of petroleum ether (60–110°) and the product was collected on a filter. Recrystallization from toluene yielded 5.05 g (34%) of white crystals, mp 141–144°, which gave a positive active halide test[14] and a negative Bratton-Marshall test for aromatic amine.[11] *Anal.* ($C_{31}H_{31}BrFNO_3S$) C, H, F.

This compound should be handled with caution since it is an extreme skin irritant.

α-[3-(3,4-Dichlorophenoxyacetamido)pyridinium]-*p*-fluorosulfonylacetanilide Bromide (23) (Method A).—A solution of 0.89 g (3.0 mmoles) of **25** and 0.97 g (3.3 mmoles) of **29** in 20 ml of Me_2CO was refluxed for 18 hr. The warm Me_2CO was decanted from the yellow oil, and the oil was rubbed with fresh Me_2CO until it solidified. The product was collected, washed (Me_2CO), and recrystallized from EtOH; yield, 1.43 g (80%) of white solid, mp 178–180°.

See Table III for additional compounds prepared by this method. Similarly, **24** was prepared from 3-acetamidopyridine;[17] yield, 0.70 g (36%) of tan crystals, mp 208–210°. *Anal.* ($C_{14}H_{14}BrFN_2O_5S$) C, H, F.

Method B was the same as A, but $CHCl_3$ was employed as solvent.

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Irreversible Enzyme Inhibitors. CLI.[1,2] Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase[3] Derived from 5-(*p*-Aminophenylbutyl)-2,4-diaminopyrimidines with a Terminal Sulfonyl Fluoride

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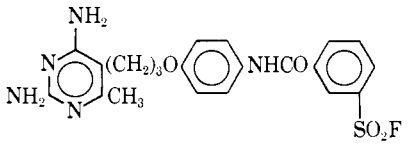
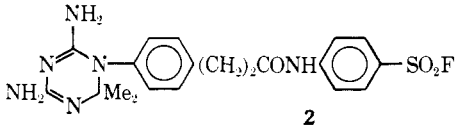
Wittig condensation of 2-acetamido-4-hydroxy-6-methylpyrimidine-5-propionaldehyde (**5**) with *p*-nitrobenzyl triphenylphosphonium bromide (**6**) proceeded in 69% yield to 2-acetamido-6-methyl-5-[4-(*p*-nitrophenyl)-3-buten-1-yl]-4-pyrimidinol (**7**) in DMF by use of 1,4-diazabicyclo[4.3.0]non-5-ene (DBN) as the base. By further transformations, **7** was converted to the key intermediate, 5-(*p*-aminophenylbutyl)-2,4-diamino-6-methylpyrimidine (**11**). 2,4-Diacetamidopyrimidine-5-carboxaldehyde (**14**) condensed smoothly with *p*-nitrocinnamyltriphenylphosphonium bromide (**15**) with DBN as the base to give 2,4-diacetamido-5-[4-(*p*-nitrophenyl)-1,3-butadien-1-yl]pyrimidine (**16**) in 84% yield. Catalytic reduction of **16** and hydrolysis afforded the key intermediate, 5-(*p*-aminophenylbutyl)-2,4-diaminopyrimidine (**17**) in 56% yield. Acylation of the arylamino group of **11** and **17** with *m*- or *p*-fluorosulfonylbenzoyl chloride gave a series of candidate irreversible inhibitors of dihydrofolic reductase. Two of these were excellent irreversible inhibitors of the enzyme from L1210 mouse leukemia and showed good specificity by showing poor inactivation of the mouse liver enzyme; however, these compounds showed poor diffusion through the L1210 cell wall.

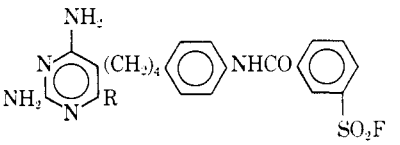
In a previous study, the 5-phenoxypropylpyrimidine (**1**) was found to be an excellent active-site-directed irreversible inhibitor[4] of dihydrofolic reductase from L1210 mouse leukemia when assayed at a $K_i = 3 \times 10^{-9}$ *M* concentration; furthermore, at 60–100K_{ii} **1** showed no inactivation of this enzyme from the liver, intestine, or spleen of the mouse.[3,5] Unfortunately, **1** showed poor penetration of the L1210 cell wall and as a result was inactive *in vivo*.[3] In contrast, **2** was an active-site-directed irreversible inhibitor of dihydrofolic reductase with little selectivity;[5] however, **2** penetrated the cell quite effectively and as a result **2** showed reproducible *in vivo* activity against L1210.[3,5] Presumably, both **1** and **2** penetrate the L1210 cell wall by passive diffusion;[6] therefore, the difference in the ability of **1** and **2** to diffuse through the cell wall was surprising. Little is known about effect of variation of structure on cell wall diffusion since assays are usually performed with intact cells or with isolated target enzymes, but seldom both; without both assays, one cannot differentiate poor inhibition from poor diffusion. A recent study on dihydrofolic reductase inhibitors for *Escherichia coli*[7] indicated that structural change could change diffusion by a factor of 2700 after the effect on the enzyme is normalized by comparing the ED_{50}/I_{5a} ratio.



1



2



3, R = CH_3
4, R = H

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **12**, 221 (1969).

(3) For the previous paper on this enzyme see B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 108 (1969), paper CXLIII of this series.

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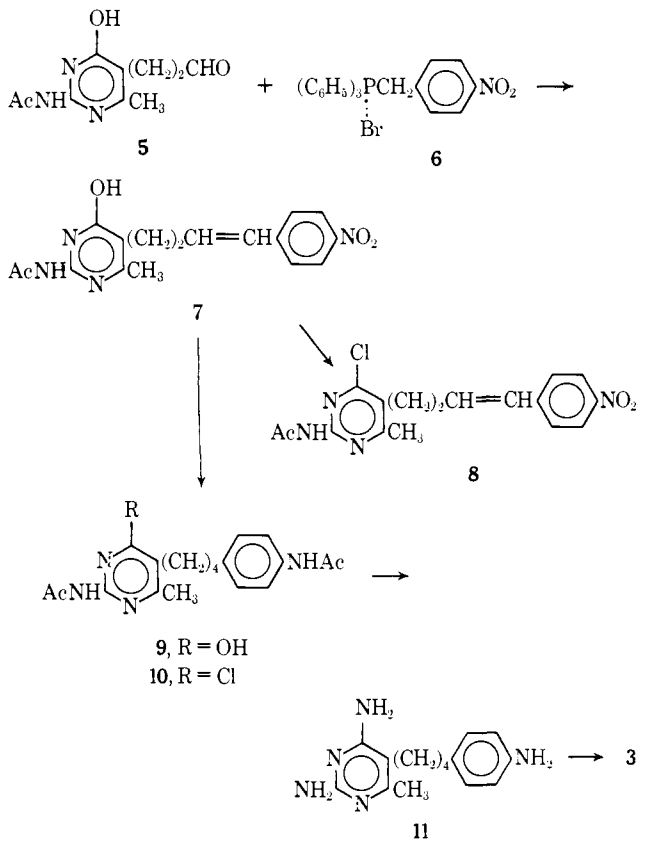
SCHEME I



The fact that **2** had a completely nonpolar bridge between the diamino heterocycle and the carboxamidophenylsulfonyl fluoride moiety, whereas **1** had a polar oxygen in the bridge, suggested that structures such as **3** and **4** should be synthesized and evaluated for specific irreversible enzyme inhibition and ability to penetrate the L1210 cell wall. The results are the subject of this paper.

Chemistry.—Wittig condensation of the pyrimidinepropionaldehyde (**5**)[8] with **6** using 1,4-diazabicyclo[4.3.0]non-5-ene (DBN)[9] as the base proceeded smoothly to **7** (Scheme I). Although replacement of the 4-OH group of **7** by Cl (**8**) with $POCl_3$ was successful, subsequent treatment of **8** with MeOH–NH_3 at 140–170° gave extensive decomposition; similarly, results were obtained with NaN_3.[3] Since this instability of **8** to NH_3 and NaN_3 was attributed to the base lability of its nitrostyryl moiety, **7** was reduced and acetylated to **9**. Conversion of **9** to **10** with $POCl_3$ proceeded without difficulty. Reaction of **10** with MeOH–NH_3 at 170° afforded both displacement of the 4-Cl and deacetylation to the desired amine (**11**). Acylation of **11** with *m*-fluorosulfonylbenzoyl chloride in DMF with DBN as the acid acceptor gave the candidate irreversible inhibitor (**3**) isolated as its hemisulfate.

2,4-Diamino-5-cyanopyrimidine (**12**) was conveniently prepared in 81–86% yield from ethoxymethylenemalononitrile and guanidine by the method of Huber.[10]

Catalytic reduction of **12** at 60–80° with a Pd–C catalyst under hydrolytic conditions in 2 *N* HCl afforded the aldehyde (**13**) in 80% yield, as suggested by Weinstock, *et al.*[11] Attempted Wittig condensation between **13** and **15**[12] failed; that this failure was due to the weak electrophilicity of the aldehyde function caused by the electron-donating amino groups was supported by the negative dimethoxybenzidine test for aldehydes. In previous analogous cases in this laboratory, the electrophilicity of primidine-6-carboxaldehydes was increased by acetylation of the ring amino groups.[12,13] Acetylation of **13** to **14** proceeded with some difficulty due to insolubility, but could be achieved in fair yield with Ac_2O–DMF (Scheme II); that the resultant aldehyde (**14**) was more electrophilic was shown by its positive dimethoxybenzidine test. Witting condensation of **14** with **15**[12] using DBN as the base, proceeded smoothly to **16** in 84% yield. Catalytic reduction in HOAc with a Pd–C catalyst, followed by HCl deacetylation, afforded **17** in 56% yield. Acylation of **17** with the appropriate fluorosulfonylbenzoyl chloride in DMF with DBN as acid acceptor afforded the candidate irreversible inhibitors **4** and **18**. Reaction of **17** with O-(*p*-nitrophenyl)-N-(*p*-fluorosulfonylphenyl)urethan[14] afforded the urea **19**.

Experimental Section

All analytical samples had uv and ir spectra compatable with their assigned structures; each moved as a single spot on tlc on Brinkmann silica gel GF and gave combustion analyses for C, H, and N or F within 0.4% of theoretical. Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected.

2-Acetamido-6-methyl-5-[4-(*p*-nitrophenyl)-3-buten-1-yl]-4-pyrimidinol (7).—To a stirred mixture of 10 g (45 mmoles) of **5**,[8] 22 g (46 mmoles) of **6**,[15] and 100 ml of DMF was added dropwise over 10 min 5.77 g (47 mmoles) of DBN. After being stirred 2 hr at ambient temperature, the mixture was heated 10 min on a steam bath, then poured into 250 ml of 1:1 *i*-PrOH–H_2O. The product was collected on a filter, washed (*i*-PrOH), and recrystallized from MeOEtOH–H_2O; yield, 10.5 g (69%) of yellow crystals, mp 181–221°. This was presumably a mixture of *cis–trans* isomers, but moved as one spot on tlc in 1:10 EtOH–$CHCl_3$. *Anal.* ($C_{17}H_{18}N_4O_4$) C, H, N.

2-Acetamido-4-chloro-6-methyl-5-[4-(*p*-nitrophenyl)-3-buten-1-yl]pyrimidine (8).—A mixture of 2.5 g (7.3 mmoles) of **7** and 10 ml of $POCl_3$ was stirred in a bath at 90° for 90 min protected from moisture, then poured into 100 ml of petroleum ether (bp 60–110°). The gum that separated was washed with 50 ml of petroleum ether, then stirred with 25 g of ice, 100 ml of 10% NaOAc, and 100 ml of $CHCl_3$ until solution of the gum was complete. The separated $CHCl_3$ layer was washed (50 ml of 10% NaOAc, 50 ml of H_2O), then dried ($MgSO_4$). Evaporation *in vacuo* gave an oil that crystallized on addition of EtOH; yield 1.70 g (65%), mp 165–183°, gave a single spot on tlc in EtOAc. Recrystallization from MeOEtOH–*i*-PrOH gave the analytical sample, mp 170–185°. *Anal.* ($C_{17}H_{17}ClN_4O_3$) C, H, N.

2-Acetamido-5-(*p*-acetamidophenylbutyl)-6-methyl-4-pyrimidinol (9).—A mixture of 5.0 g (15 mmoles) of **7**, 100 ml of MeOEtOH, and 0.2 g of PtO_2 was shaken with H_2 at 2–3 atm for 30

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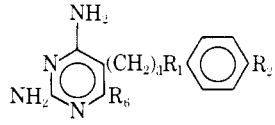
Table I

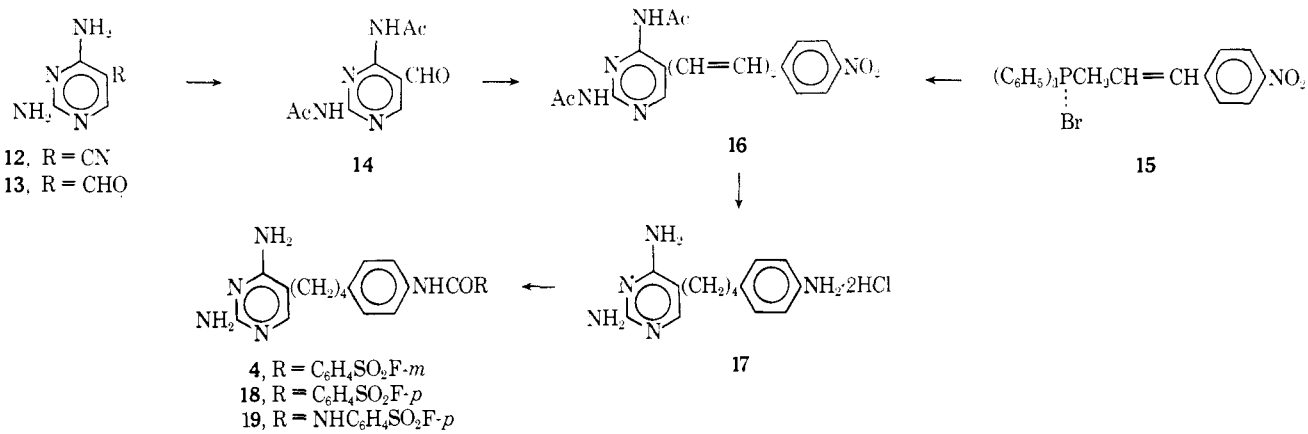
Inhibition[a] of Dihydrofolic Reductase by



No.	R_5	R_7	R_2	Enzyme source	I_{50},[b] μM	Inhib, μM	Time, min	% inactvn[c]	ED_{50},[d] μM	ED_{50}/I_{50}
1[e]	CH_3	O	$NHCOC_6H_4SO_2F$-*m*	Liver	0.019	0.6	60	6		
				L1210/DFS	0.016	0.05	60	100		
				L1210/0	0.016	0.05	60	94	2.2	140
3	CH_3	CH_2	$NHCOC_6H_4SO_2F$-*m*	L1210/0	0.011	0.070	60	95	0.68	68
				L1210/DFS		0.070	60	95		
				Liver		0.070	60	6		
4	H	CH_2	$NHCOC_6H_4SO_2F$-*m*	L1210/DFS	0.0060	0.046	60	88	7.3	1200
				L1210/0		0.046	60	80		
				Liver	0.010	0.04	60	58		
18	H	CH_2	$NHCOC_6H_4SO_2F$-*p*	Liver	0.045	0.04	60	30	2.7	59
				L1210/DFS		0.046	60	54		
19	H	CH_2	$NHCONHC_6H_4SO_2F$-*p*	Liver	0.048	0.14	60	30	5.6	120
				L1210/DFS		0.048	60	64		

[a] The technical assistance of Sharon Lafler and Diane Shea with these assays is acknowledged. [b] Concentration necessary for 50% inhibition when assayed with 6 μM dihydrofolate in 0.05 M Tris (pH 7.4) containing 0.15 M KCl as previously described.[5] [c] Incubated at 37°, then the concentration of remaining enzyme was assayed as previously described.[5] [d] Concentration for 50% inhibition of L1210/0 cell culture. [e] Data from ref 3 and 5.

Scheme II



min when reduction was complete. The filtered solution was evaporated *in vacuo*. After addition of 50 ml of C_6H_6, the evaporation was repeated. The residue was heated on a steam bath with 25 ml of Ac_2O and 5 ml of C_6H_6 for 2 hr. The cooled mixture was filtered and the product was washed with petroleum ether (bp 60–110°); yield 2.7 g (52%), mp 237–239°. Recrystallization of a sample from MeOEtOH–H_2O gave white crystals, mp 239–240°, tlc in 1:10 EtOH–$CHCl_3$. *Anal.* ($C_{19}H_{24}N_4O_3$) C, H, N.

5-(*p*-Aminophenylbutyl)-2,4-diamino-6-methylpyrimidine (11) Sesquisulfate.—Crude **10** was obtained as an oil from 1.40 g (3.93 mmoles) of **9** when treated with $POCl_3$ as described for the preparation of **8**. The crude **10** with 40 ml of MeOH saturated with NH_3 was heated in a Parr bomb at 170° for 24 hr. The solution was evaporated *in vacuo* and the residue was dissolved in 9 ml of 3 N H_2SO_4. The solution was clarified with charcoal, then *i*-PrOH was added to turbidity. On standing the solution deposited 0.92 g (56%) of white crystals which gradually decomposed over 230°; the product moved as a single spot on tlc in 1:4 EtOH–$CHCl_3$, had λ_{max} 276 mμ (pH 1) and 287 mμ (pH 13), but was contaminated $(NH_4)_2SO_4$.

2,4-Diacetamidopyrimidine-5-carboxaldehyde (14).—A mixture of 5.0 g (36 mmoles) of **13**,[11] 40 ml of Ac_2O, and 40 ml of DMF was heated on a steam bath for 6 hr with occasional mixing. The solution was kept at −5° overnight, then the separated product was collected on a filter and washed with H_2O; yield 3.9 g (49%), mp 223–224°, moved as a single spot on tlc in EtOAc and was suitable for further transformation. Recrystallization of a sample from DMF–C_6H_6 gave white crystals, mp 224–225°. *Anal.* ($C_9H_{10}N_4O_3$) C, H, N.

2,4-Diacetamido-5-[4-(*p*-nitrophenyl)-1,3-butadien-1-yl]pyrimidine (16).—To a stirred mixture of 7.90 g (36 mmoles) of **14**, 17.7 g (36 mmoles) of **15**,[12] and 75 ml of DMF cooled in an ice bath was added 4.46 g (36 mmoles) of DBN over 10 min. After 10 min, the mixture was diluted with 300 ml of C_6H_6 and vigorously stirred for 4 hr. The product was collected on a filter and washed with hot C_6H_6; yield 10.8 g (84%), moved as a single spot on tlc in EtOAc. Recrystallization of a sample from DMF gave orange crystals, mp 287–289°. *Anal.* ($C_{18}H_{17}N_5O_4$) C, H, N.

5-(*p*-Aminophenylbutyl)-2,4-diaminopyrimidine Dihydrochloride (17).—A hot solution of 6.0 g (16 mmoles) of **16** in 200 ml of HOAc was clarified with charcoal, then allowed to stand 6 hr during which time **16** separated from solution. The mixture was shaken with H_2 at 2–3 atm in the presence of 0.5 g of 10% Pd–C for 2 hr when reduction was complete. To the filtered solution was added 40 ml of 6 N HCl. The solution was heated on a steam bath for 1 hr to remove the N-Ac groups, then allowed to stand overnight. The product was collected on a filter and washed with HOAc; yield 3.0 g (56%), gradually decomposed over 280° and was homogeneous on tlc in 1:4 EtOH–$CHCl_3$. Recrystallization of a sample from dilute HCl–HOAc gave white crystals with unchanged melting point. *Anal.* ($C_{14}H_{19}N_5 \cdot 2HCl$) C, H, N.

2,4-Diamino-5-[*p*-(*m*-fluorosulfonylbenzamido)phenylbutyl]pyrimidine (4) Sulfate.—To a solution of 200 mg (0.60 mmole)

of **17** and 250 mg (2 mmoles) of DBN in 2 ml of DMF cooled in an ice bath at −10° was added 220 mg (1 mmole) of *m*-fluorosulfonylbenzoyl chloride over about 5 min with stirring. After 15 min the solution was poured into a stirred mixture of 30 ml of 1 *N* H_2SO_4 and 10 ml of $CHCl_3$. The collected product was washed with hot $CHCl_3$, then recrystallized from glacial HOAc containing a few drops of 6 *N* H_2SO_4; yield 215 mg (64%), gradually decomposed over 150° and moved as one spot on tlc in 1:4 EtOH–$CHCl_3$. *Anal.* ($C_{21}H_{22}FN_5O_3S \cdot H_2SO_4 \cdot 0.5H_2O$) C, H, F.

2,4-Diamino-5-[*p*-(*m*-fluorosulfonylbenzamido)phenylbutyl]-6-methylpyrimidine (3) Hemisulfate.—Reaction of 420 mg (1 mmole) of crude $11 \cdot 1.5H_2SO_4$ with 220 mg (1 mmole) of acid chloride, as described for **4**, gave a crude product that was recrystallized from EtOH–H_2O; yield 90 mg (17%), mp >161° with gradual decomposition. *Anal.* ($C_{22}H_{24}FN_5O_3S \cdot 0.5H_2SO_4 \cdot H_2O$) C, H, F.

The *p*-benzamide (**18**) was prepared as described for **4**; yield 130 mg (38%), mp 211–218° dec. *Anal.* ($C_{21}H_{22}FN_5O_3S \cdot H_2SO_4 \cdot H_2O$) C, H, F.

By reaction of **17** with O-(*p*-nitrophenyl) N-(*p*-fluorosulfonylphenyl)carbamate,[14] as described for the preparation of **4**, was obtained **19** in 46% yield, mp >140° with gradual decomposition. *Anal.* ($C_{21}H_{23}FN_6O_3S \cdot H_2SO_4 \cdot 0.5H_2O$) C, H, F.

Enzyme Results and Discussion

Replacement of the ether linkage in the bridge of **1** by methylene (**3**) gave little change in the ability of the compound to inactivate mouse L1210 dihydrofolic reductase, nor was specificity changed since **3** showed no significant inactivation of the mouse liver enzyme (Table I). Unfortunately, penetration through the L1210 cell wall was still poor with **3** since there was little change in ED_{50}[16] or the normalized ED_{50}/I_{50} compared to **1**. The ED_{50}/I_{50} = 68 for **3** should be compared with the ED_{50}/I_{50} = 0.003 for **2**.[3,5]

When the 6-methyl group of **3** was replaced by H, the resultant **4** was still an excellent irreversible inhibitor of L1210 dihydrofolic reductase, but showed perceptible inactivation of the mouse liver enzyme; however, **4** was even less effective than **3** against intact L1210 cells in culture. Even though **18** and **19** were less effective on the L1210 enzyme and less specific than **4**, these two compounds were assayed against L1210 cell culture; again, penetration was poor.

Since such high specificity against L1210 dihydrofolic reductase is obtained with **1** and **3**, further studies would be warranted to see if cell penetration can be improved. Variants at the 6 position of the pyrimidine, the oxypropyl bridge between the pyrimidine and inside phenyl, as well as the bridge between the two benzene rings are under continued investigation.

(16) We wish to thank Dr. Florence White of the CCNSC for the L1210 cell culture data.

2,4-Diaminopyrimidines. The Cyclization of 6-Phenacylthio and Related Derivatives to Thieno[2,3-*d*]pyrimidines and Thiazolo[3,2-*c*]pyrimidines[1]

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2,4-Diamino-5- and -6-substituted thieno[2,3-*d*]pyrimidines have been prepared from 2,4-diamino-6-mercaptopyrimidine plus α-halo ketones. The ease of cyclization of the intermediate pyrimidyl sulfides (Pyr-SCHR'COR) varies dramatically with the R and R' substituents. When R = *p*-bromophenyl and R' = H, cyclization can be effected in low yield at 200° in inert medium. On the other hand, with R = methyl and R' = benzyl, cyclization proceeds spontaneously at room temperature in slightly acidic medium. In concentrated sulfuric acid, where R = *p*-bromophenyl and R' = H, the isomeric thiazolo[3,2-*c*]pyrimidinium sulfate is readily produced. This compound is stable only as the cation. In alkali, the pyrimidine ring opens with loss of its 2-carbon atom. The 2,4-diaminothieno[2,3-*d*]pyrimidines are weak bases, with pK_a values below 5. A bulky R' group and small R substituent favors activity as a dihydrofolate reductase inhibitor, but slightly acidic solutions are required for maximum activity. The low pK_a values of these compounds militate against wide utility, since the protonated species is required for enzyme binding.

Our laboratories have been engaged for many years in chemotherapy studies based on the inhibition of folate biosynthesis and function.[2] Many derivatives of 2,4-diaminopyrimidine have been found to be potent inhibitors of the enzyme dihydrofolate reductase, which plays a major role in folic metabolism by catalyzing the reduction of dihydrofolate to its active cofactor form, tetrahydrofolate. This cofactor is involved in at least 15 biosynthetic transfer reactions of one-carbon fragments involved in amino acid and nucleic acid synthesis.[3]

Impetus to the search for new compounds which block the action of this enzyme has been given by the finding that dihydroreductases from microbial *vs.* mammalian sources differ greatly in their binding capacity for different diaminopyrimidines and related compounds.[4] For example, the antibacterial agent trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine][5] is bound 50,000 times more strongly to bacterial than to mammalian enzymes; this provides a sound explanation for its therapeutic effectiveness.[6]

(1) This paper was presented in part at the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966.

(2) See, for example, G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 416 (1965), and references cited therein.

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