Inhibition of Adenosine Deaminase by Alcohols Derived from Adenine Nucleosides†

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ABSTRACT: A novel series of inhibitors of adenosine deaminase from calf intestine have been synthesized. These compounds were obtained by oxidative cleavage of the sugar ring of pyranosyl nucleosides with sodium metaperiodate followed by reduction of the dialdehydes to the alcohols with sodium borohydride. In this manner, alcohols derived from 9-α-D-mannopyranosyladenine (**2**), 9-β-D-xylopyranosyladenine (**3**), 9-α-D-arabinopyranosyladenine (**4**), 9-α-L-rhamnopyranosyladenine (**5**), 9-β-D-fucopyranosyladenine (**6**), and 9-β-L-fucopyranosyladenine (**7**) were prepared. In addition, the alcohol derived from adenosine (**1**) was also studied. None of the alcohols were substrates for adenosine deaminase. With the exception of **2**, all of these compounds were competitive inhibitors. The best inhibitor was **5** with a K_i of 4.4×10^{-6} M. The data indicated a preference for the R configuration at the carbon atom which was originally the anomeric carbon of the nucleoside. The compounds appeared to be binding to a specific hydroxyl binding site and in the cases of **5**, **6**, and **7**, to be binding to a methyl binding site on the enzyme. In this region of the molecule the S configuration was preferred. However, a methyl group was not a distinct requirement, because **3** was almost as good an inhibitor as **5**. Reasons as to why these compounds are inhibitors of adenosine deaminase rather than substrates are discussed.

Nucleoside antibiotics have been isolated and analogs of nucleosides have been prepared which have potential uses as antitumor and antimicrobial agents. A number of these compounds are nucleosides having adenine or cytosine as the base. For example, 9-β-D-arabinofuranosyladenine is converted to a triphosphate which acts as a competitive inhibitor of DNA polymerase (Furth and Cohen, 1967). This nucleoside is a substrate for adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) (Cory and Suhadolnik, 1965; Frederiksen, 1966) and its biological activity is minimized by deamination to the hypoxanthine analog (Brink and LePage, 1964). This type of inactivation of potentially useful drugs has inspired the synthesis of nucleoside analogs which would either resist deamination or would themselves act as inhibitors of the deaminase. As a result, an organized study of the structural requirements for adenosine deaminase to bind to a substrate or an inhibitor came into being (Cory and Suhadolnik, 1965; Frederiksen, 1966; Bloch *et al.*, 1967; Wolfenden *et al.*, 1969; Simon *et al.*, 1970) and due to its commercial availability, many of these studies have been performed with the enzyme obtained from calf intestinal mucosa.

A study of the mode of binding of the ribofuranose ring to adenosine deaminase has received special attention from Schaeffer and his coworkers, who have studied the inhibitory properties of various 9-hydroxyalkylpurines (Schaeffer and Bhargava, 1965; Schaeffer *et al.*, 1965; Schaeffer and Vince, 1967). With the structures of some of the better inhibitors in mind, we set out to prepare and study the properties of a number of alcohols derived from adenine nucleosides.

Experimental Section

General Materials and Methods. Melting points were obtained as corrected values on a Kofler micro hot stage. Elementary analyses were performed by the Baron Consulting Co., Orange, Conn., or by the Spang Microanalytical Laboratory, Ann Arbor, Mich. Ultraviolet spectra were recorded on a Beckman DK-2 spectrophotometer and optical rotations were determined in 100-mm semimicro tubes using a Rudolph polarimeter. Paper chromatography was performed by a descending technique on Whatman No. 1 paper. Mobilities are recorded as R_{Ad} values, which correspond to the ratio of the distance that the alcohol moved to that which adenine moved. Spots were located with an ultraviolet lamp (254 nm). The solvent systems used were (A) 5% aqueous disodium hydrogen phosphate (B) 1-butanol–water (86:14, v/v). All evaporations were carried out under reduced pressure on a rotary evaporator at a bath temperature of 40–45°.

Nucleosides. Adenosine (General Biochemicals) was recrystallized three times from water until chromatographically pure. 9-β-D-Xylopyranosyladenine was synthesized as previously reported (Lerner, 1969) and 9-β-D-fucopyranosyladenine was supplied from a previous preparation (Lerner, 1971). 9-α-L-Rhamnopyranosyladenine was prepared according to the directions of Baker and Hewson (1957) and the trialcohol, 2-*O*-[1(*R*)-(9-adenyl)-2-(hydroxy)ethyl]glycerol (**1**), which was derived from adenosine, was supplied from a previous preparation (Lerner, 1970).

9-α-D-Mannopyranosyladenine. The procedure used to prepare this compound was as described previously (Lerner and Kohn, 1964). When crystallized from concentrated aqueous solutions, needlelike crystals were obtained which appeared to be the hemihydrate, mp 240.5–241.5°. *Anal.* Calcd for $C_{11}H_{15}N_5O_5 \cdot 0.5H_2O$: C, 43.13; H, 5.23; N, 22.87. Found: C, 43.36; H, 5.02; N, 23.20. Recrystallization of this nucleoside from dilute solutions gave the familiar monohydrate plates (mp 146–148°) which were used in the preparation of **2**.

9-α-D-Arabinopyranosyladenine. Tri-*O*-benzoyl-β-D-arabinopyranosyl bromide (30.4 g) (Fletcher and Hudson, 1950) was reacted with 27.4 g of 6-benzamidochloromercuripurine by the procedure of Davoll and Lowy (1951). After removal of the blocking groups with methanolic sodium methoxide, the product was crystallized from water: 9.5 g (58%), mp

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167–169°, $[\alpha]_D^{24}$ −35.5° (*c* 2.79, H_2O). Martinez *et al.* (1969) reported mp 167–168° and $[\alpha]_D$ −34° for a sample prepared by a somewhat different route.

9-β-L-Fucopyranosyladenine. Tetra-*O*-acetyl-α-L-fucopyranose was prepared in 72% yield under the acid-catalyzed conditions described for the D form (Lerner, 1971): mp 91–93°, $[\alpha]_D^{26}$ −119° (*c* 0.32, $CHCl_3$) (lit. mp (Leaback *et al.*, 1969) 93°, $[\alpha]_D^{20}$ −116° (*c* 1, $CHCl_3$)). This acetate (14.5 g, 43.4 mmoles) was reacted with 6-benzamidochloromercuripurine (25.9 g, 54.5 mmoles) in a mixture containing 6.2 ml of titanium tetrachloride, 25.9 g of Celite-545, and 1.2 l. of 1,2-dichloroethane. A yellow foam (20.7 g) was obtained which was converted to the picrate (17.4 g, 63%) in refluxing ethanolic picric acid. A small sample was recrystallized from ethanol–ethyl acetate (mp 220–221° dec). *Anal.* Calcd for $C_{23}H_{24}N_8O_{14}$: C, 43.40; H, 3.80; N, 17.61. Found: C, 43.42; H, 3.95; N, 17.47.

The picrate ion was removed with Bio-Rad Ag2-X8 (CO_3^{-2}) resin in 1.4 l. of 80% aqueous acetone. 9-(2,3,4-Tri-*O*-acetyl-β-L-fucopyranosyl)adenine was crystallized from ethyl acetate in two crops, 11.8 g (87%). Recrystallization afforded an analytical sample containing 1 mole of ethyl acetate as solvate: mp 238–239°, $[\alpha]_D^{26}$ +5.9° (*c* 1.5, acetone). *Anal.* Calcd for $C_{17}H_{21}N_5O_7 \cdot C_4H_8O_2$: C, 50.90; H, 5.90; N, 14.14. Found: C, 51.18; H, 6.09; N, 14.16.

The blocked nucleoside (8.4 g) was treated at reflux with 280 ml of 0.1 N methanolic sodium methoxide. 9-β-L-Fucopyranosyladenine (5.34 g, 95%) was crystallized from methanol (5–7 ml). After recrystallization the product effervesced at 110° and melted at 171–172° to a viscous liquid: $[\alpha]_D^{25}$ −11.1° (*c* 2.33, H_2O), $\lambda_{max}^{H_2O}$ 258 nm (ε 15,000). *Anal.* Calcd for $C_{11}H_{15}N_5O_4 \cdot 0.5CH_3OH$: C, 46.45; H, 5.77; N, 23.56. Found: C, 45.95; H, 5.77; N, 23.51.

2-O-[1(S)-(9-Adenyl)-2-(hydroxy)ethyl]glycerol (**2**). To a stirred suspension of 9-α-D-mannopyranosyladenine (4.46 g, 15 mmoles) in 65 ml of water was added sodium metaperiodate (6.63 g, 30 mmoles) in small portions while the temperature was maintained near 20° with an ice bath. The resulting solution was stored in the dark overnight and then poured into 700 ml of ethanol. The mixture was stirred for 0.5 hr, filtered, and the filtrate was evaporated to a syrup which was dissolved in 400 ml of water. This solution was added, dropwise, to a solution of sodium borohydride (2.88 g) in 50 ml of water and stored overnight. The basic solution was brought to neutrality with Bio-Rad AG50-X8 (H^+) resin and the water was evaporated. Absolute methanol was evaporated several times from the residue to remove boric acid as methyl borate. The syrup was dissolved in 50 ml of methanol, 150 ml of 10% methanolic picric acid was added, and the flask was chilled in ice for 1 hr. The dried material weighed 1.6 g. Recrystallization from methanol gave crystals which melted slowly above 180° with slow decomposition: $[\alpha]_D^{23}$ −48.3° (*c* 1.79, dimethylformamide). *Anal.* Calcd for $C_{16}H_{18}N_8O_{11}$: C, 38.54; H, 3.64; N, 22.50. Found: C, 38.42; H, 3.73; N, 22.27.

The picrate (1.15 g) was suspended in water (700 ml) and treated with Bio-Rad AG2-X8 (CO_3^{-2}) resin. Evaporation of the water and lyophilization yielded a white hygroscopic powder which required further drying under high vacuum in a drying pistol over P_2O_5 at 65°: $[\alpha]_D^{25}$ −62.6° (*c* 2.57, H_2O), uv $\lambda_{max}^{H_2O}$ 259 nm (ε 14,500), R_{Ad} 1.81 (A) and 0.61 (B). *Anal.* Calcd for $C_{10}H_{15}N_5O_4$: C, 44.61; H, 5.62; N, 26.01. Found: C, 44.88; H, 5.65; N, 25.72.

Tri-O-benzoate of **2**. A solution containing 0.3 g of **2** in pyridine was treated with benzoyl chloride as described previously for the *R* enantiomer **1** (Lerner, 1970). The picrate was prepared in refluxing ethanol and recrystallized from the same solvent to afford 0.13 g, mp 164–166°. *Anal.* Calcd for $C_{37}H_{30}N_8O_{14}$: C, 64.01; H, 4.68; N, 12.03. Found: C, 63.68; N, 4.71; N, 11.79.

The picrate ion was removed with a resin as described above and the tribenzoate was crystallized from methanol in a 70% yield, mp 144–145°. *Anal.* Calcd for $C_{31}H_{27}N_5O_7$: C, 64.01; H, 4.68; N, 12.03. Found: C, 63.68; H, 4.71; N, 11.79.

2-O-[1(R)-(9-Adenyl)-2-(hydroxy)ethyl]ethanediol (**3**). To a suspension of 5.34 g (0.02 mole) of 9-β-D-xylopyranosyladenine in 160 ml of water was added 8.56 g (0.04 mole) of sodium metaperiodate in small portions over a period of 5 min while the temperature was maintained near 20° with an ice bath. The mixture was stirred for 12 hr, protected from light, at which time complete solution finally occurred and the flask was stored for another 24 hr. The volume was reduced to *ca.* 75 ml by evaporation, the solution was poured into 350 ml of ethanol, and the mixture was stirred vigorously for 1.25 hr. After filtration, the solvents were removed by evaporation and the residue, which was not very soluble in water, was dissolved in warm 50% aqueous methanol and cooled. A solution containing 11 g of sodium borohydride in 150 ml of water was added dropwise, the mixture was stored at room temperature for 18 hr, and neutralized with Bio-Rad AG50-X8 (H^+) resin. After removal of the resin and solvents, a picrate was prepared by addition of 100 ml of 10% methanolic picric acid to a solution of the dialcohol in 200 ml of methanol. Crystallization was aided by chilling of the flask in an ice bath for 2 hr; yield 7.59 g (81%). Recrystallization was accomplished by dissolving the picrate in 1500 ml of boiling methanol followed by concentration of the boiling solution to *ca.* 450 ml; 6.58 g. This material started to sublime between 160 and 170°, forming tiny needles on the cover slip which began to decompose above 245°, $[\alpha]_D^{26}$ +24.8° (*c* 2.34, dimethylformamide). It also slowly decomposes when exposed to light. *Anal.* Calcd for $C_{15}H_{16}N_8O_{10}$: C, 38.46; H, 3.44; N, 23.93. Found: C, 38.66; H, 3.49; N, 23.60.

The picrate (5.94 g) was suspended in 1 l. of water and treated with an anion-exchange resin as described for the preparation of **2**. The lyophilized powder was dried further in a drying pistol to afford 2.12 g of an extremely hygroscopic material: $[\alpha]_D^{23}$ +34.5° (*c* 3.05, H_2O), R_{Ad} 1.75 (A) and 0.91 (B). *Anal.* Calcd for $C_9H_{13}N_5O_3$: C, 45.18; H, 5.47; N, 29.28. Found: C, 44.93; H, 5.50; N, 29.51.

Di-O-benzoate of **3**. Benzoylation of **3** (206 mg) was carried out in the same manner as for **2** and a picrate (203 mg) was isolated. This was not characterized, but was converted to the dibenzoate which was crystallized from ethanol (mp 124–126.5°) to a viscous syrup which solidified, turned into needles and melted again at 142–143°. *Anal.* Calcd for $C_{23}H_{21}N_5O_5$: C, 61.74; H, 4.73; N, 15.65. Found: C, 61.98; H, 4.90; N, 15.46.

2-O-[1(S)-(9-Adenyl)-2-(hydroxy)ethyl]ethanediol (**4**). 9-α-D-Arabinopyranosyladenine (4.0 g, 14 mmoles) was dissolved in 90 ml of water, sodium metaperiodate (6.6 g, 31 mmoles) was added, and the subsequent steps were carried out as described for the preparation of **2**. A picrate was prepared (7.4 g, 99%) which was recrystallized from methanol and exhibited the same melting behavior as the *R* enantiomer **3**, $[\alpha]_D^{23}$ −27.5° (*c* 1.6, dimethylformamide). *Anal.* Calcd for $C_{15}H_{16}N_8O_{10}$: C, 38.46; H, 3.44; N, 23.93. Found: C, 38.28; H, 3.59; N, 23.95.

The picrate moiety was removed as described above to give **4** as a lyophilized, dried powder: $[\alpha]_D^{23}$ −36.8° (*c* 2.67, H_2O), uv $\lambda_{max}^{H_2O}$ 258 nm (ε 14,800), R_{Ad} 1.81 (A) and 0.93 (B). *Anal.*

Calcd for $C_9H_{13}N_5O_3$: C, 45.18; H, 5.47; N, 29.28. Found: C, 44.91; H, 5.58; N, 29.01.

Di-O-benzoate of **4**. The dialcohol **4** (0.3 g) was converted into the dibenzoate picrate as described for **3**. Recrystallization from ethanol gave 0.19 g (25%) which softened at 153°, melted slowly above 203° with sublimation, and decomposed above 220°. *Anal.* Calcd for $C_{29}H_{24}N_8O_{12}$: C, 51.48; H, 3.57; N, 16.57. Found: C, 51.72; H, 3.88; N, 16.33.

The picrate ion was removed as described before to afford a 60% yield of the dibenzoate, which crystallized from ethanol, mp 139–141°. *Anal.* Calcd for $C_{23}H_{21}N_5O_5$: C, 61.74; H, 4.73; N, 15.65. Found: C, 61.86; H, 4.89; N, 15.73.

2(S)-O-[1(R)-(9-Adenyl)-2-(hydroxy)ethyl]propanediol (**5**). 9-α-L-Rhamnopyranosyladenine (4.2 g, 15 mmoles) was treated with sodium metaperiodate (6.6 g, 30 mmoles) in 60 ml of water and subsequent steps were carried out as described for the preparation of **2**. A picrate of **5** was isolated (5.88 g, 81%) and a sample was recrystallized from methanol. The crystals became opaque at 173–174°, formed small clusters of needles at 275° and decomposed above 280°, $[\alpha]_D^{23}$ +22.7° (*c* 1.11, dimethylformamide). *Anal.* Calcd for $C_{16}H_{18}N_8O_{10}$: C, 39.84; H, 3.76; N, 23.23. Found: C, 40.01; H, 3.95; N, 23.27.

The dialcohol **5** was generated from the picrate (5.77 g) as described for **2** and crystallized from absolute ethanol: 2.1 g (69%), mp 206–209°, $[\alpha]_D^{23}$ +80.6° (*c* 1.76, H_2O), uv $\lambda_{max}^{H_2O}$ 258 nm (ε 14,100), R_{Ad} 1.78 (A) and 1.21 (B). *Anal.* Calcd for $C_{10}H_{15}N_5O_3$: C, 47.42; H, 5.97; N, 27.66. Found: C, 47.39; H, 5.98; N, 27.39.

2(R)-O-[1(R)-(9-Adenyl)-2-(hydroxy)ethyl]propanediol (**6**). 9-β-D-Fucopyranosyladenine (1.0 g, 3.36 mmoles) was dissolved in 20 ml of water and treated with 1.44 g (6.72 mmoles) of sodium metaperiodate in a manner similar to the preparation of **5** except that it was not necessary to form a picrate. After removal of boric acid with methanol, a solid formed which was recrystallized from aqueous ethanol to afford 0.75 g (88%) of **6**, mp 219–221°. Recrystallization from water gave cubic crystals: mp 219–221°, $[\alpha]_D^{26}$ +55° (*c* 0.5, H_2O), uv $\lambda_{max}^{pH\ 7.6}$ 258 nm (ε 14,750), R_{Ad} 1.68 (A) and 1.21 (B). *Anal.* Calcd for $C_{10}H_{15}N_5O_3$: C, 47.42; H, 5.97; N 27.66. Found: C, 47.20; H, 5.99; N, 27.42.

2(S)-O-[1(S)-(9-Adenyl)-2-(hydroxy)ethyl]propanediol (**7**). 9-β-L-Fucopyranosyladenine (1.5 g, 5.3 mmoles) was treated in a manner similar to the D form. In the last step, a clear, colorless syrup was obtained which solidified after several evaporations of absolute ethanol. Recrystallization from aqueous ethanol afforded 0.98 g (76%) of **7**. One recrystallization from water gave cubic crystals: mp 221–222°, $[\alpha]_D^{26}$ −54° (*c* 0.5, H_2O), uv $\lambda_{max}^{pH\ 7}$ 258 nm (ε 15,000), R_{AD} 1.71 (A) and 1.24 (B). *Anal.* Calcd for $C_{10}H_{15}N_5O_3$: C, 47.42; H, 5.97; N, 27.66. Found: C, 47.36; H, 6.05; N, 27.68.

Enzyme Assay and Methods. Calf intestinal adenosine deaminase (type I) was purchased from Sigma Chemical Co. and contained 190 units/mg of protein. One unit is defined as the amount of enzyme which will deaminate 1 μmole of adenosine to inosine per minute at pH 7.5 and 25°. The assay method used was based upon that of Kaplan (1955). A Cary Model 14 spectrophotometer was used to measure the rate of change in absorbance at 265 nm in 0.05 M phosphate buffer (pH 7.6) at 25°. The cuvette contained 3.0 ml of a solution of adenosine and the alcohol in buffer and the reaction was begun by addition of 0.1 ml of an enzyme solution containing 0.3 unit/ml. Changes in absorbance with time were linear for at least 2 min. The Michaelis constant for adenosine and the inhibitor constants were determined by the reciprocal plot method of Lineweaver and Burk (1934). In order to determine the ability of the alcohols **1**–**7** to act as substrates for the enzyme, solutions of the alcohol (*ca.* 5×10^{-5} M) were treated with the enzyme solution and when no change in absorbance occurred even after several hours, the experiment was repeated using stock solutions of enzyme with concentrations increased 50-fold.

Results

Synthesis of Alcohols Derived from Nucleosides. The synthetic route chosen to the alcohols consisted of periodate oxidation of a preformed nucleoside and reduction of the resulting dialdehyde with sodium borohydride. This route has been reported previously for the preparation of trialcohol **1** as an analytical sample derived from adenosine (Lerner, 1970). The starting material used in the preparation of each alcohol is rather arbitrary from a theoretical point of view, but the choice of parent nucleoside was dictated by the simplest and most inexpensive preparation. For example, **2** can be prepared from 9-α-D-ribofuranosyladenine, 9-α-D-arabinofuranosyladenine, or 9-α-D-altropyranosyladenine, but the preparation of each of these substances is a major undertaking. The structures of the starting nucleosides in a Haworth representation and the structures of the alcohols derived from them in Fischer projection formulas are represented in Chart I.

Purification of the alcohols **2**–**5** entailed preparation of their crystalline picrates, followed by removal of the picrate ion with an anion-exchange resin. As in the previous case of **1** (Lerner, 1970), **2**–**4** could not be induced to crystallize, but they were obtained as analytically pure powders after lyophilization. Due to their extremely hygroscopic nature, these powders required further drying in a drying pistol under high vacuum and elevated temperature. In each case a crystalline benzoate derivative was prepared. Dialcohols **5**, **6**, and **7** were easily crystallized; moreover, **6** and **7** did not require purification as picrates.

The synthesis of 9-β-L-fucopyranosyladenine, the nucleoside needed for the preparation of **6**, was not successful in our hands by a procedure recently described in the literature (Fischer *et al.*, 1969). This synthesis was successfully accomplished using the procedure previously used to obtain the D form (Lerner, 1971).

The structure of each of the alcohols was based upon the known structures of the starting nucleosides, the known course of the chemical reactions, and elementary analyses. Moreover, the ultraviolet and infrared spectra of these new compounds had peaks similar to that of the parent nucleosides. All of the enantiomers prepared in this project had identical physical properties, as expected, except for the sign of the optical rotation. Because of the inavailability of D-rhamnose, the enantiomer of **5** was not prepared.

Inhibitory Effects of Alcohols Derived from Nucleosides. The alcohols **1**–**7** were not substrates for adenosine deaminase. With the exception of **2**, they were inhibitors of this enzyme. Compound **2** did not exhibit any inhibition even at very high concentrations relative to the concentration of substrate. Lineweaver–Burk plots were linear in all cases and demonstrated competitive kinetics. The inhibitor constants are given in Table I.

The data reported in Table I indicate that there are three levels of inhibition occurring, with **3** and **5** binding much better to the enzyme than **4** and **6**, which in turn are binding much better than **1** and **7**. Between the strongest inhibitor (**5**)

TABLE I: Inhibition of Adenosine Deaminase by Alcohols Derived from Nucleosides.[a]

Alcohol	Configuration: Proximal Carbon	Configuration: Distal Carbon	K_i[a] (M)
1	*R*		1.7×10^{-4}
2	*S*		
3	*R*		1.2×10^{-5}
4	*S*		4.7×10^{-5}
5	*R*	*S*	4.4×10^{-6}
6	*R*	*R*	6.8×10^{-5}
7	*S*	*S*	1.4×10^{-4}

[a] Under the same conditions the K_m for adenosine was 4.7×10^{-5} M.

and the weakest inhibitor (**1**) in this series of compounds there was a 40-fold difference in ability to bind. The results show that the *R* configuration is the preferred arrangement of groups at the carbon atom bonded to the purine nitrogen (henceforth called the *proximal* carbon). These conclusions are demonstrated by comparison of the inhibitor constants of **3** to **4** and of **6** to **7**. Moreover, **2** which is the enantiomer of **1** does not bind at all. If the oxygen bridge connecting the two alcoholic moieties of the molecule is bonded to an asymmetric carbon (henceforth called the *distal* carbon) then the *S* configuration is preferred. The presence of a methyl group at the distal carbon increases the ability of the alcohols to bind to the enzyme. The *S* configuration at the proximal carbon in itself does not result in an inactive compound, but is dependent upon what groups are present and their arrangement at the distal carbon. Thus, a methyl group bonded to a distal carbon with an *S* configuration will overcome the disadvantage of having an *S* configuration at the proximal carbon, as shown by the activity of **7** but a comparison to **4** and **5** shows that the presence of the methyl group offers no advantage over its absence unless the proximal carbon has the *R* configuration. When compared to nucleoside analogs which have been evaluated to be powerful competitive inhibitors of adenosine deaminase (K_i values 0.2–0.9 $\times 10^{-5}$ M), **3** and **5** can be considered to be in the same range of potency.

Discussion

A number of publications have appeared which have attempted to determine the structural requirements for a compound to act either as a substrate or as an inhibitor of adenosine deaminase. The present work, in addition to bringing to attention some new competitive inhibitors, has attempted to throw further light on this subject. At the outset of this investigation it could easily be predicted that alcohols **1–7** would not be substrates for the enzyme because no compounds were known to be substrates for adenosine deaminase that did not have the pentofuranose ring and a hydroxyl group at either C-5′ or C-3′ cis to the adenine ring (Bloch *et al.*, 1967). However, it was recently shown that 9-hydroxyethoxymethyladenine (**8**, Chart II) is a substrate with a K_m of 1.3×10^{-4} M (Schaeffer *et al.*, 1971). Since a compound such as **3** can also assume a conformation identical with **8**, a question arises. Why is it not a substrate and, indeed, why then does it act as

CHART I

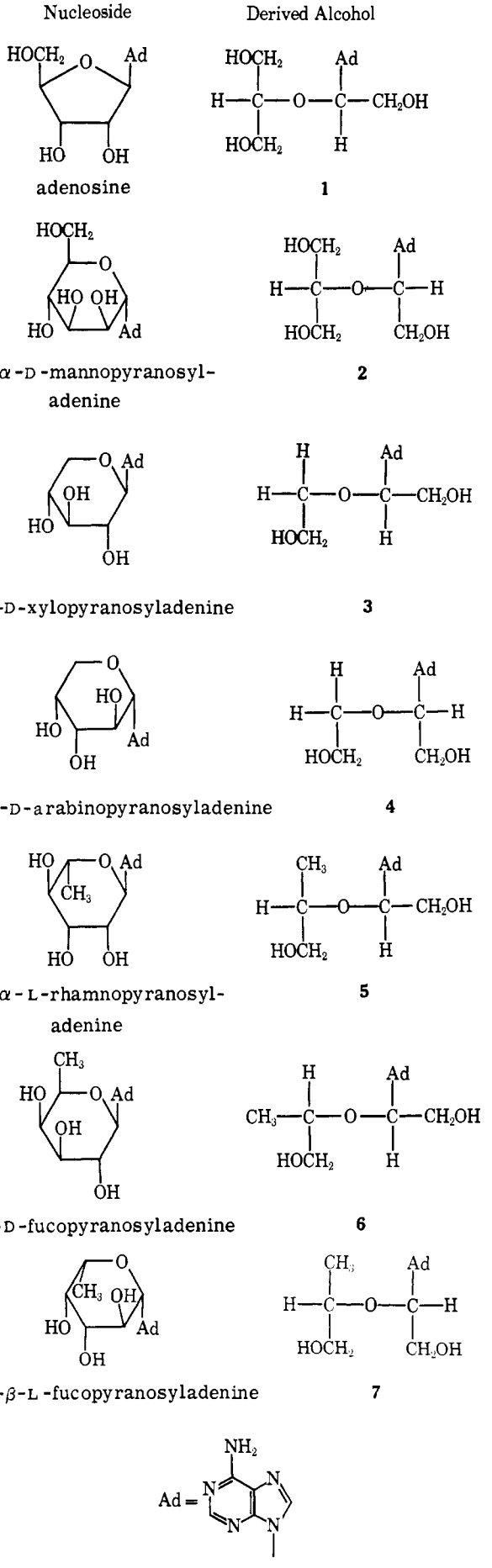
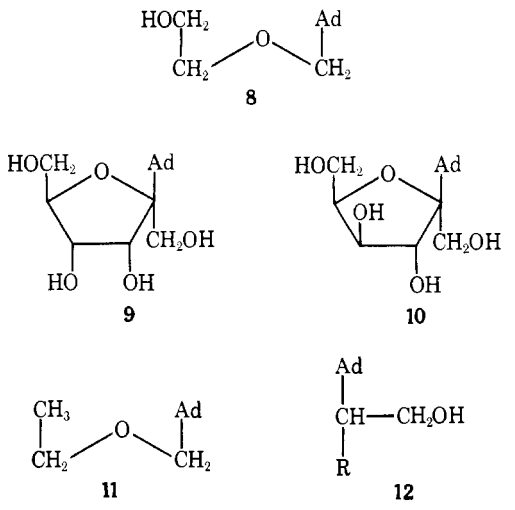


CHART II



R = CH_3, C_2H_5, etc.

an inhibitor? An examination of Dreiding stereomodels revealed a number of interesting points when the structures of **3** and **8** were compared to the structures of psicofuranine (**9**) and 9-β-D-fructofuranosyladenine (**10**). These nucleosides are neither substrates nor inhibitors of calf intestinal adenosine deaminase (Cory and Suhadolnik, 1965; Frederiksen, 1966) even though the nucleosides without the C-1′ hydroxymethyl group (adenosine and 9-β-D-xylofuranosyladenine, respectively) are substrates. The explanation for this is that there is a "tight fit" for the pentofuranose ring at the enzyme active site (Schaeffer and Bhargava, 1965) and that there is no bulk tolerance for the hydroxymethyl group at the C-1′ position (Cory and Suhadolnik, 1965). If **3** assumed the adenosine-like conformation proposed for **8**, this hydroxymethyl group would be in the same position as the C-1′ hydroxymethyl group of **9** or **10**. Since this represents an unfavorable situation, **3** and the other alcohols having the *R* configuration (**1**, **5**, and **6**) probably do not fit properly in the active site and therefore will not be deaminated.

The inhibitory activity of the alcohols can be explained by simply allowing a change in conformation so that the hydroxymethyl group at the proximal carbon was interacting with the hydroxyl binding site described by Schaeffer and coworkers in their investigations of the binding of various 9-hydroxyalkyladenines (Schaeffer and Bhargava, 1965; Schaeffer *et al.*, 1965). This hydroxyl binding site is reported to be two or three carbons removed from the purine ring, however, it is unknown as to whether the hydroxyl group donates the hydrogen or the oxygen in binding to the enzyme. This same binding site is obviously not required for substrate activity since it has been shown that the 2′- and 3′-hydroxyls of adenosine are not required for substrate activity (Cory and Suhadolnik, 1965; Frederiksen, 1966; Bloch *et al.*, 1967).

Examination of the distal carbon does not reveal an obvious reason as to why **3** binds more than ten times better to adenosine deaminase than **1**. No unfavorable steric interactions have been proposed in this region except for adenosine itself, where substituents at the 5′-carbon atom yield inactive substances (Bloch *et al.*, 1967). Some steric interaction appears supported at first by the tenfold increase in binding exhibited by **3**; even **4** which has an *S* configuration binds quite well whereas **2** does not bind at all. However, substitution of a methyl group for a hydroxymethyl group, such as in **5**, resulted in a 40-fold increase in binding over **1** and a distinct increase in binding when compared to **3**, as long as the configuration at the distal carbon was *S*. Even **7**, which has an *S* configuration at the proximal carbon was as good an inhibitor as **1**. This would indicate that there is a methyl binding site in this region and that the inhibitors **5**, **6**, and **7** are probably bridging the hydroxyl binding site and the methyl binding region. It has been shown that 9-alkyladenines such as 9-propyladenine and 9-butyladenine will significantly inhibit adenosine deaminase (Schaeffer and Vogel, 1965). An inhibitor closely related in structure to 9-butyladenine and to the substrate **8** is 9-ethoxymethyladenine (**11**, Chart II) in which the substitution of a methyl group for a hydroxymethyl group produced an inhibitor rather than a substrate (Schaeffer *et al.*, 1971).

The present work supports the previous findings of a hydroxyl binding site and a methyl binding site and demonstrates the importance of utilizing both of these regions in the design of an inhibitor of adenosine deaminase. The lack of a rigid structure in these compounds and the ability to assume a number of conformations enable them to take advantage of available binding sites even if the configurations may be somewhat unfavorable. Recently, a report has appeared in which a series of adenine derivatives having an alkyl chain and a branched hydroxymethyl group (**12**, Chart II) have been prepared and found to be excellent inhibitors of adenosine deaminase (Schaeffer and Schwender, 1971). Unfortunately, these compounds are racemic mixtures, for it would be of interest to correlate reactivity with configuration at the asymmetric carbon atom. Some caution should be exercised in interpreting the results of all of these investigations since nothing is known about possible conformational changes in the enzyme upon exposure to these various compounds.

Data have been presented in support of a hypothesis which states that there are certain similarities at or near the active sites of enzymes of nucleic acid metabolism, and that an inhibitor of adenosine deaminase may, in fact, be an inhibitor of other reactions in nucleic acid biosynthesis (Murray *et al.*, 1969). Therefore, the compounds prepared here will be submitted to a program of antimicrobial and antitumor testing.

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Purification, Crystallization, and Subunit Structure of Allosteric Adenosine 5′-Monophosphate Nucleosidase†

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ABSTRACT: Adenosine monophosphate nucleosidase (adenosine monophosphate phosphoribohydrolase, EC 3.2.2.4) has been purified from *Azotobacter vinelandii* strain OP. The purified enzyme preparation was homogeneous as judged by analytical ultracentrifugation and polyacrylamide gel electrophoresis. Following purification, the enzyme was crystallized from ammonium sulfate solutions. The molecular weight of the enzyme was approximately 370,000 as determined from gel filtration and sedimentation velocity experiments, while a molecular weight of approximately 325,000 was obtained from the Archibald approach to equilibrium method. Results of polyacrylamide gel electrophoresis in sodium dodecyl sulfate or 9 M urea, as well as analysis of amino acid composition, suggest that the enzyme consists of similar polypeptide chains having a molecular weight of approximately 57,000. The crystalline enzyme differs from previously described preparations of AMP nucleosidase in that the substrate- or salt-induced association–dissociation reaction does not occur.

Adenosine monophosphate nucleosidase catalyzes the hydrolysis of AMP[1] to yield ribose 5-phosphate and adenine:

$$\text{AMP} \xrightarrow{\text{MgATP}} \text{adenine} + \text{ribose 5-phosphate}$$

The enzyme was first described by Hurwitz *et al.* (1957) who showed that both magnesium and ATP were required for catalysis, and that ATP is not altered by the enzyme. Studies have indicated that the enzyme exhibits Michaelis–Menten kinetics with substrate, but that plots of initial velocity as a function of MgATP are strongly sigmoidal (Yoshino *et al.*, 1967; Schramm and Hochstein, 1971). Thus the enzyme may be considered as being allosteric.[2]

Recent reports of Yoshino (1970) and Ogasawara *et al.* (1970) have described the preparation of homogeneous AMP nucleosidase from *Azotobacter vinelandii*. However the enzyme obtained by these workers was unstable in the absence of high salt concentrations and could not be completely stabilized even in the presence of 0.4 M K_2SO_4. The molecular weights of this preparation were reported to be 240,000 in the presence of substrate, activator or high ionic strength and 120,000 in their absence. These results differ from those obtained by Schramm and Hochstein (1971) with an 80-fold-purified preparation of this enzyme, which showed apparent molecular weights of 360,000 and 180,000 under conditions similar to those described above. With this latter preparation a 240,000 molecular weight form appeared only after prolonged ageing of the 360,000 molecular weight enzyme (Schramm and Hochstein, 1971). In order to resolve these discrepencies, physical and chemical studies were performed on a

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[1] Molecules carrying charges such as $MgATP^{2-}$, ATP^{4-}, and AMP^{2-} have been written without their respective charges. Other abbreviations are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMSF, phenylmethylsulfonyl fluoride.

[2] "Allosteric" refers to enzymes which show (a) marked activation or inhibition by naturally occurring compounds other than substrates or products, and (b) a sigmoidal plot of initial velocity as a function of either substrate, activator, or inhibitor concentration.