Irreversible Enzyme Inhibitors. CVIII.[1,2] 6-(*p*-Chloroacetylanilinomethyl)-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase

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The title compound (**10a**) was synthesized by reductive condensation of 5-(*p*-chlorophenyl)-2,4-diaminopyrimidine-6-carboxaldehyde (**7**) with 2-(*p*-aminophenyl)-2-chloromethyl-1,3-dioxolane (**8a**) followed by hydrolysis of the ketal blocking group. Three higher homologs (**10**) were also synthesized from the appropriate 2-(*p*-aminophenylalkyl)-2-chloromethyl-1,3-dioxolane (**8**). The title compound rapidly inactivated the dihydrofolic reductase from Walker 256 rat tumor, rat liver, and mouse leukemia L1210/FR8; the enzyme from pigeon liver was inactivated perceptibly slower. That reversible complex formation between the enzyme and the inhibitor was a necessary prerequisite for inactivation was shown by the failure of *p*-amino-α-chloroacetophenone to inactivate dihydrofolic reductase under conditions that led to rapid inactivation with **10a**.

Considerable difficulty was encountered in the design of the first active-site-directed irreversible inhibitor[3,4] of dihydrofolic reductase until the discovery[5] of a hydrophobic bonding region adjacent to the active site was explored.[6] The first successful type of active-site-directed irreversible inhibitor for this enzyme was the 4-pyrimidinol **1**, where the 5-phenylbutyl group was complexed to the hydrophobic bonding region and the 6-phenethyl group projected into a polar region that could then be covalently linked to the inhibitor;[7] this hydrophobic bonding region is adjacent to either the 4 or 8 position of dihydrofolate (**3**), the substrate, when it is complexed to the enzyme.[7-9]

Since the rate of inactivation by an active-site-directed irreversible enzyme inhibitor is dependent upon the concentration of reversible enzyme–inhibitor complex, which in turn is dependent upon the dissociation constant (K_i) of the complex,[10] **1** was considered to be too poor a reversible inhibitor to be effective *in vivo*; a concentration of 4×10^{-5} *M* **1** is necessary for an effective rate of inactivation.[7] Attention was therefore turned to the 2,4-diaminopyrimidine type of inhibitor, since these are 300–3000 times more potent reversible inhibitors than the corresponding 2-amino-4-pyrimidinols[6] and should be able to inactivate dihydrofolate in the 10^{-6} to 10^{-8} *M* region. The conversion of an effective active-site-directed inhibitor such as **1** to its diamino counterpart **2** was not expected to be the answer, based on reversible binding data.[7,9] Indeed **2** did not inactivate dihydrofolic reductase from pigeon liver, although it was a good reversible inhibitor as anticipated.[11] Similarly, the 5-chlorophenylpyrimidine (**4**) was a good reversible inhibitor, but not an irreversible inhibitor of pigeon liver dihydrofolic reductase;[11] surprisingly, **4** could slowly inactivate *Escherichia coli* dihydrofolic reductase, but **2** did not.[12] From reversible binding data it is believed that **2** and **4** project the phenethyl group to the left, but **1** projects the phenethyl group to the right as indicated.[11]

Two of several approaches to the solution of this enigma have proved to be successful. The first approach was to extend the leaving group through the hydrophobic bonding region until a polar region was encountered; the synthesis and evaluation of active-site-directed irreversible inhibitors of dihydrofolic reductase of type **5** have been recently described.[21] The second approach was to use a side chain at the 6 position of **4** that had a more flexible ground-state conformation and was more polar. Such a side chain is present in **6** which might complex in conformation **6b** similar to **1** and not conformation **6a** similar to **4**; the results of such studies are the subject of this paper.

Chemistry.—The synthesis of **6** and its analogs (**10**) by reductive condensation of the 2,4-diaminopyrimidine-6-carboxyaldehyde (**7**) and an appropriately substituted aniline (**8**) appeared to be a likely route, particularly since methods for synthesis of **7** and **8** were available from earlier studies in this laboratory (Scheme I). The aldehyde **7**[11] was condensed with the aniline containing a blocked chloromethyl ketone side chain (**8**)[13] in DMF; then the resultant anil was reduced with sodium borohydride in methanol[13,14] to give the desired blocked products **9**. The dioxolane blocking group of **9** was removed by hydrolysis with dilute hydrochloric acid, affording the candidate active-site-directed irreversible inhibitor **10**.

Enzyme Results.—The 6-anilinomethylpyrimidine (**10a** = **6**) showed rapid inactivation of the dihydrofolic reductase from pigeon liver (Table I). Determinations of the I_{50} of **10a** were erratic due to a mixture of reversible and irreversible inhibitions; our older method

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

(2) (a) For paper CVII of this series see B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **10**, 1129 (1967); (b) for the previous paper on dihydrofolic reductase see B. R. Baker and G. J. Loureus, *ibid.*, **10**, 1113 (1967); paper CV of this series.

(3) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.

(4) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964), a review.

(5) B. R. Baker, B.-T. Ho, and D. V. Santi, *ibid.*, **54**, 1415 (1965).

(6) For a review on the mode of binding of inhibitors to dihydrofolic reductase see ref 3, Chapter X.

(7) B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **55**, 1417 (1966); paper LXVII of this series.

(8) B. R. Baker, T. J. Schwan, J. Novotny, and B.-T. Ho, *ibid.*, **55**, 302 (1966).

(9) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 308 (1966).

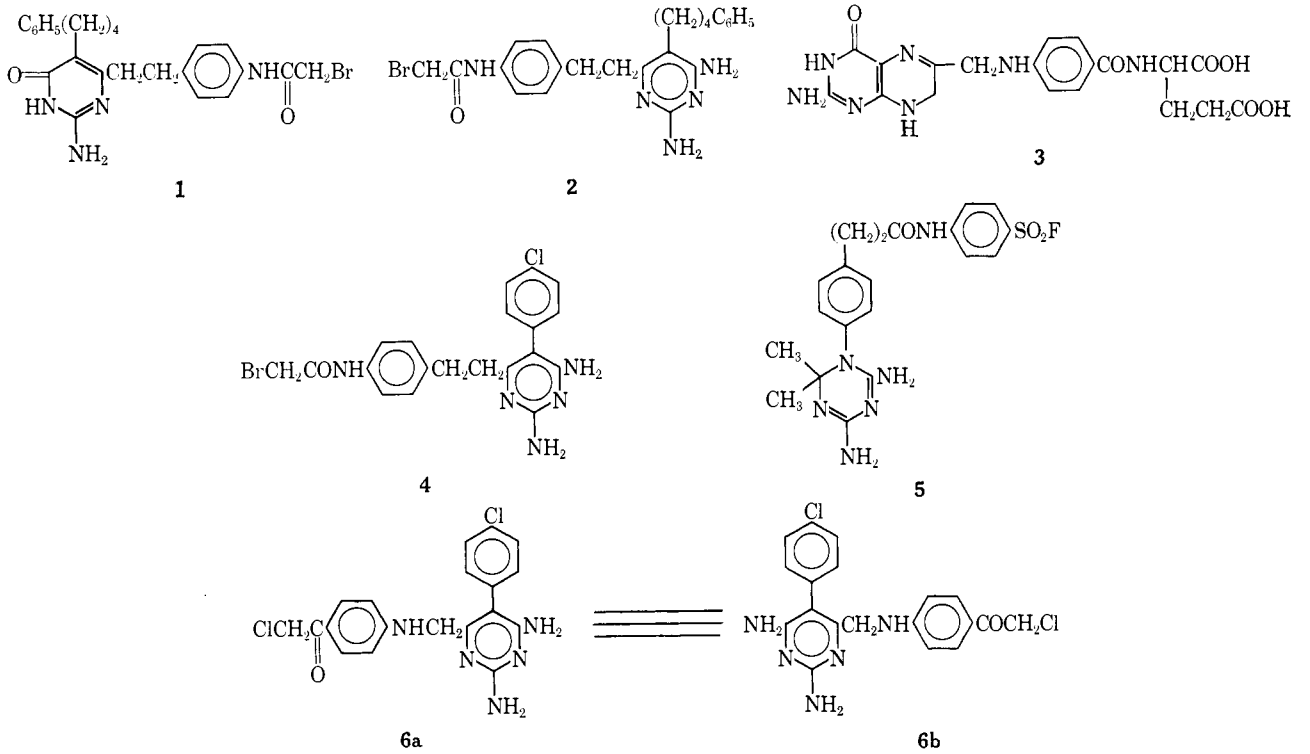
(10) For the kinetics of irreversible enzyme inhibition see ref 3, Chapter VIII.

(11) B. R. Baker and J. H. Jordaan, *J. Heterocyclic Chem.*, **4**, 31 (1967); paper LXXXIII of this series.

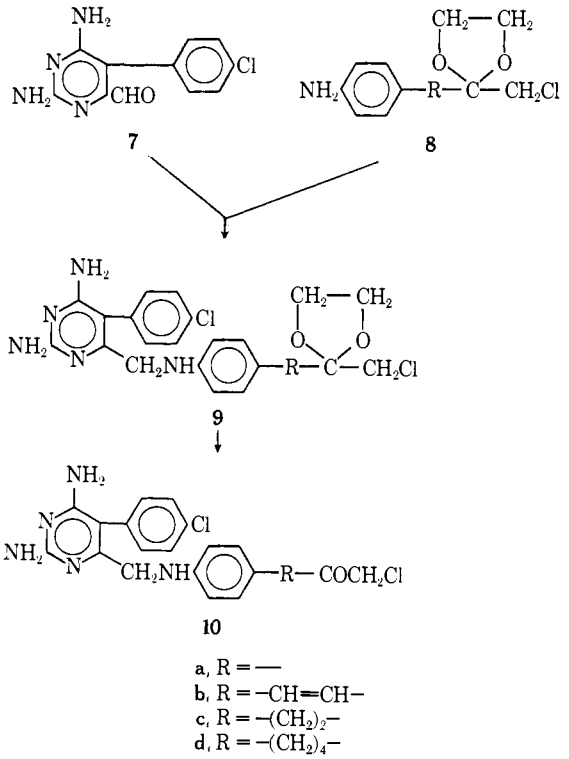
(12) B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **56**, 660 (1967); paper LXXXVIII of this series.

(13) B. R. Baker and J. H. Jordaan, *J. Med. Chem.*, **8**, 35 (1965).

(14) The feasibility of this reaction was first shown by condensation of **7** with aniline by Dr. J. H. Jordaan in this laboratory.



SCHEME I



a, R = —
b, R = —CH=CH—
c, R = —$(CH_2)_2$—
d, R = —$(CH_2)_4$—

allowed considerable contact time between the enzyme and the inhibitor before the substrate was added.[15] With the newly devised methods,[2b] which allows only a short contact time between enzyme and inhibitor, consistent I_{50} results were obtained; the true[2b] I_{50} was three to ten times higher than that observed by the old method.[15] Since the dihydrofolic reductases from Walker 256 rat tumor and L1210/FR8 mouse leukemia were also inactivated by **10a**, these two enzymes were studied more extensively than the pigeon liver enzyme.

At a concentration of **10a** of 25 μM, which is sufficient to convert about 96% of the total enzyme to a reversible complex,[10] 85% of the L1210 enzyme was inactivated in 30 min at 37°. Time studies on the half-life of inactivation of the L1210 enzyme were then performed. At 6 and 1.8 μM **10a**, sufficient to convert 87 and 65% of the enzyme, respectively, to a reversible complex, the half-lives of inactivation were about 2 and 5 min, respectively.

That the inactivation of the L1210 enzyme did not proceed by a random bimolecular process[10] was clearly indicated by the failure of 25 μM *p*-amino-α-chloroacetophenone (**11**) to inactivate the enzyme (Table I); therefore, it is highly probable that **10a** inactivates the enzyme by formation of a covalent bond within the reversible complex between **10a** and the enzyme, the so-called active-site-directed mechanism of irreversible enzyme inhibition.[3]

In over 40 incubation runs with **10a** and the four sources of enzymes, the total inactivation did not exceed 80–85%; thus it is possible that when reaction between the enzyme and the inhibitor is complete, the resultant modified enzyme still has 15–20% residual activity. Such a phenomenon has previously been observed with chymotrypsin.[10,16] In order to establish whether or not a modified enzyme is obtained, further studies with more purified enzyme would be warranted.

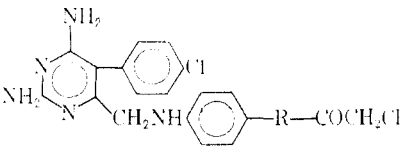
At the low concentration of 0.3 μM, **10a** showed little inactivation of the L1210 enzyme (Table I); this concentration of inhibitor is sufficient to convert 25% of the total enzyme to a reversible complex[10] and should therefore inactivate the enzyme at about one-third the rate seen with 6 μM **10a**, providing the compound does not decompose in the incubation mixture.

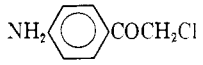
(15) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964).

(16) W. B. Lawson and H. Schramm, *Biochemistry*, **4**, 377 (1965).

TABLE I

INHIBITION[a] OF DIHYDROFOLIC REDUCTASES BY



			Reversible		Irreversible				
No.	—R—	Enzyme source[b]	I_{50},[c] μM	Estd K_i $\times 10^6$ M[d]	Inhibitor concn, μM	TPNH concn, μM	% EI[e]	Time, min	% inactiv	$t_{1/2}$, min[f]
10a (6)	...	L1210	5.8	0.96	25	30	96	30	85	
					6	30	87			2
					1.8	30	65	15	84	4–6
					0.3	30	25	30	0–9	
		W256	5.0	0.83	25	0		30	80	<2
					1.8	0				<2
					25	60	97	60	85	3
					1.8	30	69			3
					0.45	30	36	120	38	
		Rat liver	2.3	0.40	1.8	0		2	80	<2
					1.8	30	82	12	78	<2
		Pigeon liver	5.0	0.83	25	0		60	70	
					25	60	97	60	80	5–7
					1.8	30	69	120	0–12	
10b	—CH=CH—	L1210	7.5	1.2	25	30	93	60	36, 46	
		W256	6.0	1.0	25	0		60	27	
					25	60	96	60	13	
					0.7	30		120	0	
10c	$-CH_2CH_2-$	L1210	8.0	1.3	25	30	96	60	0	
		W256	1.0	0.16	5	0		120	0	
					5	60	97	120	0	
10d	$-(CH_2)_4-$	L1210	3.5	0.60	17.5	30	97	60	0	
		W256	0.33	0.055	1.65	0		120	0	
					1.65	60	97	120	0	
11		L1210	Large	Large	25	30	~0	60	0	
		W256	Large	Large	25	0	~0	10	0	

[a] The technical assistance of Barbara Baine and Jean Reeder is acknowledged. [b] W256 = Walker rat tumor; L1210 = mouse leukemia L1210/FR8. [c] Inhibitor concentration necessary for 50% inhibition of the enzyme in the presence of 6 μM dihydrofolate and 30 μM TPNH (12 μM with pigeon liver) at pH 7.4 when measured as previously described.[2b] [d] Calculated from $K_i = K_m (I_{50}/[S])$ where $K_m = 1 \times 10^{-6} M$ and $[S] = 6 \times 10^{-6} M$; this equation is valid when $K_m > 4[S]$.[5,6] [e] Per cent of enzyme reversibly complexed; calculated from $[EI] = [E_t]/(1 + K_i/[I])$. [f] $t_{1/2}$ = time for half-inactivation at 37° determined as previously described.[2b]

That such a decomposition is detectable with 1.8 μM **10a** was seen; the total inactivation was variable, being between 50 and 85%. When the inactivation stopped at 50%, addition of 1.8 μmoles/l. more of **10a** dropped the enzyme content to 14%. Furthermore, when the crude dihydrofolic reductase preparation was incubated with 1.8 μM **10a** for 30 min, which inactivated the dihydrofolate, then additional enzyme was added, no inactivation of the second aliquot of enzyme occurred. That this decomposition of **10a** was not due to buffer or foreign protein was shown by preincubating **10a** with 3.5 mg/ml of bovine serum albumin in buffer; after 30 min, the preincubation mixture still showed 50% inactivation of L1210 dihydrofolic reductase at 1.8 μM **10a**. Thus, this decomposition of **10a** is due to some other material in the crude enzyme, or is due to a dihydrofolic reductase catalyzed decomposition of **10a**; studies with purified enzyme could differentiate between these two possibilities.

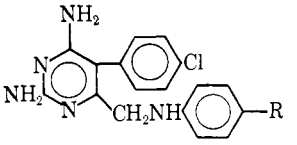
Whether or not inactivation of the L1210 enzyme occurs in the absence of TPNH cannot be determined due to the instability of this enzyme in the absence of TPNH.

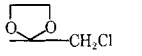
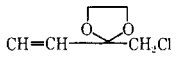
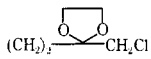
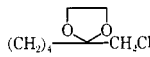
A similar rapid inactivation of dihydrofolic reductase from Walker 256 enzyme with **10a** was observed (Table I); the half-life was about 3 min in the presence of TPNH, but less than 2 min in the absence of TPNH. It is probable that the difference in these rates with and without TPNH is significant, although with an earlier compound showing slower inactivation, definite protection against inactivation by TPNH was seen.[7] Also notable with the Walker 256 was that 25 μM **11** failed to show any inactivation of the enzyme, again eliminating the possibility of inactivation by a random bimolecular process, but supporting the active-site-directed mechanism.

Dihydrofolic reductase from rat liver was rapidly inactivated by 1.8 μM **10a** in the presence or absence of TPNH; in less than 2 min, the enzyme was 80% inactivated. The dihydrofolic reductase from pigeon liver was also inactivated by **10a**, but perceptibly slower than the rat liver enzyme. With 25 μM **10a**, the pigeon liver enzyme in the presence of TPNH was inactivated with a half-life of about 6 min. At the lower concentration of 1.8 μM, the difference in inactivation of the liver enzyme was more noticeable; the pigeon liver enzyme only occasionally showed slight inactivation, whereas the rat liver was maximally inactivated in less than 2 min. The failure of 1.8 μM **10a** to inactivate the pigeon liver enzyme is probably

TABLE II

PHYSICAL CONSTANTS OF



No.	R	Method	% yield	Mp, °C dec	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N	λmax, mμ pH 1	λmax, mμ pH 13
9a		A	38[a]	178–179	54.2[g]	4.74	15.1	54.4	4.35	15.1	280	256, 293
9b		A	37[b]	183–184	58.4	4.90	14.8	58.2	5.02	14.7	280	293
9c		A	30[a]	140–142	57.3[c]	5.44	14.5	57.6	5.62	14.2	276	291
9d		A	30[a]	146–148	59.6	5.81	13.9	59.9	5.86	13.8	276	291
10a	$COCH_2Cl \cdot HCl$	B	80[d]	180–182	49.7[e]	4.40	15.0	49.5	4.61	14.7	325	340
10b	$CH{=}CHCOCH_2Cl$	B	85[f]	>300	56.8[e]	4.76	15.7	57.1	5.10	16.0	276, 372	293, 398
10c	$(CH_2)_2COCH_2Cl \cdot HCl$	B	85[f]	150–152	53.1[c]	4.85	14.7	53.4	5.08	14.5	275	290
10d	$(CH_2)_4COCH_2Cl \cdot HCl$	B	86[f]	175–177	55.7	5.25	14.2	55.6	5.20	14.0	275	290

[a] Recrystallized from EtOH–H_2O. [b] Recrystallized from EtOH. [c] Hemihydrate. [d] Recrystallized from EtOH by addition of 0.1 *N* HCl. [e] Monohydrate. [f] Recrystallized from methoxyethanol–H_2O.

due to a combination of decomposition of the **10a** coupled with its slower rate of inactivation of the enzyme.

When the distance between the chloromethyl ketone group and the pyrimidine ring was increased, as in **10c** and **10d**, reversible inhibition was changed little with the L1210 enzyme; in contrast, the two compounds failed to irreversibly inhibit the L1210 enzyme. Similarly, **10c** and **10d** failed to inactivate the Walker 256 enzyme, but reversible inhibition was somewhat better than with **10a**. Insertion of the vinyl group into **10a** to give **10b** resulted in little change in reversible inhibition, but irreversible inhibition was considerably less effective with **10b**.

Discussion

6-(*p*-Chloroacetylanilinomethyl)-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine (**10a**) is an irreversible inhibitor of dihydrofolic reductase that does not inactivate the enzyme by a random bimolecular process, else *p*-amino-α-chloroacetophenone (**11**) should have also inactivated the enzyme. Therefore, a complex between **10a** and the enzyme is necessary for inactivation. There are at least two ways in which an enzyme can become inactivated within the enzyme–inhibitor complex: (a) a neighboring-group reaction can occur within the complex,[7,10] or (b) the complex causes a conformational change that exposes a group on the enzyme to bimolecular attack.[10,17] These two mechanisms can usually be distinguished kinetically,[10] but since **10a** inactivates the enzyme with such rapidity, this is difficult to do experimentally in this case.

Although absolute rate ratios have not been obtained, it is clear (Table I) that the order of decreasing rate of inactivation is rat liver > Walker 256 > L1210 >> pigeon liver. That differences in the rates of inactivation by **10a** should exist is predictable by the bridge principle of specificity.[4,18,19] An irreversible inhibitor that is a close analog of the substrate and covalently links to the enzyme within the active site (endo mechanism) would not be expected to show isozyme specificity.[18] However, an irreversible inhibitor that either covalently links the enzyme outside of the active site (exo mechanism) or partially complexes outside the active site and covalently links inside the active site could show isozyme specificity.[18] Evolutionary changes in an enzyme are much more apt to have occurred outside the active site than inside the active site;[18] therefore, an inhibitor such as **10a** that does utilize a part of the enzyme outside the active site could show isozyme specificity.

With **10a** there is insufficient difference between the rate of inactivation of the Walker 256 rat tumor enzyme and the rat liver enzyme to be of chemotherapeutic use. However, it should be possible to build into the molecule further specificity by utilization of the bridge principle of specificity[4,18] as has been previously done with the lactic dehydrogenase isozymes.[19] Such an isozyme specificity study with **10a** may be on the borderline of usefulness since a concentration of $2\text{–}25 \times 10^{-6}$ *M* would be required; it would be more desirable to have inhibition related to **10a** which could operate at 10^{-7} to 10^{-9} *M* by being better reversible inhibitors. Studies of both types are currently being pursued.

Experimental Section[20,21]

6-[*p*-(2-Chloromethyl-1,3-dioxolan-2-yl)anilinomethyl]-5-*p*-(chlorophenyl)-2,4-diaminopyrimidine (9a). Method A.—A solution of 500 mg (2 mmoles) of **7**[12] and 430 mg (2 mmoles) of **8a**[13] in 5 ml of DMF was stirred for 30 min, then diluted with

(17) T. Inagami, *J. Biol. Chem.*, **240**, 3453 (1965).

(18) See ref 3, Chapter IX.

(19) (a) B. R. Baker, *J. Med. Pharm. Chem.*, **5**, 654 (1962); *Biochem. Pharmacol.*, **11**, 1155 (1962); (b) B. R. Baker and R. P. Patel, *J. Pharm. Sci.*, **53**, 714 (1964).

(20) Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Tlc was performed on Brinkmann silica gel GF and spots were detected by visual examination under ultraviolet light. All analytical samples had ir and uv spectra compatible with their assigned structures and each moved as a single spot on tlc. Compounds of structure **10** gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen.[21]

(21) B. R. Baker, D. V. Santi, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocyclic Chem.*, **3**, 425 (1966).

30 ml of MeOH. $NaBH_4$ (1.20 g) was added over a period of 45 min; then the mixture was stirred at ambient temperature for 15 hr. The nearly clear solution was clarified by filtration, then spin-evaporated *in vacuo* to about 10 ml, and diluted with 70 ml of H_2O. The product was collected on a filter and washed with water. Two recrystallizations from aqueous EtOH gave 340 mg (38%) of light yellow crystals, mp 178–179° dec. See Table II for additional data.

6-(*p*-Chloroacetylanilinomethyl)-5-(*p*-chlorophenyl)-2,4-diaminopyrimidine (10a) Hydrochloride. Method B.—A mixture of 180 mg (0.4 mmole) of **9a** and 10 ml of 0.1 *N* HCl was refluxed with stirring for 1 hr, then cooled to 0° for several hr. The product was collected on a filter and washed with 2 ml of ice water. Recrystallization from EtOH by addition of 0.1 *N* HCl gave 146 mg (80%) of white plates, mp 180–182° dec. See Table II for additional data.

2,2′-Hydrazobis(5-nitrothiazoles) and Analogs, a New Type of Antiprotozoal Agents

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A series of 1,2-diacyl-1,2-bis(5-nitro-2-thiazolyl)hydrazines and several bis(5-nitro-2-thiazolyl) derivatives have been prepared and tested for antiprotozoal activity. Some of the compounds show a very strong *in vitro* but no *in vivo* activity.

The heterocyclic nitro compounds belong to one of the most thoroughly investigated, versatile, and useful systems in the services of chemotherapy of infectious diseases. The best examples are the nitrofurans, exhibiting pronounced trypanocidal, coccidistatic, and very strong antibacterial activity.[2] Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is today the drug of choice in the systemic treatment of trichomoniasis.[3] This fact has led to the preparation of a great number of substituted 5-nitroimidazoles as potential chemotherapeutic agents.[4] 2-Amino-5-nitropyridine,[5,6] 2-amino-5-nitropyrimidine,[6] and a number of 4-nitropyrazoles[7] and nitropyrroles[8] show marked trichomonacidal activity. The 2-amino-5-nitrothiazole nucleus seems to possess one of the broadest profiles of antiparasitic activity, ranging from trichomonal and helminthic infections, especially schistosomiasis, to histomoniasis and amebiasis.[9] 2-Acetamido-5-nitrothiazole has also a suppressive action on infections with *Trypanosoma cruzi* in mice.[10] These results encouraged the study of a further number of 5-nitrothiazoles with different substituents at the 2-amino group.[11,12]

One common feature found in many chemotherapeutic agents is their symmetrical structure. These molecules have been described as "dumb-bell" shaped[13] or as "butterfly structures."[14] Typical examples are the aromatic diamidines used in the treatment of trypanosomiasis[15] and leishmaniasis[16] and the derivatives of 4,4′-diaminodiphenyl sulfone, used in the therapy of all forms of leprosy.[17] Bis(4,6-diaminoquinaldine) derivatives show a very marked antitrypanosomal[18] and antibacterial activity,[19] polymethylenebisquinolinium and -isoquinolinium salts possess a wide bacteriostatic[19] and fungistatic profile,[20] while diaminodiphenoxyalkanes are considered potential schistosomicides.[21]

The combination of these two important features, nitro heterocyclic compounds and symmetrical molecules, led us to consider the investigation of a new type

(1) (a) To whom inquiries should be addressed. (b) Deceased.

(2) (a) F. Hawking in "Experimental Chemotherapy," Vol. I, R. J. Schnitzer and F. Hawking, Ed., Academic Press Inc., New York, N. Y., 1963, p 197; (b) L. P. Joyner, S. F. M. Davies, and S. B. Kendal, *ibid.*, p 460; (c) H. E. Paul and M. F. Paul, *ibid.*, Vol. II, 1964, p 307.

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(4) (a) Rhone-Poulenc S.A., French Patents 1,379,787 (1964), M 3270 (1965), M 3342 (1965); (b) Netherlands Patent 6,411,717 (1965); (c) Merck and Co., Netherlands Patents 6,409,117 (1965), 6,409,120 (1965), 6,413,815 (1965); (d) Belgian Patents 660,836 (1965), 661,262 (1965); (e) May and Baker Ltd., Belgian Patents 639,372 (1964), 639,469 (1964); (f) U. S. Patent 3,236,856 (1966); (g) Carlo Erba S.p.A., Belgian Patent 667,262 (1965).

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