Enzyme Inhibitors. XX. Studies on the Hydrophobic and Hydroxyl Binding Regions of Adenosine Deaminase. 9-(2-*m*-Bromoacetamidophenethyl)adenine, a New Irreversible Inhibitor of Adenosine Deaminase[1]

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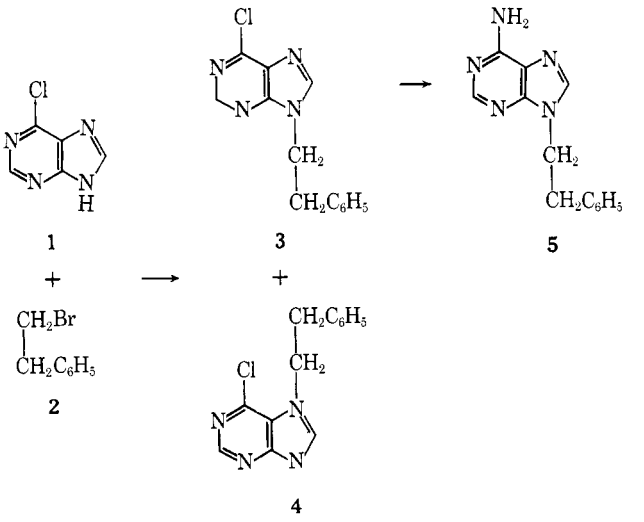
9-(*o*- and *p*-bromoacetamidobenzyl)adenines irreversibly inactivated calf intestinal mucosa adenosine deaminase rapidly, whereas the corresponding *meta* isomer irreversibly inactivated the enzyme very slowly. In order to study the effect of structure on the reversible and irreversible inhibition of adenosine deaminase, a variety of 6-substituted purines were synthesized that were substituted at the 9 position by a phenethyl, a β-hydroxyphenethyl, or a *meta*-substituted phenethyl group. The phenethyl derivatives were generally weaker reversible inhibitors than the corresponding benzyl analogs. However, 9-(*m*-bromoacetamidophenethyl)adenine (**24**) was found to cause a rapid irreversible inactivation of adenosine deaminase. Evidence is presented that the irreversible inactivation of adenosine deaminase by **24** is not a random bimolecular process but proceeds by a first-order reaction (k_2) in an initial reversible enzyme–inhibitor complex. A kinetic analysis of the irreversible inactivation by **24** of this enzyme revealed that the K_i of **24** = 72×10^{-6} *M* and that the apparent first-order rate constant (k_2) for the alkylation of the enzyme is 28×10^{-2} min^{-1}.

Several recent publications have suggested that adenosine deaminase has a hydrophobic region and a hydroxyl binding site that are involved when certain inhibitors bind to this enzyme.[2,3] In an attempt to prepare compounds which could bind to the hydrophobic region or to both the hydrophobic and hydroxyl binding regions, we decided to prepare some 9-phenethyl-, 9-(*m*-nitrophenethyl)-, and 9-(β-hydroxyphenethyl)-6-substituted purines and evaluate them as inhibitors of adenosine deaminase. Furthermore, it has been reported previously, that 9-(*m*-bromoacetamidobenzyl)adenine was an irreversible inhibitor of adenosine deaminase but the rate of irreversible inactivation was very low when compared with the rates of irreversible inactivation of this enzyme by 9-(*o*- or *p*-bromoacetamidobenzyl)adenines.[4] Therefore, we were interested in the preparation of some 9-(*meta*-substituted phenethyl)-6-substituted purines and in particular 9-(*m*-bromoacetamidophenethyl)adenine to determine if the longer chain length would allow the compound to bridge more easily to a nucleophilic site on the enzyme with the result that the irreversible inhibition of adenosine deaminase would occur at a much higher rate of reaction. This paper describes the synthesis and biological evaluation of this series of compounds (Table I).

Chemistry.—The synthesis of these compounds was accomplished by modification of procedures which have previously been employed for the preparation of some 9-substituted-6-substituted purines.[5,6] Thus, condensation of 6-chloropurine (**1**) with 2-bromoethylbenzene (**2**) in the presence of triethylamine gave a mixture of 9-phenethyl-6-chloropurine (**3**) and 7-phenethyl-6-chloropurine (**4**) (Chart I). Treatment of **3** with methanolic ammonia at 85° for 19 hr gave 9-(2-phenethyl)adenine (**5**).

CHART I



The synthesis of the 9-(β-hydroxyphenethyl)purines was accomplished as outlined in Chart II. 5-Amino-4,6-dichloropyrimidine (**6**) on condensation with α-(aminomethyl)benzyl alcohol (**7**) gave the desired pyrimidine (**8**) which on reaction with triethyl orthoformate and hydrochloric acid formed 9-(β-hydroxyphenethyl)-6-chloropurine (**9**). The 6-substituted derivatives (**10–14**) were prepared by allowing **9** to react with ammonia, methylamine, dimethylamine, aqueous formic acid, and thiourea.

Finally, the 9-(*meta*-substituted phenethyl)purines were prepared by the condensation of **6** with 2-(*m*-nitrophenyl)ethylamine (**15**) which resulted in the formation of **16**. Treatment of **16** with triethyl orthoformate and HCl gave **17** which was converted into **18–22** in the usual manner. Catalytic hydrogenation of 9-(*m*-nitrophenethyl)adenine (**18**) with Pd–C gave **23** in good yield. When **23** was allowed to react with bromoacetic anhydride, acetic anhydride, or phenyl chloroformate, the corresponding acylated derivatives (**24–26**) were obtained in moderate yields (Chart III).

(1) This investigation was supported by Grant T-337A from the American Cancer Society, by a Public Health Service research grant (5-R01-GM-09775-05), by a research career program award (5-K3-CA-18718-05) from the National Cancer Institute, and a training grant (5-T1-GM-555-05) from the Division of Medical Sciences, U. S. Public Health Services, Bethesda, Md.

(2) H. J. Schaeffer and D. Vogel, *J. Med. Chem.*, **8**, 507 (1965).

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(5) J. A. Montgomery and C. Temple, Jr., *J. Am. Chem. Soc.*, **83**, 630 (1961).

(6) C. Temple, Jr., C. L. Kussner, and J. A. Montgomery, *J. Med. Pharm. Chem.*, **5**, 866 (1962).

TABLE I

7- AND 9-(SUBSTITUTED PHENETHYL)-6-SUBSTITUTED PURINES

Compd	Recrystn solvent[a]	Mp, °C	Yield, %	Ultraviolet λ_{max}, mμ (ε × 10⁻⁴) 1 N HCl–cosolvent	H_2O–cosolvent	1 N NaOH–cosolvent	Cosolvent	Formula	Analyses
3	A	102–103	50.1	266 (0.944)	266 (0.975)	266 (0.984)	10% EtOH	$C_{13}H_{11}ClN_4$	C, H, Cl, N
4	B	160–162	11.7	267 (0.825)	268 (0.807)	270 (0.820)	10% EtOH	$C_{13}H_{11}ClN_4$	C, H, Cl, N
5	C	176–177	62.4	261 (1.36)	263 (1.36)	263 (1.40)	10% EtOH	$C_{13}H_{13}N_5$	C, H, N
9	D	141–142	50.6	264 (1.01)	264 (1.03)	264 (1.01)	10% EtOH	$C_{13}H_{11}ClN_4O$	C, H, Cl, N
10	B	225–226	63.3	258 (1.36)	259 (1.32)	260 (1.57)	10% EtOH	$C_{13}H_{13}N_5O$	C, H, N
11	E	188–189	49.7	263 (1.53)	265 (1.44)	266 (1.60)	10% EtOH	$C_{14}H_{15}N_5O$	C, H, N
12	E	179–180	66.8	267 (1.83)	273 (1.78)	274 (1.99)	10% EtOH	$C_{15}H_{17}N_5O$	C, H, N
13	B	287–289	60.8	249 (1.12)	249 (1.21)	254 (1.31)	None	$C_{13}H_{12}N_4O_2$	C, H, N
14	..	313–314	91.7	322 (2.00)	316 (2.03)	310 (2.04)	10% DMSO	$C_{13}H_{12}N_5OS$	C, H, N, S
17	B	212–213	74.0	267 (1.53)	267 (1.53)	267 (1.57)	10% EtOH	$C_{13}H_{10}ClN_5O_2$	C, H, Cl, N
18	B	236–237	70.8	261 (1.96)	262 (1.99)	261 (2.18)	4% EtOH	$C_{13}H_{12}N_6O_2$	C, H, N
19	F	225–227	60.0	264 (2.07)	267 (2.00)	267 (2.11)	10% EtOH	$C_{14}H_{14}N_6O_2$	C, H, N
20	B	175.5–176.5	76.5	269 (2.42)	274 (2.35)	274 (2.51)	4% EtOH	$C_{15}H_{16}N_6O_2$	C, H, N
21	B	256–257	60.1	253 (1.60)	251 (1.57)	257 (1.95)	10% EtOH	$C_{13}H_{11}N_5O_3$	C, H, N
22	..	293–295	95.5	324 (2.06)	323 (2.23)	313 (2.19)	10% DMSO	$C_{13}H_{11}N_5O_2S$	C, H, S
23	..	219–221	82.7	260 (1.34)	263 (1.40)	263 (1.40)	10% EtOH	$C_{13}H_{14}N_6$	C, H, N
24	..	154 dec	51.5	257 (2.01)	257 (2.02)	257 (2.02)	8% DMSO	$C_{17}H_{17}BrN_6O_5$	C, H, Br, N
25	G	212–213	34.5	250 (1.86)	250 (1.77)	250 (1.79)	10% EtOH	$C_{15}H_{16}N_6O$	C, H, N
26	..	192 dec	24.0	260 (1.46)	260 (1.47)	260 (1.63)	5% DMSO	$C_{22}H_{20}N_6O_6$	C, H, N

[a] Recrystallization solvents: A, C_6H_{14}; B, EtOH; C, C_6H_6; D, C_6H_6–C_6H_{14}; E, EtOAc; F, *i*-PrOH; G, MeOH.

CHART II

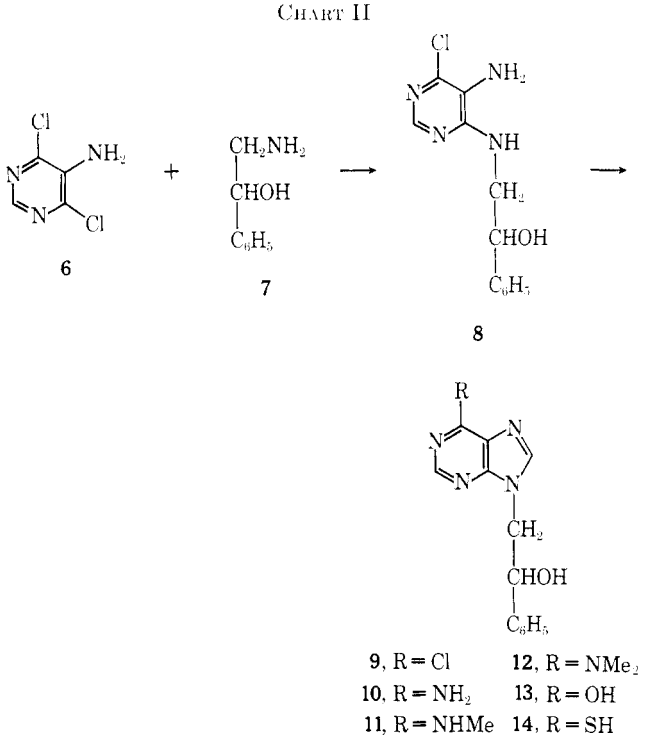
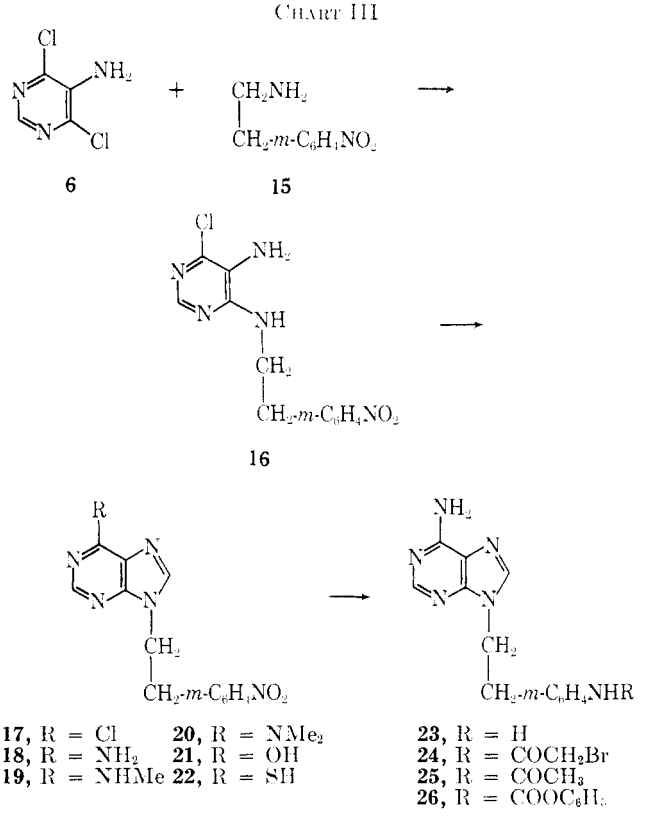


CHART III



Experimental Section[7]

9- and 7-(Phenethyl)-6-chloropurines (3 and 4).—A solution of 2.31 g (15.0 mmoles) of **1**, 3.10 g (16.7 mmoles) of **2**, and 1.68 g (16.6 mmoles) of triethylamine in 50 ml of DMF was stirred at room temperature for 98 hr. The resulting mixture was filtered, and the filtrate was evaporated *in vacuo* to give a semisolid. A $CHCl_3$ solution of the crude material was chromatographed on a column of neutral alumina (61.0 g), and **3** was eluted with $CHCl_3$ (250 ml).

After the 9 isomer had been removed from the column, **4** was eluted from the alumina column with 200 ml of 5% MeOH–$CHCl_3$. After removal of the solvent *in vacuo*, 719 mg (18.5%) of the crude **4** was obtained; mp 130–152°.

General Method 1. Preparation of 5, 10–12, 18–20.—A mixture of 0.500–1.00 mmole of 9-(substituted phenethyl)-6-chloropurine in 20% MeOH–NH_3, 40% aqueous methylamine–EtOH (2:1), or 25% aqueous dimethylamine–EtOH (2:1) was heated in a stainless steel bomb at 51–92° for 19–25 hr. The mixture was evaporated *in vacuo* to give **5**, **10**, **11**, **12**, and **19**. In the case of **18** and **20**, the solid which precipitated from the reaction mixture was collected by filtration. In the case of **10**, liquid NH_3 (15 ml) was employed in place of MeOH–NH_3.

General Method 2. Preparation of 8 and 16.—A solution of 20.0–36.8 mmoles of **6**, 28.5–36.2 mmoles of **7** or **15**, and 22.0–37.8

(7) The infrared spectra were determined on a Perkin-Elmer Model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer Model 202 spectrophotometer; the enzyme studies were done on a Gilford Model 2000 spectrophotometer. The melting points, unless noted otherwise, were taken in open capillary tubes on a Mel-Temp and are corrected. The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

mmoles of triethylamine in 70–125 ml of 1-propanol was heated under reflux for 21–22 hr. Evaporation of the volatile materials *in vacuo* followed by trituration of the residue with water gave crude **8** and **16**, respectively.

General Method 3. Preparation of 13 and 21.—A solution of 0.660–0.750 mmole of the chloropurine in 10 ml of 88% aqueous formic acid was heated under reflux for 20 min. Evaporation of the volatile materials *in vacuo* gave crude **13** and **21**, respectively.

General Method 4. Preparation of 14 and 22.—A mixture of 0.660–0.750 mmole of the 9-(substituted phenethyl)-6-chloropurine and 0.660–0.840 mmole of thiourea in 4–5 ml of 1-propanol was heated under reflux for 0.75–1 hr. The mixture was cooled, and the product was collected by filtration to give **14** and **22**, respectively.

4-Chloro-5-amino-6-(β-hydroxyphenethylamino)pyrimidine (8).—A solution of 3.28 g (20.0 mmoles) of **6**, 3.91 g (28.5 mmoles) of **7**, and 2.23 g (22.0 mmoles) of triethylamine in 70 ml of 1-propanol was heated under reflux for 21 hr. Evaporation of the volatile materials *in vacuo* followed by trituration of the residual oil with H_2O produced 5.36 g of crude solid, mp 167–170°. Recrystallization of the crude product from EtOH gave 4.43 g (83.6%) of the analytical sample: mp 170–172°; λ_{max} [in mμ ($\epsilon \times 10^{-4}$)] (0.1 *N* HCl–10% EtOH) 302 (1.29), (H_2O–10% EtOH) 289 (0.975) and 262 (0.935), (0.1 *N* NaOH–10% EtOH) 289 (0.945) and 262 (0.902); ν (in cm^{-1}) (KBr) 3400 (OH), 3250, 3100, and 1690 (NH), 1570 and 1550 (sh) (C=N and C=C). *Anal.* ($C_{12}H_{13}ClN_4O$) C, H, Cl, N.

9-(β-Hydroxyphenethyl)-6-chloropurine (9).—To a suspension of 3.94 g (14.9 mmoles) of **8** in 100 ml of triethyl orthoformate was added 1.37 ml (16.4 mmoles) of concentrated HCl. After stirring the mixture for 48 hr, the volatile materials were evaporated *in vacuo*. Recrystallization of the residue from benzene–hexane gave 728 mg of pure product, mp 141–142°. The filtrate was evaporated *in vacuo*, and the residue was dissolved in $CHCl_3$ and chromatographed on a column of neutral alumina (150.0 g). The contaminating materials were eluted with $CHCl_3$ (1 l.); **9** was eluted with $CHCl_3$ containing 5% MeOH (400 ml).

β-m-Nitrophenethylamine Hydrochloride (15).—To a mixture of 4.85 g (30.0 mmoles) of *m*-nitrobenzyl cyanide and 2.27 g (60.0 mmoles) of $NaBH_4$ in 100 ml of cold redistilled diglyme was added 12.75 ml (80.0 mmoles) of $BF_3 \cdot O(C_2H_5)_2$ over a period of 35 min. The reaction mixture was stirred at room temperature for 1.5 hr before the excess B_2H_6 was destroyed. After filtration, dry HCl was bubbled into the solution to produce the HCl salt. The volatile materials were removed *in vacuo*, and the residue was dissolved in 50 ml of H_2O. The pH of this solution was adjusted to 13 with 10% aqueous NaOH. The amine was extracted with $CHCl_3$ (three 50-ml portions), and the extract was dried ($MgSO_4$). Addition of dry HCl to the extract produced 4.8 g (79%) of the product, mp 197–206°. Two recrystallizations of the crude material from absolute EtOH gave the analytical sample:[8] yield 1.66 g (27.2%); mp 209–211°; λ_{max} [in mμ ($\epsilon \times 10^{-3}$)] (pH 1) 268 (7.34), (pH 7) 269 (7.08), (pH 13) 272 (7.00); ν (in cm^{-1}) (KBr) 2950 and 1600 (NH_3^+), 1530 and 1350 (NO_2). *Anal.* ($C_8H_{11}ClN_2O_2$) C, H, N.

4-Chloro-5-amino-6-(β-nitrophenethylamino)pyrimidine (16) was prepared in the same manner as **8**. Recrystallization of the crude product from MeOH gave 7.62 g (71.8%) of pure material: mp 174–175°; λ_{max} [in mμ ($\epsilon \times 10^{-4}$)] (0.1 *N* HCl–4% EtOH) 282 (1.46), (H_2O–4% EtOH) 267 (1.50), (0.1 *N* NaOH–4% EtOH) 267 (1.50); ν (in cm^{-1}) (KBr) 3500, 3400, 3290, and 1630 (NH), 1580 (C=N and C=C), 1520 and 1340 (NO_2). *Anal.* ($C_{12}H_{12}ClN_5O_2$) C, H, Cl, N.

9-(m-Nitrophenethyl)-6-chloropurine (17).—To a suspension of 7.18 g (24.4 mmoles) of **16** in 150 ml of triethyl orthoformate was added 2.24 ml (27.9 mmoles) of concentrated HCl over a period of 5 min. This mixture was stirred at room temperature for 162 hr. The precipitated product was collected by filtration; yield 6.90 g (93.4%), mp 210–212°.

9-(β-m-Aminophenethyl)adenine (23).—A solution of 284 mg (1.00 mmole) of **18** in 50 ml of HOAc containing 66 mg of 5% Pd–C was hydrogenated for 1 hr at an initial pressure of 4.2 kg/cm^2. The catalyst was removed by filtration through a Celite pad, and the filtrate was evaporated *in vacuo*. The crude product was dissolved in 10 ml of 1 *N* HCl and filtered, and the filtrate was adjusted to pH 13 with 1 *N* NaOH. The precipitated solid was collected by filtration to give **23**.

9-(β-m-Bromoacetamidophenethyl)adenine Oxalate (24).—To a cold solution of 169 mg (0.659 mmole) of **23** in 5 ml of THF and 0.6 ml of 10% aqueous HOAc was added dropwise 256 mg (0.983 mmole) of bromoacetic anhydride in 1 ml of THF. After stirring the solution at 0° for 30 min, 20 ml of 5% aqueous $NaHCO_3$ was added portionwise. The off-white precipitate was collected by filtration and dried *in vacuo* at room temperature to give 151 mg (61.2%) of material. The precipitated material was dissolved in 10 ml of THF and filtered, and 2.3 ml of 1 *M* oxalic acid in THF was added to the chilled filtrate. The white precipitate (**24**) was collected by filtration.

9-(β-m-Acetamidophenethyl)adenine (25).—To a cold solution of 254 mg (1.00 mmole) of **23** in 0.50 ml of HOAc and 5.0 ml of THF was added 158 mg (1.54 mmoles) of $O(Ac)_2$ in 2.0 ml of THF. The solution was stirred at 0° for 0.5 hr and the precipitate was collected by filtration to give **25**.

9-(β-m-Phenoxycarbonylaminophenethyl)adenine Oxalate (26).—To a solution of 103 mg (0.405 mmole) of **23** in 20 ml of *p*-dioxane was added 72.9 mg (0.720 mmole) of triethylamine followed by 86.3 mg (0.552 mmole) of phenyl chloroformate. The mixture was stirred at room temperature for 1 hr and filtered, and the filtrate was concentrated to *ca.* 2 ml. To the chilled filtrate was added 15 ml of H_2O, and the crude product was collected by centrifugation. Precipitation of the crude material in MeOH with H_2O gave 64.0 mg (42.3%) of material. Oxalic acid (2 ml of 1 *M* in THF) was added to a chilled solution of the precipitated material in THF. The precipitate was collected by centrifugation to give **26**.

Reagents and Assay Procedure.—Adenosine deaminase (Type I, calf intestinal mucosa) was purchased from the Sigma Chemical Co. The assay procedure for the reversible inhibitors has been described previously and is a modification of the procedure of Kaplan[9] based on the work of Kalckar.[10] The method used to study the irreversible inactivation of adenosine deaminase at 37° has been described earlier[4,11] and is a modification of a published procedure.[12] Since **24** was isolated and purified as its oxalate salt, it was necessary to determine that oxalic acid was not the active agent which caused the irreversible inactivation of adenosine deaminase. When oxalic acid was incubated with adenosine deaminase at 37°, it was found that oxalic acid did not cause an irreversible inactivation of this enzyme.

Chemical Reactivity of Iodoacetamide and 24.—These experiments were performed by a modification of the procedures described in the literature[11,13] except that DMSO was employed in place of 2-methoxyethanol.

Results and Discussion

When this series of compounds was evaluated as reversible inhibitors of adenosine deaminase, it was found that those compounds with a 6-chloro, 6-dimethylamino, 6-hydroxy, or 6-mercapto group were essentially noninhibitory when tested at 0.12 m*M* concentrations. The 6-methylamino derivatives (**11** and **19**) are very weakly inhibitory but even those compounds with a 6-amino group are weaker reversible inhibitors of this enzyme than is 9-benzyladenine whose $([I]/[S])_{0.5} = 1.5$ (see Table II). The weaker reversible inhibition of these 9-substituted adenines relative to 9-benzyladenine suggests that the hydrophobic region on adenosine deaminase has limited bulk tolerance for the phenyl group attached to C-2 of the ethyl chain. Furthermore, since **10** is a weaker inhibitor of this enzyme than is **5**, it is clear that it is not

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(9) N. O. Kaplan, *Methods Enzymol.*, **2**, 473 (1955).

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(12) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

(13)(a) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, **27**, 1435 (1955); (b) T. J. Bardos, N. Datta-Gupta, P. Hebborn, and D. J. Triggle, *J. Med. Chem.*, **8**, 167 (1965); (c) B. R. Baker and J. H. Jordaan, *J. Heterocyclic Chem.*, **2**, 21 (1965).

TABLE II
INHIBITION INDEX AND PARTIAL INHIBITION OF ADENOSINE DEAMINASE BY SOME 9-(SUBSTITUTED) ADENINES

Compd	Ad-CH_2CHR-*m*-$C_6H_4R_2$ R_1	R_2	m*M* concn for 50% inhib	$([I]/[S])_{0.5}$
5	H	H	0.33 ± 0.04	5.0 ± 0.6
10	OH	H	0.54 ± 0.02	8.2 ± 0.3
18	H	NO_2	0.22 ± 0.03[a]	3.4 ± 0.5
23	H	NH_2	0.51 ± 0.07	7.8 ± 1.1
25	H	$NHCOCH_3$	0.34 ± 0.02	5.2 ± 0.4
26	H	$NHCOOC_6H_5$	0.16 ± 0.01	2.5 ± 0.1

[a] Because of poor solubility, enzyme inhibition was determined in 0.05 *M* phosphate buffer containing 20% DMSO.

possible to bind to both the hydroxyl binding region and the hydrophobic region with a β-hydroxyphenethyl group at the 9 position of adenine. Studies are underway in an attempt to prepare compounds that can bind to both regions.

Our second reason for preparing this series of compounds was to determine the effect of structure on the irreversible inhibition of adenosine deaminase. It has previously been found that 9-(*m*-bromoacetamidobenzyl)adenine (**27**) causes an irreversible inactivation of adenosine deaminase but that the rate of inactivation is very low. It is possible that the longer chain length of **24** would allow the bromoacetamide moiety to bridge more readily to a nucleophilic group on the enzyme which would cause a more rapid irreversible inactivation.

When **24** was incubated with adenosine deaminase, a rapid and irreversible inactivation of the enzyme occurred. In addition it was found that the *m*-acetamido derivative (**25**) was not an irreversible inhibitor of the enzyme. Thus, the alkylating activity of **24** is essential for the irreversible inhibition, although mere alkylating activity is not sufficient since iodoacetamide, even at higher concentrations, does not inactivate adenosine deaminase. Furthermore, the phenoxycarbonylamino derivative (**26**) was not an irreversible inhibitor of adenosine deaminase; thus, the amino acid on the enzyme which is alkylated by **24** is not acylated by **26** under the conditions of these experiments.

We have previously presented evidence that the irreversible inactivation of adenosine deaminase by the *ortho* and *para* isomers of 9-(bromoacetamidobenzyl)adenines (**28**, **29**) is not a random, bimolecular process.[4] Rather, the irreversible inactivation occurs by the initial formation of a reversible enzyme–inhibitor complex in which the irreversible, first-order reaction between the enzyme and the inhibitor occurs (see eq 1). For reactions that proceed as outlined in eq

$$\begin{array}{ccccc} E + I & \rightleftharpoons & E\cdots I & \xrightarrow{k_2} & E\cdots I + X^- \\ & & \vdots & & \diagdown \\ & & R & & R \\ & & \vdots & & \\ & & X & & \end{array} \qquad (1)$$

1, a kinetic method has been developed for the evaluation of the reversible enzyme–inhibitor constant (K_i) and of the first-order rate constant (k_2) for the alkylation of the enzyme by the irreversible inhibitor.[14]

(14) (a) A. R. Main, *Science*, **144**, 992 (1964); (b) A. R. Main and F. Iverson, *Biochem. J.*, **100**, 525 (1966); (c) A. R. Main and F. L. Hastings, *Science*, **154**, 400 (1966).

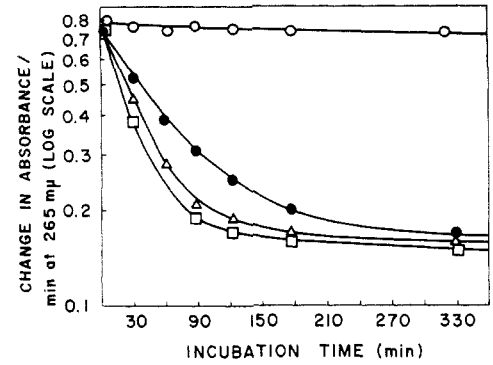
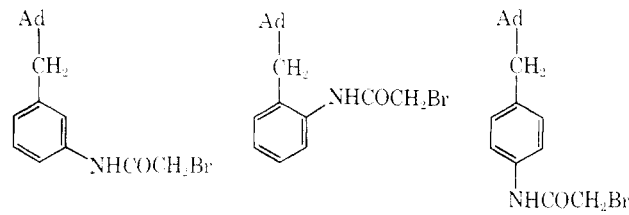


Figure 1.—Irreversible inactivation of adenosine deaminase: ○, enzyme control; □, 9-(*m*-bromoacetamidophenethyl)adenine (0.10 m*M*); △, 9-(*m*-bromoacetamidophenethyl)adenine (0.08 m*M*); ●, 9-(*m*-bromoacetamidophenethyl)adenine (0.05 m*M*).

The equation to describe such a process is shown in eq 2, where k_{obsd} equals the observed first-order rate constant for enzyme inactivation, K_i is the dissociation

$$\frac{1}{k_{obsd}} = \frac{K_i}{k_2}\frac{1}{[I]} + \frac{1}{k_2} \qquad (2)$$

constant of the reversible enzyme–inhibitor complex, k_2 is the first-order rate constant for the alkylation of the enzyme by the inhibitor, and [I] is the concentration of the inhibitor. Therefore, if a series of irreversible enzyme inactivations were performed at various inhibitor concentrations, a plot of $1/k_{obsd}$ *vs.* $1/[I]$ would give a straight line with a slope of K_i/k_2 and an intercept of $1/k_2$. Recently Main and co-workers have derived related equations for the irreversible inhibition of esterases.[14]



27 28 29

In Figure 1 are shown some of the results of the irreversible inactivations of adenosine deaminase by several concentrations of **24**. Interestingly, it was found that **24** caused only an 80 ± 3% inactivation of adenosine deaminase, whereas **28** and **29** caused >95%. That the enzyme has been completely alkylated by **24** was shown in the following way. The irreversible inactivation of adenosine deaminase by **24** was performed until no further decrease in rate was observed at which time an additional volume of freshly prepared inhibitor (**24** or **29**) was added. Upon incubation of this solution, no further decrease in the rate of enzymic activity was observed. However, when the enzyme was irreversibly inactivated with **24** until no further decrease in the rate was observed and then a freshly prepared solution of **28** was added, an additional decrease in the rate of enzymic activity was observed. Based on these results we postulate that **24** and **29** alkylate the same amino acid, whereas **28** alkylates a different amino acid on the enzyme. Therefore, when the alkylation of the enzyme by **24** is complete, there

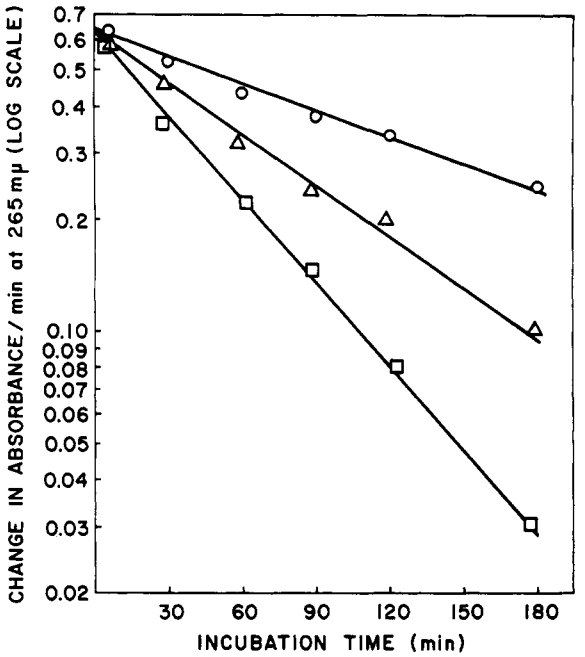


Figure 2.—Half-life periods for the irreversible inactivation of adenosine by 9-(*m*-bromoacetamidophenethyl)adenine: O, 0.015 m*M*; △, 0.015 m*M*; □, 0.050 m*M*.

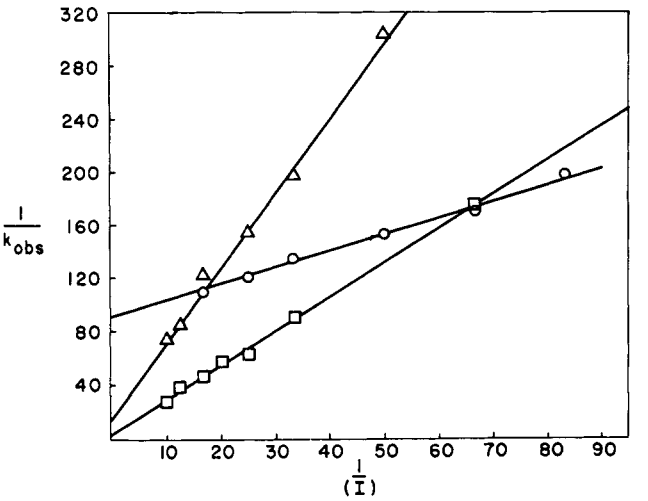


Figure 3.—Plot of $1/k_{obsd}$ for irreversible inactivation of adenosine deaminase *vs.* 1/[I]: △, 9-(*o*-bromoacetamidobenzyl)-adenine; □, 9-(*m*-bromoacetamidophenethyl)adenine; O, 9-(*p*-bromoacetamidobenzyl)adenine.

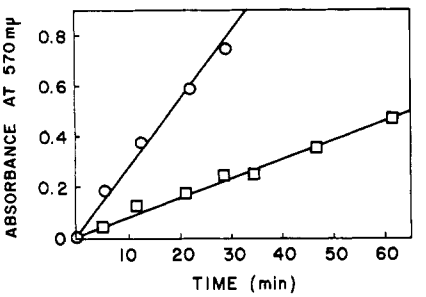


Figure 4.—Comparative chemical reactivity of some alkylating agents with 4-(*p*-nitrobenzyl)pyridine: O, 9-(*m*-bromoacetamidophenethyl)adenine (0.135 m*M*); □, iodoacetamide (0.135 m*M*).

is a residual enzyme activity of 20%.[15] The observation of residual activity in the alkylated enzyme has a bearing on the mechanism of inhibition. One could argue that the formation of a covalent bond between the inhibitor and the enzyme would cause a conformational change in the enzyme with the result that there no longer exists an attaction of the substrate to the active site of the enzyme. However, at the present time, we believe that our results are better rationalized in the following way. In the initial reversible enzyme–inhibitor complex, a covalent bond is formed between the inhibitor and the enzyme. In the case where the enzyme has been alkylated by **28** or **29**, the adenine moiety of the inhibitor is either juxtapositioned over the active site of the enzyme or the adenine moiety still forms a reversible complex with the active site. In either case, for steric reasons the substrate can no longer approach the active site of the enzyme. However, when adenosine deaminase has been irreversibly inactivated by **24**, the additional CH_2 group in the 9 substituent of the inhibitor does not allow the adenine moiety to be ideally juxtapositioned over the active site of the enzyme. Therefore, there remains a residual enzyme activity of 20%.

In order to evaluate the half-life period for the irreversible inactivation of adenosine deaminase by **24**, the velocity of the enzymic reaction at infinite time was subtracted from the observed velocity of the enzymic reactions for the various timed intervals. These data were replotted as shown in Figure 2 and give a reasonably linear relationship from which the half-life period was evaluated. The apparent first-order rate constants (k_{obsd}) for the irreversible inactivation of this enzyme were calculated from the half-life periods, and a complete listing of these data are given in Table III. Finally, a plot of $1/k_{obsd}$ *vs.* 1/[I] for **24** is shown in Figure 3. By regression analysis, it was found that for **24** the slope of the line was 2.54 and the intercept on the $1/k_{obsd}$ axis was 3.52. A *t* test on the intercept was significant at better than 95% level. For comparison, a plot of some related data for **28** and **29** is included on Figure 3. From these data it can be calculated that for **24** the $K_i = 72 \times 10^{-5}$ *M* and $k_2 = 28 \times 10^{-2}$ min^{-1}. We have previously reported that for **28** the $K_i = 43 \times 10^{-5}$ *M* and $k_2 = 7.7 \times 10^{-2}$ min^{-1} and for **29** the $K_i = 1.4 \times 10^{-5}$ *M* and $k_2 = 1.1 \times 10^{-2}$ min^{-1}. It is clear that the alkylation reaction (k_2) between **24** and the enzyme is 25 times faster than in the case of **29** and the enzyme and ap-

TABLE III

KINETIC CONSTANTS FOR THE IRREVERSIBLE INHIBITION OF ADENOSINE DEAMINASE BY 9-(*m*-BROMOACETAMIDOPHENETHYL)ADENINE (**24**)

m*M* concn of **24**	k_{obsd}[a] × 10^3
0.100	35.8
0.080	26.6
0.060	22.0
0.050	17.5
0.040	15.8
0.030	11.1
0.015	5.75

[a] k_{obsd} is in min^{-1}.

(15) Other investigators have observed residual enzyme activity upon total alkylation. See, for example, W. B. Lawson and H. J. Schramm, *Biochemistry*, **2**, 252 (1963), and F. J. Kézdy, J. Feder, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 1009 (1967).

proximately 3.6 times faster than in the case of **28** and the enzyme. Comparative chemical reactivities using 4-(*p*-nitrobenzyl)pyridine as the nucleophilic reagent reveal that **24** is 3.5 times more reactive than is iodoacetamide (Figure 4). It has previously been reported that when 4-(*p*-nitrobenzyl)pyridine is used as the nucleophilic reagent, the chemical reactivities of **29** and **28** relative to iodoacetamide are 3.0 and 5.6, respectively. Therefore, the increased rate of alkylation of the enzyme by **24** cannot be attributed to its increased chemical reactivity. The differences in the rates of irreversible inactivation (k_2) of adenosine deaminase by **24** and **29** may be rationalized by assuming that in the reversible E···I complex, the alkylating group of the inhibitors is juxtapositioned in a different steric orientation on the enzyme.

Irreversible Enzyme Inhibitors. CIX.[1,2] Candidate Irreversible Inhibitors of Dihydrofolic Reductase Derived from 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazine. III[3]

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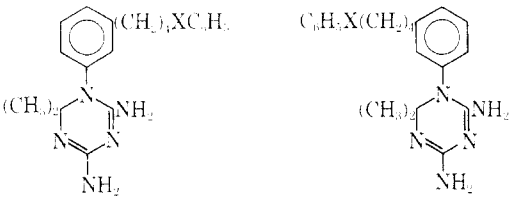
Nineteen derivatives of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazine bridged from its phenyl group to N-phenylbromoacetamide, maleanilic acid, and 1-chloro-2-alkanone were synthesized and evaluated as active-site-directed irreversible inhibitors of dihydrofolic reductase from pigeon liver, Walker 256 rat tumor, and L1210/FR8 mouse leukemia. Although the bromoacetamido group of most of these compounds appeared to be in contact with the enzyme surface, no irreversible inhibition occurred. These negative results were most probably due to the incapability of the juxtaposed enzymic nucleophilic group to react with these halomethyl and α,β-unsaturated carbonyl groups on the inhibitor when the inhibitor was complexed to the enzyme, since similarly positioned sulfonyl fluoride groups have been shown to inactivate the dihydrofolic reductases by the active-site-directed mechanism.

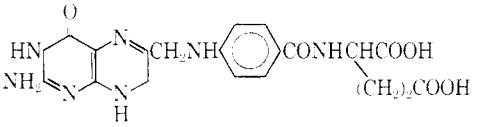
One of the major projects in this laboratory has been the design and synthesis of active-site-directed irreversible inhibitors[5,6] for dihydrofolic reductase;[7] such irreversible inhibitors that operate by the exo mechanism—that is, the inhibitor is reversibly complexed to the active site of the enzyme but covalently binds to the enzyme outside the active site—have considerable potential for species-specific or tissue-specific inhibition.[8]

Previous studies had indicated that the 1-phenyl group of dihydro-*s*-triazines such as **1** was complexed to dihydrofolic reductase by a hydrophobic interaction.[7,9,10] Furthermore, this hydrophobic bonding region[11] was not part of the active site but was just adjacent to the region on the enzyme where the 4 or 8 position of dihydrofolate (**3**), the substrate, resides.[7,12] Compounds of type **1** and **2** were then selected for investigation to determine how far the hydrophobic bonding region extended from the region where the



1a, X = CH_2
2a, X = O

1b, X = CH_2
2b, X = O



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1-phenyl group was complexed. The phenylamyl group of **1** gave a 16-fold increment in binding over the parent 1-phenyl-*s*-triazine;[13] the butyl part on **1** contributed only threefold in binding. The phenoxybutyl group of **2** had complexing ability equal to the phenylamyl group of **1**,[14] indicating that the terminal phenyl group was complexed to a relatively polar region on the enzyme.[7] With compounds of type **1**, the actual complex with the enzyme would have a preference for either conformation **1a** or **1b**, but not likely both.

When all of these studies are combined, then logical candidates for exo-type active-site-directed irreversible

(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, P. C. Huang, and A. L. Pogolatti, Jr., *J. Med. Chem.*, **10**, 1134 (1967).

(3) For the previous papers on candidate irreversible inhibitors derived from 1-phenyl-*s*-triazines see (a) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of the complete series; (b) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **56**, 28 (1967), paper LXX of this series.

(4) G. J. L. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship.

(5) 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.

(6) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

(7) For a review see ref 5, Chapter X.

(8) For a discussion of the evolutionary changes in enzymes outside the active site and utilization of such changes for chemotherapy, see ref 5, Chapter IX.

(9) For a discussion of hydrocarbon interactions with enzymes consisting primarily of hydrophobic bonding and van der Waals forces, see ref 5, Chapter II.

(10) B. R. Baker and B.-T. Ho, *J. Heterocyclic Chem.*, **2**, 335 (1965).

(11) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965).

(12) (a) B. R. Baker, T. S. Schwan, J. Novotny, and B.-T. Ho, *ibid.*, **55**, 295 (1966); (b) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 308 (1966).

(13) B. R. Baker, B.-T. Ho, and G. J. Lourens, *ibid.*, **56**, 737 (1967), paper LXXXVI of this series.

(14) B. R. Baker and G. J. Lourens, *ibid.*, **56**, 871 (1967), paper LXXXVII of this series.