pyr 6 H), 7.91 (s, 1, Ar OH), 11.52 (br, 1, NH$^+$); MS *m/e* 299, 284, 256. UV cation (0.01 N HCl) λ_{max} 222 nm (ϵ 50 300), 266 (9900); anion (0.1 N NaOH) sh 240 (18 300), 288 (5600). Anal. ($C_{18}H_{25}N_3O$·HBr) C, H, Br, N.

2,4-Diamino-3-bromo-5-(3,4,5-trimethoxybenzyl)pyridine (30d). Compound **25a** (0.5 g, 1.4 mmol) was methylated in 20 mL of Me$_2$SO, using KO-*t*-Bu (0.17 g, 1.5 mmol) and MeI (0.09 mL, 1.45 mmol). After the mixture had stirred at room temperature for 3 h, HOAc was added and the solvent removed in vacuo. The residue was dissolved in CHCl$_3$ and extracted with 0.5 N NaOH to remove residual starting material. The CHCl$_3$ layer was dried with MgSO$_4$, filtered, and evaporated to give 0.46 g (88.5%) of **30d**, which was slightly impure by TLC. Column purification (CHCl$_3$–MeOH, 100:1) gave 0.32 g (62%) of **30d**: mp 154–155.5 °C; MS *m/e* 369, 367, 354, 352, 338, 336, 322, 320, 288, 181. UV cation (0.1 N HCl) λ_{max} 204 nm (ϵ 46 200), 222 (47 100), 278 (8900); neutral species (0.01 N NaOH) 215 (49 800), sh 278 (3200). Anal. ($C_{15}H_{18}BrN_3O_3$) C, H, N.

A trace of di-N-methylated trimethoxybenzylpyridine was obtained from the column as well: MS *m/e* 397, 395, 382, 380, 316, 181.

2,4-Diamino-5-(3,4,5-trimethoxybenzyl)pyridine Hydrobromide (31d). Compound **30d** (0.31 g, 0.84 mmol) was hydrogenated as for **29a** to give 0.24 g of **31d**·HBr (77.4%): mp 167–169 °C (H_2O); NMR (Me$_2$SO-d_6) δ 3.63 (s, 5, CH$_2$, OMe), 3.73 [s, 6, (OMe)$_2$], 5.87 (s, 1, pyr 3 H), 6.60 (s, 2, Ar), 6.91 (br s, 2, NH$_2$), 7.08 (br s, 2, NH$_2$), 7.28 (s, 1, pyr 6 H), 11.59 (br, 1, NH$^+$); MS *m/e* 289, 274, 258. UV cation (0.1 N HCl) λ_{max} 222 nm (ϵ 51 800), 265 (9500); neutral species (0.01 N NaOH) sh 220 (47 400), 277 (2900). Anal. ($C_{15}H_{19}H_3O_3$·HBr) C, H, Br, N.

2,4-Diamino-3-fluoropyridine (22). 2,4-Diaminopyridine (17) was fluorinated with F_2 gas in the following manner. The F_2 was diluted with N_2 until the mixture would not ignite a cotton swab dipped in acetone and would blow out a paper match flame. The gas mixture was passed into 2 L of HOAc at 18 °C, and aliquots were removed to determine F_2 content. After 4 h, the solution was 0.039 N in F_2. Then, 1530 mL (59.9 mmol of F_2, 25% excess) was quickly added in 3 min to 17 (5.2 g, 47.6 mmol) in HOAc (300 mL) at 18 °C.[9] The reaction was immediate, and the solvent was evaporated in vacuo. Then H_2O and NaOH were added to pH 11, and the product was extracted into CHCl$_3$, from which was separated 2.13 g (35.2%) of **22**: mp 115–124 °C; NMR (Me$_2$SO-d_6) δ 5.42 (br s, 2, NH$_2$), 5.58 (br s, 2, NH$_2$), 5.98 (t, 1, pyr 5 H, J = 6 Hz), 7.27 (d, 1 pyr 6 H, J = 6 Hz) (the triplet at 5.98 is due to coupling between 5H–6H and 5H–3F); MS *m/e* 127, 100, 99. The product was used directly in the next reaction.

Reaction of 22 with Phenolic Mannich Base (3a). Compound **22** (2.46 g, 19.4 mmol) was treated with 4.53 g (21.5 mmol) of **3a** in the manner used with **20**. Column purification (CHCl$_3$–MeOH, 40:1) gave 1.41 g (24.8%) of a mixture of **2,4-diamino-3-fluoro-5-(4-hydroxy-3,5-dimethoxybenzyl)pyridine (23a)** and **2(or 4)-amino-3-fluoro-4(or 2)-[(4-hydroxy-3,5-dimethoxybenzyl)amino]pyridine (24a)**. Upon crystallization of the mixture from EtOH, **23a** precipitated first: yield 0.67 g (11.7%); mp 229–230 °C dec; NMR (Me$_2$SO-d_6) δ 3.55 (s, 2, CH$_2$), 3.65 [s, 6, (OMe)$_2$], 5.26 [br s, 4, (NH$_2$)$_2$], 6.44 (s, 2, Ar), 7.16 (s, 1, pyr 6 H), 7.97 (s, 1, Ar OH); MS *m/e* 293, 278, 262, 167, 140, 127. UV cation (0.01 N HCl) λ_{max} 205 nm (ϵ 47 200), sh 215 (45 000), sh 259 (8900), 276 (10 400); approximate neutral species (pH 8.7, phosphate) 208 (51 500), sh 240 (13 700), 272 (4100); anion (0.1 N NaOH) sh 250 (12 600), sh 283 (6400). Anal. ($C_{14}H_{16}FN_3O_3$·0.2H_2O) C, H, N.

Compound **24a** was recovered from the filtrate of **23a** as the hydrochloride salt: yield 0.62 g (9.7%); mp 202–203 °C (EtOH/Et$_2$O); NMR (Me$_2$SO-d_6) δ 3.73 [s, 6, (OMe)$_2$], 4.39 (d, 2, NHC*H*$_2$, J = 6 Hz), 6.46 (t, 1, pyr 5 H, J = 7 Hz), 6.82 (s, 2, Ar), 7.40 (br s, 2, NH$_2$), 7.55 (d, 1, pyr 6 H, J = 7 Hz), 8.20 (br t, 1, N*H*CH$_2$, J = 6 Hz), 8.2 (br, 1, Ar OH), 13.02 (br, 1, NH$^+$); MS *m/e* 293, 167, 127. UV cation (0.01 N HCl) λ_{max} 205 nm (ϵ 51 700), sh 228 (28 800), 281 (17 000); neutral species (pH 8.7, phosphate) 205 (52 500), 246 (15 200), sh 280 (4100); anion (0.1 N NaOH) 254.5 (20 000). Anal. ($C_{14}H_{16}FN_3O_3$·HCl) C, H, N.

Dihydrofolate Reductase Assays. Dihydrofolate reductase partially purified from rat liver [27] and affinity column purified from *E. coli*[28] was assayed as described by Baccanari et al.,[28] in phosphate buffer. Compounds were incubated for 5 min with enzyme and buffer at 37 °C before NADPH and then FH$_2$ were added to initiate the reactions. The inhibitory potencies are reported as the concentration required to reduce the reaction rate by 50% (IC$_{50}$), as calculated from titrations of three to six concentrations of compound or as the percent inhibition observed at the highest concentration tested.

Acknowledgment. The authors are grateful for the continued encouragment offered by Dr. George H. Hitchings during the course of this investigation and for helpful discussions with Dr. Peter Goodford, Wellcome Research Laboratories, Beckenham, England. The opportunity offered by Dr. David Matthews to view and study his three-dimensional models of dihydrofolate reductase was deeply appreciated. The enzyme assays vs. dihydrofolate reductase enzymes were carried out by Robert Ferone and staff; Sheila Smith conducted the studies with the plasmid enzyme. The ^{13}C NMR spectra and interpretations were determined by Ronald Crouch. We thank Pauline Baker for excellent technical assistance with UV and pK_a determinations and chromatographic separations.

Supplementary Material Available: Carbon-13 NMR spectra were used to determine which isomers were correct for compounds **36** and **37**, based on calculations using model compounds. A table of C-13 shift data is available (1 page). Ordering information is given on any current masthead page.

(27) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, 1, 126 (1965).

(28) D. Baccanari, A. Phillips, S. Smith, D. Sinski, and J. Burchall, *Biochemistry*, 14, 5267 (1975).