# Enzyme Inhibitors VII

## Syntheses of *trans*-4-(6-Substituted-9-purinyl)cyclohexylcarbinols and Evaluation as Inhibitors of Adenosine Deaminase

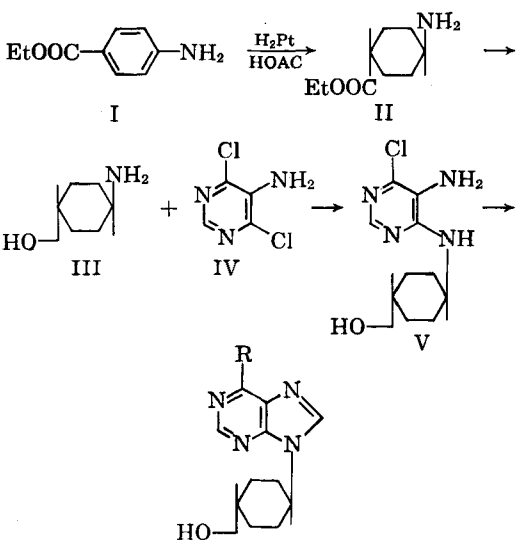
By HOWARD J. SCHAEFFER and EUGENE ODIN

A series of *trans*-4-(6-substituted-9-purinyl)cyclohexylcarbinols were prepared by the following procedure. Ethyl *p*-aminobenzoate gave, on catalytic hydrogenation, *trans*-4-ethoxycarbonylcyclohexylamine which, on reduction with sodium and ethanol, generated *trans*-4-hydroxymethylcyclohexylamine. Condensation of this amine with 5-amino-4,6-dichloropyrimidine, followed by ring closure of the resultant substituted pyrimidine, gave *trans*-4-(6-chloro-9-purinyl)cyclohexylcarbinol (VI). Displacement of the 6-chloro group by a number of nucleophilic reagents gave a variety of nucleoside analogs. Enzymatic evaluation of these analogs established that those compounds with a 6-amino and a 6-methylamino group inhibited adenosine deaminase and that the hydroxymethyl group did not make a significant contribution to binding to the enzyme.

MANY ENZYMES are known which utilize purine nucleosides or purine nucleotides as their normal substrates (1, 2); but relatively little is known about which atoms or functional groups of the substrates are important for binding to the enzyme. The authors have been interested in this problem and have been studying the inhibition of adenosine deaminase by a variety of nucleoside analogs. From these studies (3, 4) it has been found that (*a*) a compound with a 6-amino group on the purine nucleus is a more effective inhibitor than one with a 6-methylamino group, which in turn is a more effective inhibitor than a compound with a 6-dimethylamino group, (*b*) the purine nucleus must bear a 9-substituent for effective inhibition, (*c*) a hydroxyl group on the 9-substituent can increase the binding to the enzyme if the hydroxyl group is located in the proper position, and (*d*) there is a relatively tight fit by the enzyme around the periphery of the ribofuranosyl portion of the substrate. In a previous study, the syntheses and enzymatic evaluation of some *cis*-(6-substituted-9-purinyl)-cycloalkylcarbinols were reported (5). This paper describes the preparation and evaluation of some *trans*-4-(6-substituted-9-purinyl)cyclohexylcarbinols as inhibitors of adenosine deaminase.

### DISCUSSION

**Chemistry.**—The reaction sequence which was used for the synthesis of this series of compounds (Scheme I) is a modification of the method employed previously. This method involves the condensation of an appropriately substituted amine with 5-amino-4,6-dichloropyrimidine to give a 5-amino-4-substituted amino-6-chloropyrimidine, which on ring closure will form a 6-chloro-9-substituted purine.




Scheme I

The 6-chloro group of this purine is readily displaceable by nucleophilic reagents; thereby a variety of 6-substituted analogs can be synthesized. As the first step in this synthetic scheme, it was necessary to prepare *trans*-4-hydroxymethylcyclohexylamine. It was shown previously that when *p*-aminobenzoic acid was hydrogenated with a platinum catalyst in aqueous solution, the principal product of reaction was *cis*-4-carboxycyclohexylamine. However, when ethyl *p*-aminobenzoate (I) was hydrogenated with a platinum catalyst in glacial acetic acid, the principal product of reaction was *trans*-4-ethoxycarbonyl-

Received September 14, 1964, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo.

Accepted for publication November 2, 1964.

This investigation was supported by research grant CA-06388-03 and research career program award 5-K3-CA-18718-03 from the National Cancer Institute, and by training grant 5-T1-GM-555 from the Division of Medical Sciences, U. S. Public Health Service, Bethesda, Md., and by grant T-337 from the American Cancer Society.

The authors express their appreciation to Mrs. B. Hoppe for the measurement of the enzymatic reactions.

cyclohexylamine (II).[1] When II was reduced with sodium and ethanol, a moderate yield of the desired *trans*-4-hydroxymethylcyclohexylamine (III) was obtained. Condensation of III with 5-amino-4,6-dichloropyrimidine (IV) proceeded smoothly, and a 77% yield of V was obtained. Treatment of V with triethyl orthoformate and hydrochloric acid (6) resulted in a facile ring closure to give *trans*-4-(6-chloro-9-purinyl)cyclohexylcarbinol (VI). When VI was allowed to react with ammonia, methylamine, dimethylamine, aqueous hydrochloric acid, thiourea, and sodium methoxide, the corresponding 6-substituted analogs (VII–XII) were produced in good yields. The details of these reactions are given under *Experimental*.

## EXPERIMENTAL[2]

**Ethyl trans-4-Aminocyclohexanecarboxylate (II).**—A mixture of 49.6 Gm. (300 mmoles) of I and 1.0 Gm. of platinum oxide in 200 ml. of glacial acetic acid was hydrogenated in a Parr hydrogenator at an initial pressure of 60.0 p.s.i. and at room temperature. After 68 hr., the theoretical amount of hydrogen was consumed, and the catalyst was removed by filtration through a Celite pad. The solvent was removed *in vacuo*, and the residual oil was dissolved in 150 ml. of chloroform and washed with concentrated ammonium hydroxide (4 × 20 ml.). The chloroform solution was dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. The crude product on distillation gave 36.4 Gm. (70.8%) of the pure material (II), b.p. 56–58°/0.08 mm. $\bar{\nu}$ in cm.$^{-1}$ (film): 3400 ($NH_2$); 1730 (C=O). For analysis, the compound was converted to its hydrochloride salt, m.p. 153°.

*Anal.*[3]—Calcd. for $C_9H_{18}ClNO$: C, 52.05; H, 8.73; Cl, 17.07; N, 6.74. Found: C, 52.08; H, 8.71; Cl, 17.13; N, 6.81.

**trans-4-Aminocyclohexylcarbinol (III).**—To a stirred cold solution of 36.3 Gm. (212 mmoles) of II in 480 ml. of absolute ethanol, was added gradually 37.6 Gm. of metallic sodium in small pieces over a period of 1.25 hr. The mixture was heated under reflux for 4 hr., then allowed to remain at room temperature overnight. After the mixture was diluted with 300 ml. of distilled water and the alcohol was evaporated *in vacuo*, a precipitate formed in the aqueous solution at room temperature, which was collected by filtration after cooling the mixture in an ice bath. The solid residue was recrystallized from benzene and gave the analytical sample (III); yield, 9.03 Gm. (32.9%), m.p. 145°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3370 (OH); 3100 (NH).

*Anal.*—Calcd. for $C_7H_{15}NO$: C, 65.07; H, 11.70; N, 10.84. Found: C, 64.85; H, 11.56; N, 10.62.

The hydrochloride salt was prepared in the usual way and after recrystallization from a mixture of ethanol and ether had a melting point of 178°.

[1] The assignment of the *trans*-configuration is based on the fact that this compound and all of the compounds prepared from it are different from the corresponding compounds prepared from the *cis*-4-ethoxycarbonylcyclohexylamine which was synthesized by Patel, R. K., and Gisvold, O., This Journal, **42**, 321(1953).

[2] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra and enzyme rates were determined on a Perkin-Elmer 4000A spectrophotometer. The melting points were determined on a Kofler Heizbank and are corrected.

[3] The analyses were performed by Galbraith Microanalytical Laboratories, Inc., Knoxville, Tenn.

*Anal.*—Calcd. for $C_7H_{16}ClNO$: C, 50.75; H, 9.74; Cl, 21.40. Found: C, 50.93; H, 9.90; Cl, 21.24.

**trans-4-(5-Amino-6-chloro-4-pyrimidinylamino)cyclohexylcarbinol (V).**—A solution of 4.00 Gm. (31.0 mmoles) of III, 5.08 Gm. (31.0 mmoles) of 5-amino-4,6-dichloropyrimidine (IV), and 3.13 Gm. (31.0 mmoles) of triethylamine in 100 ml. of butanol-1 was heated under reflux for 42 hr., then the volatile materials were removed *in vacuo*. One recrystallization of the crude product from ethanol and water gave the analytical sample (V); yield, 6.16 Gm. (77.4%), m.p. 206°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3400 (OH); 3310 ($NH_2$). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$): pH 1, 303 (14.1); pH 7, 291 (10.6), 263 (10.8); pH 13, 291 (11.0), 263 (11.2).

*Anal.*—Calcd. for $C_{11}H_{17}ClN_4O$: C, 51.46; H, 6.67; Cl, 13.81; N, 21.82. Found: C, 51.45; H, 6.77; Cl, 13.95; N, 21.59.

**trans-4-(6-Chloro-9-purinyl)cyclohexylcarbinol (VI).**—To a stirred solution of 2.24 Gm. (8.71 mmoles) of V in 35 ml. of triethyl orthoformate at room temperature was added 0.50 ml. (6.00 mmoles) of concentrated hydrogen chloride, over a 10-min. period. After 1.25 hr., the volatile materials were removed *in vacuo*, and the oily residue was dissolved in a 20% solution of ammonia in methanol and allowed to stand overnight at 0°. The solvent was evaporated *in vacuo*, and recrystallization of the crude material from water gave the analytical sample (VI); yield, 1.05 Gm. (45.1%), m.p. 164°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3380 (OH); 1590, 1550 (C=C and C=N). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$) pH 1, 267 (10.0); pH 7, 267 (10.0); pH 13, 267 (10.1).

*Anal.*—Calcd. for $C_{12}H_{15}ClN_4O$: C, 54.03; H, 5.67; Cl, 13.29; N, 21.01. Found: C, 53.81; H, 5.70; Cl, 13.16; N, 21.26.

**trans-4-(6-Amino-9-purinyl)cyclohexylcarbinol (VII).**—A solution of 82.5 mg. (0.308 mmole) of VI in 13 ml. of methanolic ammonia was heated in a stainless steel bomb at 60° for 16 hr. The solvent was evaporated *in vacuo*, and the residual solid was recrystallized from water; yield, 54 mg. (66%), m.p. 210°. Two recrystallizations of the crude product from water gave the analytical sample (VII), m.p. 230°; yield, 24.8 mg (33.5%). $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3350, 3160 (OH and $NH_2$); 1660 ($NH_2$); 1590, 1570 shoulder (C=C and C=N). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$): pH 1, 260 (17.7); pH 7, 261 (18.1); pH 13, 261 (18.0).

*Anal.*—Calcd. for $C_{12}H_{17}N_5O$: C, 58.27; H, 6.93; N, 28.32. Found: C, 58.41; H, 7.11.; N, 28.11.

**trans-4-(6-Methylamino-9-purinyl)cyclohexylcarbinol (VIII).**—A solution of 204 mg. (0.760 mmole) of VI in 2.5 ml. of ethanol and 12.5 ml. of 40% methylamine in water was heated in a stainless steel bomb at 83° for 48 hr. The volatile materials were removed *in vacuo*, and the solid residue was recrystallized from ethyl acetate to give the analytical sample (VIII); yield, 100 mg. (51.0%), m.p. 170°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3300 (OH and NH); 1620 and 1570 (C=C and C=N). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$): pH 1, 265 (19.0); pH 7, 269 (18.3); pH 13, 269 (18.1).

*Anal.*—Calcd. for $C_{13}H_{19}N_5O$: C, 59.75; H, 7.33; N, 26.80. Found: C, 59.52; H, 7.28; N, 26.52.

**trans - 4 - (6 - Dimethylamino - 9 - purinyl)cyclohexylcarbinol (IX).**—A solution of 201 mg. (0.75 mmole) of VI in 2.5 ml. of ethanol and 10 ml. of aqueous dimethylamine (25%) was heated in a stainless steel bomb at 85° for 41 hr. The volatile materials were removed *in vacuo*, and the residual solid was recrystallized from benzene to give the analytical sample (IX); yield, 159 mg. (77.2%), m.p. 175°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3250 (OH); 1590 and 1560 (sh) (C=C and C=N). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$): pH 1, 270 (20.1); pH 7, 277 (20.6); pH 13, 277 (20.7).

*Anal.*—Calcd. for $C_{14}H_{21}N_5O$: C, 61.06; H, 7.69; N, 25.43. Found: C, 60.94; H, 7.80; N, 25.62.

**trans - 4 - (6 - Hydroxy - 9 - purinyl)cyclohexylcarbinol (X).**—A solution of 297 mg. (1.11 mmoles) of VI in 7.5 ml. of 1 *N* hydrochloric acid was heated under reflux for 20 min., then evaporated *in vacuo* to dryness. A solution of the residual solid in 5% sodium hydroxide was decolorized with charcoal, filtered through a Celite pad, and precipitated in an ice bath with 5% hydrochloric acid and gave the analytical sample (X); yield, 140 mg. (50.9%), m.p. 345–350° dec. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3450 (OH); 1680 (C=O enol); 1590, 1540 (C=C and C=N). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$): pH 1, 250 (13.0); pH 7, 250 (13.2); pH 13, 255 (14.9).

*Anal.*—Calcd. for $C_{12}H_{16}N_4O_2$: C, 58.05; H, 6.50; N, 22.57. Found: C, 58.21; H, 6.42; N, 22.29.

**trans - 4 - (6 - Mercapto - 9 - purinyl)cyclohexylcarbinol (XI).**—A solution of 513 mg. (1.92 mmoles) of VI and 146 mg. (1.92 mmoles) of thiourea in 10 ml. of propanol-1 was heated under reflux for 30 min., and then cooled in an ice bath. The solid which precipitated was collected by filtration. Two recrystallizations of the crude material from methanol gave the analytical sample (XI); yield, 252 mg. (49.8%), m.p. 322–325° dec. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3430 (OH); 2800–2000 (acidic hydrogen); 1590 and 1525 (C=C and C=N). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$): pH 1, 323 (20.8); pH 7, 322 (23.1); pH 13, 312 (20.7).

*Anal.*—Calcd. for $C_{12}H_{16}N_4OS$: C, 54.52; H, 6.10; N, 21.20; S, 12.13. Found: C, 54.20; H, 6.25; N, 20.88; S, 11.85.

**trans - 4 - (6 - Methoxy - 9 - purinyl)cyclohexylcarbinol (XII).**—To a solution of 296 mg. (1.11 mmoles) of VI in 15 ml. of absolute methanol was added 162 mg. (3.0 mmoles) of sodium methoxide, and the mixture was heated under reflux for 1.5 hr. The mixture then was cooled in an ice bath, acidified to pH 6 with 6 *N* hydrochloric acid, and the solvent evaporated *in vacuo*. One recrystallization of the solid residue from water gave the pure sample (XII); yield, 212 mg. (72.9%), m.p. 149°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3340 (OH); 1585 and 1560 (C=C and C=N). $\lambda_{max.}$ in m$\mu$ ($\epsilon \times 10^{-3}$): pH 1, 253 (11.9); pH 7, 253 (12.2); pH 13, 253 (12.5).

*Anal.*—Calcd. for $C_{13}H_{18}N_4O_2$: C, 59.52; H, 6.92; N, 21.36. Found: C, 59.26; H, 6.72; N, 21.59.

**Reagents and Assay Procedure.**—Adenosine and adenosine deaminase were purchased from the Sigma Chemical Co. The general method of assay has been described previously (7). The enzymatic reactions were performed in 0.05 *M* phosphate buffer at pH 7.6 at 25°. The substrate and all inhibitors also were prepared in 0.05 *M* phosphate buffer at pH 7.6. For the assay, the cell contained a total volume of 3.1 ml., which was 0.066 m*M* with respect to adenosine. The ratio of the millimolar concentration of the inhibitor to the millimolar concentration of the substrate for 50% inhibition, [*I*/*S*] 0.5, was used to compare the inhibitory properties of the various compounds. To determine the concentration of inhibitor required for 50% inhibition, a plot of $V_0/V$ *versus* $I$ was made, where $V_0$ = initial velocity of uninhibited enzymatic reactions, $V$ = initial velocity of the inhibited enzymatic reaction at various inhibitor concentrations, and $I$ = the various concentrations of inhibitor (8).

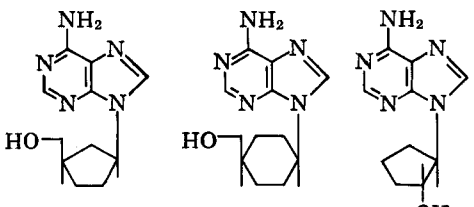
TABLE I.—PARTIAL INHIBITION AND 50% INHIBITION OF ADENOSINE DEAMINASE BY CERTAIN NUCLEOSIDE ANALOGS

| Compd.[a] | % Inhibition by 0.12 m*M* Concn. of Inhibitor | [*I*/*S*] 0.5 |
|---|---|---|
| VII | 21 | 6.8 ± 0.1 |
| VIII | 14 | 8.6 ± 0.2 |
| XIII | 21 | 6.6 ± 0.2 |
| XIV | 17 | 7.5 ± 0.7 |
| XV | 36 | 3.6 ± 0.2 |

[a] The concentration of adenosine in all experiments was 0.066 m*M*.

## RESULTS

Enzymatic evaluation of the nucleoside analogs (VI–XII) revealed that the 6-amino and the 6-methylamino derivatives (VII and VIII) were inhibitory, whereas the remaining compounds were inactive against adenosine deaminase when tested at concentrations two to three times that of the substrate. The percentage inhibition of the enzymatic reaction by a 0.12 m*M* solution of the inhibitor is given in Table I as well as the extrapolated [*I*/*S*] 0.5. For purposes of comparison, similar data also are given for the three related compounds, *cis*-3-(6-amino-9-purinyl)cyclopentylcarbinol (XIII), *cis*-4-(6-amino-9-purinyl)cyclohexylcarbinol (XIV), and *trans*-2-(6-amino-9-purinyl)cyclopentanol (XV).




XIII XIV XV

## DISCUSSION

Previous studies have shown that the substituent at the 6-position of the purine nucleus is critical for inhibition of adenosine deaminase and that in general the effectiveness of inhibition decreases in the following order: 6-amino > 6-methylamino > 6-dimethylamino (4). In the present study, a similar order of effectiveness of inhibition was

observed since VII > VIII > IX. However, the main interest in the synthesis of this series of compounds was to determine if the *trans*-hydroxymethyl group of the cyclohexyl nucleus could bridge to a binding point which could not be reached by the corresponding *cis*-derivative. An examination of Table I reveals that VII is approximately as effective an inhibitor as XIII or XIV, compounds whose hydroxymethyl groups are *cis*. Since it has been established previously that the hydroxymethyl groups of XIII and XIV contribute little to binding to the enzyme (5), the *trans* hydroxymethyl group of VII does not make a significant contribution to the binding of this compound to the enzyme.

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# Rapid Method for the Determination of Mixtures of *p*-Hydroxybenzoate Esters by Gas Chromatography

By J. L. LACH and J. S. SAWARDEKER

**A procedure for the separation and quantitative determination of mixtures of the *p*-hydroxybenzoate esters is described employing vapor phase chromatography.**

THE ALKYL *p*-hydroxybenzoate esters more commonly known as the parabens constitute one of the most important group of pharmaceutical preservatives. The role of parabens as preservatives has been reviewed in the literature from time to time by Aalto *et al.* (1), Barkeley (2), Neidig and Burrell (3), Sokol (4), and Gottfried (5). However, the literature cites only a few general methods for the detection and the estimation of the parabens (6–8). Furthermore, none of these methods are useful for the determination of exact quantities of individual parabens in the presence of other parabens when used in combinations. Even the U.S.P. method fails to permit such an analysis. The analysis of the parabens is complicated by the fact that the total paraben concentrations employed as preservatives seldom exceed 0.2% of the formulation. The most promising assay for these parabens has been developed by Higuchi and co-workers (9). Their procedure is based on the preliminary extraction of these parabens from the formulation, separation of the component esters by partition chromatography, and their subsequent determination by ultraviolet spectrophotometry.

The increasingly important role of the parabens as preservatives emphasized the need for a rapid method of their determination. This report deals with the qualitative and quantitative determination of the parabens by gas chromatography. Although these parabens were gas chromatographed directly, the separation of methyl paraben from the ethyl paraben offered considerable difficulty even though the propyl and the butyl parabens were well resolved. We have, therefore, converted the hydroxyl groups on the *p*-hydroxybenzoates to the corresponding ethers and gas chromatographed the trimethylsilyl derivatives. The method has the advantage of speed and accuracy and is applicable over a wide range of concentrations.

Received August 6, 1964, from the College of Pharmacy, State University of Iowa, Iowa City.
Accepted for publication November 4, 1964.

## EXPERIMENTAL

**Apparatus and Materials.**—A F & M model 500 linear programmed high-temperature chromatograph with model 1609 flame ionization attachment, equipped with Minneapolis Honeywell Y143 recorder and model 201 Disc Integrator was used.

A 2-ft. copper tube packed with Diatoport S (diatomaceous earth specially treated and silanized, offered by F & M Scientific Co.) and coated with 2% SE-30 and a 4-ft. copper tube packed with the same support and coated with 10% butanediol-succinate was used.

Hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories,