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# Substrate Specificity of Adenosine Deaminase—Function of the 5′-Hydroxyl Group of Adenosine

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Nucleosides in which the adenine ring has been moved from the 1′ position to the 5′ position are resistant to degradation by the enzyme, adenosine deaminase. This study provides further evidence for the importance of the 5′-hydroxyl group as a structural requirement for significant substrate activity.

## INTRODUCTION

The ubiquitous enzyme adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the hydrolytic deamination of adenosine to inosine. This deamination is an important factor in limiting the usefulness of adenosine analogs in chemotherapy (*1*). The design of synthetic analogs of adenosine that would be more resistant to degradation by adenosine deaminase should take into account the structural requirements for overall substrate activity. Determination of these structural requirements has been reported in a number of investigations (*2–20*). In general, substrate binding and significant substrate activity require the presence of the adenine ring (*3, 8–11*). A number of structural changes are permitted in the sugar moiety and the minimum requirements appear to be a tetrahydrofuran ring bearing the 5′-$CH_2OH$ (*3, 4–7, 9, 17–20*). The stereochemical requirement is $\beta$-D or $\alpha$-L with respect to the anomeric position and the 5′-$CH_2OH$ (*6, 9, 12–14*). Dramatic changes in substrate activity occur when the 5′-$CH_2OH$ is altered (*6, 12*). We wish to report that examination of a class of nucleosides called "reversed" nucleosides where the purine ring has been moved from the 1′ position to the 5′ position shows that these are remarkably stable compared to adenosine toward the deaminase.

## METHODS AND MATERIALS

### *General Methods*

The melting points reported were uncorrected and were taken on a Thomas–Hoover melting point apparatus equipped with a microscope. The 60-MHz $^1H$ nmr spectra were taken on a Varian A-60 nmr spectrometer. The 90-MHz $^1H$ nmr spectra and the $^{13}C$ nmr spectra were recorded on a Bruker HX-90E pulse Fourier



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transform nmr spectrometer. Lyophilizations were done on a Labconco Freeze Dry 5 lyophilization unit. Elemental analyses were performed by the University of Iowa Micoranalytical Service. Ultraviolet absorption spectra and all ADA kinetic assays were done on a Cary Model 118CX recording UV-VIS spectrophotometer. All kinetic measurements were conducted at 25.0 ± 0.1°C. This temperature was maintained through use of a Forma Scientific Masterline Model 2095 constant temperature water bath and circulator. All assay solutions (with the exception of the ADA solution) were preincubated in the water bath for at least 30 min prior to use. Water from the water bath was circulated through the thermostatable cell holder in the Cary 118 sample compartment. The ADA solution was made up in ice-cold buffer (0.05 *M* potassium phosphate, pH 7.40), and the resulting solution was kept on ice until use. The ADA solutions were used within 2 hr after dilution of a stock suspension of ADA crystals in 3.2 *M* $(NH_4)_2SO_4$. The deaminations of the adenine nucleosides by ADA were followed spectrophotometrically at 265 nm. Solutions of substrates of appropriate concentrations in 0.05 *M* potassium phosphate buffer (pH 7.40) were used. The deamination reactions were initiated by addition of ADA solution, the concentration of which had been previously determined to give a workable reaction rate. Photolyses were carried out in a Rayonet photochemical reactor. Purine was purchased from ICN Pharmaceuticals, Cleveland, Ohio. The purine ribofuranoside, Sephadex, and adenosine deaminase (Type I from calf intestinal mucosa) were purchased from Sigma Chemical Company, St. Louis, Missouri.

*Data Processing*

The kinetic data were fitted to the linear transformation of the Michaelis–Menten equation that follows by means of a linear least-squares analysis:

$$1/v = (K_m/V_{max})(1/s) + 1/V_{max}$$

(the Lineweaver–Burk plot). All calculations were performed on an IBM 360/65 digital computer or on a CDC Cyber digital computer with programs written in FORTRAN IV by the authors. Plots presented are derived from computer-generated Versatec electrostatic plots, using subroutines analogous to those available in the CalComp system. Double precision was used throughout. In addition, the nonparametric statistical analysis of Cornish-Bowden and Eisenthal (*21, 22*) was employed using a FORTRAN IV program written by these authors and modified by the authors of this work to generate Versatec plots.

*Synthesis*

*5'-(Purin-9-yl)-5'-deoxy-(α),β-*D*-ribofuranose* (*3*). A suspension of purine (250 mg, 2.08 mmol) and sodium hydride (100 mg, 2.08 mmol of a 50% oil dispersion) in 7 ml of DMF was stirred at 25°C for 75 min and then at 55°C for 15 min. A solution of methyl 2,3-*O*-isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside in 13 ml dry DMF was added to it, and the solution was heated at 95°C for 15 hr. The reaction mixture was then stripped of solvent under reduced pressure and the resulting solid was extracted with hot $CHCl_3$. The filtered extract solution was

TABLE 1

$^{13}C$ NUCLEAR MAGNETIC RESONANCE DATA FOR NUCLEOSIDES AND PHOTOADDUCTS

| Compound | Solvent (concn) | Chemical shifts (δ) from TMS | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C2 | C4 | C5 | C6 | C8 | C1′ | C2′ | C3′ | C4′ | C5′ | $OCH_3$ | $CH_2OH$ |
| 1 α | $D_2O$ (0.1 *M*) | 153.1 | 149.7 | 118.7 | 156.0 | 143.9 | 97.3 | 71.9 | 71.4 | 80.9 | 46.0 | — | — |
| 1 β | $D_2O$ (0.1 *M*) | 153.1 | 149.7 | 118.7 | 156.0 | 143.9 | 102.0 | 75.8 | 72.2 | 80.5 | 46.5 | — | — |
| 2 | $D_2O$ (0.1 *M*) | 151.6 | 149.4 | 118.5 | 154.8 | 144.0 | 109.1 | 74.9 | 72.3 | 80.6 | 46.3 | 56.3 | — |
| 3 α | $D_2O$ (0.15 *M*) | 152.6 | 152.0 | 133.5 | 148.2 | 149.4 | 97.4 | 72.2 | 71.4 | 80.6 | 46.3 | — | — |
| 3 β | $D_2O$ (0.15 *M*) | 152.6 | 152.0 | 133.5 | 148.2 | 149.4 | 102.0 | 75.7 | 72.2 | 80.3 | 46.3 | — | — |
| 4 α | $CH_3OD$ (0.05 *M*) | 135.6 | 135.6 | 117.7 | 67.2 | 149.2 | 97.9 | 72.7 | 72.2 | 82.9 | 47.8 | — | 56.8 |
| 4 β | $CH_3OD$ (0.05 *M*) | 135.6 | 135.6 | 117.7 | 67.2 | 149.2 | 103.5 | 77.1 | 73.6 | 82.1 | 48.2 | — | 56.8 |
| 5 | $D_2O$ (0.25 *M*) | 152.6 | 151.2 | 134.4 | 148.7 | 146.6 | 89.2 | 74.6 | 71.2 | 86.4 | 62.2 | — | — |
| 6 | $CH_3OD$ (0.14 *M*) | 134.8 | 135.4 | 119.7 | 67.0 | 148.8 | 91.4 | 75.9 | 73.0 | 88.3 | 63.8 | — | 56.6 |
| Adenosine (*32*) | DMSO-$d_6$ (0.9 *M*) | 152.4 | 149.1 | 119.4 | 156.1 | 140.1 | 88.1 | 73.6 | 70.8 | 86.0 | 61.8 | — | — |

evaporated to dryness and the resulting yellow oil was chromatographed on preparative silica gel PF 254 plates using 5% $CH_3OH/CH_2Cl_2$ for development. The major product was the 9-substituted purine (306 mg, 52%), mp 67.5–69°C: uv $\lambda_{max}$ ($CH_2Cl_2$) 264 nm; ($CH_3OH$, pH 9) 263 nm; $^1H$ nmr δ $Me_4Si$ ($CDCl_3$) 1.30 (s, 3H), 1.46 (s, 3H), 3.42 (s, 3H), 4.21–5.05 (m, 6H), 8.30 (s, 1H), 9.00 (s, 1H), 9.20 (s, 1H).

*Anal.* Calcd for $C_{14}H_{18}N_4O_4$: C, 54.89; H, 5.92; N, 18.29. Found: C, 54.68; H, 5.99; N, 17.97.

The 7-substituted purine was also isolated as a minor product (26%), and could easily be distinguished from the 9-isomer by its uv spectrum: $\lambda_{max}$ ($CH_2Cl_2$) 270 nm; ($CH_3OH$, pH 9) 267 nm (see Refs. (*23, 24*) for uv data comparison of 7- and 9-substituted purines). Further confirmation of structure was provided by $^{13}C$ nmr data (Table 2).

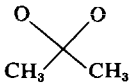
Deprotection of the major product (306 mg) was carried out in 10 ml of 0.18 *M* HCl at 80°C for 2.75 hr. After cooling, the solution was passed through a Dowex 1-X8 column ($HCO_3^-$ form) and lyophilized to give **3** as a highly hygroscopic amorphous solid (265 mg, ~100%): uv $\lambda_{max}$ ($H_2O$) 264.0 nm (log ε 3.84). $^{13}C$ and $^1H$ nmr spectral data provided excellent structural confirmation (Tables 1 and 3). The ratio of α to β forms was 1 : 6.

*Anal.* Calcd. for $C_{10}H_{12}N_4O_4 \cdot \frac{2}{3}H_2O$: C, 45.46; H, 5.09; N, 21.20. Found: C, 45.28; H, 5.03; N, 21.26.

*5′-(Purin-9-yl)-5′-*deoxy-(α),β-D-*ribofuranose methanol photoadduct (4).* A solution of 80 mg (0.32 mmol) of **3** in 170 ml of oxygen-free dry methanol was photolyzed in a Rayonet photochemical reactor with 2537-Å lamps for 50 min

TABLE 2

$^{13}C$ NUCLEAR MAGNETIC RESONANCE DATA DIFFERENTIATING 7- AND 9-SUBSTITUTION IN SUBSTITUTED PURINES

| Compound | Solvent (concn) | C2 | C4 | C5 | C6 | C8 | C1′ | C2′ | C3′ | C4′ | C5′ |  |  | $OCH_3$ | $CH_3$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7-methyl purine (*33*) | DMSO-$d_6$ (0.6 *M*) | 152.0 | 159.8 | 125.7 | 140.7 | 149.7 | — | — | — | — | — | — | — | — | 31.6 |
| Protected **3** 7-isomer[a] | $CDCl_3$ (0.5 *M*) | 153.2 | 160.7 | 125.3 | 140.2 | 148.5 | 110.5 | 84.9 | 84.6 | 81.8 | 49.4 | 113.2 | 24.9<br>26.4 | 56.1 | — |
| 9-Methyl purine | DMSO-$d_6$ (1.3 *M*) | 151.8 | 151.3 | 133.4 | 147.4 | 147.4 | — | — | — | — | — | — | — | — | 29.3 |
| Protected **3** 9-isomer[a] | $CDCl_3$ (0.5 *M*) | 152.1 | 150.9 | 133.4 | 148.1 | 145.2 | 109.8 | 84.6 | 84.1 | 81.5 | 46.4 | 112.4 | 24.5<br>25.9 | 55.3 | — |

[a] Protecting groups were β-methyl acetal and 2′,3′-*O*-isopropylidene (see Methods and Materials.

TABLE 3

$^1$H NUCLEAR MAGNETIC RESONANCE DATA FOR NUCLEOSIDES AND PHOTOADDUCTS

| Compound | Solvent (concn) | Chemical shifts (δ) and multiplicities | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | H2 | H6 | H8 | H1′ | H2′ | H3′ | H4′ | H5′ | $OCH_3$ | $CH_2OH$ | $NH_2$ |
| 1 α and β | $D_2O$ (0.2 *M*) ext TMS | 8.58(s) or 8.52(s) | — | 8.58(s) or 8.52(s) | 5.72(d) and 5.85(s) | 4.35 to 5.64 (m) | | | 4.30(q) | — | — | — |
| 2 | DMSO-$d_6$ (1 *M*) | 8.16(s) or 8.09(s) | — | 8.16(s) or 8.09(s) | 4.64(s, br) | 3.39 to 4.26 (m) | | | | 3.22(s) | — | 7.20(s) |
| 3 α and β | $D_2O$ (0.15 *M*) ext TMS | 9.52(s) | 9.65(s) | 9.16(s) | 6.08(s, br) and 6.06(d) | 4.42 to 5.87 (m) | | | | — | — | — |
| 4 α and β | $CD_3OD$ (0.2 *M*) | 7.32(d) or 7.00(s) | 3.76(t) | 7.32(d) or 7.00(s) | 5.13(d) and 5.06(s, br) | 3.51 to 4.56 (m) | | | | — | 3.88 | — |
| 5 | $D_2O$ (0.25 *M*) ext TMS | 9.21(s) | 9.36(s) | 9.04(s) | 6.53(d) | 4.63 to 5.86 (m) | | | 4.31(m) | — | — | — |
| 6 | $CD_3OD$ (0.2 *M*) | 7.53(s) or 7.04(s) | ~3.71(t) | 7.53(s) or 7.04(s) | 5.64(d) | 4.10 to 4.65 (m) | | | 3.68(m) | — | 3.71 | — |
| Adenosine | DMSO-$d_6$ (0.2 *M*) | 8.34(s) or 8.18(s) | — | 8.34(s) or 8.18(s) | 5.88(d) | 4.68(q) | 4.14(q) | 3.96(q) | 3.62(m) | — | — | 7.33(s) |

(*25, 26*). The reaction was monitored by following the change in the uv spectrum from 262 to 296 nm as the reaction progressed. The methanol was then removed carefully under reduced pressure and room temperature to give 73 mg (81%) of a pale yellow viscous material. The compound was unstable and its structure and purity was established by its mass spectrum ($M^+$ 284) and by its $^{13}C$ and $^1H$ nmr spectra (Tables 1 and 3).

*Nebularine–methanol photoadduct* (*6*). A solution of 81 mg (0.32 mmol) of nebularine **5** was photolyzed as described above for **3** to give 95 mg (~100%) of a colorless, unstable oil (*27*). Its structure confirmation was provided by its mass spectrum ($M^+$ 284) and by NMR data (Tables 1 and 3).

*5′-(6-Aminopurin-9-yl)-5′-deoxy-(α),β-*D*-ribofuranose* (*1*). This compound was prepared as previously described (*28*) and purified by gel permeation chromatography on a column of Sephadex G-25–80. The nucleoside **1** was obtained as a white solid: mp 181–182°C (lit. (*28*) mp 168–169°C); uv $\lambda_{max}$ ($H_2O$) 259.7 nm (log ε 4.17); $^{13}C$ and $^1H$ nmr data (Tables 1 and 3).

*Methyl 5′-(6-aminopurin-9-yl)-5′-deoxy-β-*D*-ribofuranoside* (*2*). The protected reversed nucleoside methyl 5′-(6-aminopurin-9-yl)-2′,3′-*O*-isopropylidene-5′-deoxy-β-D-ribofuranoside (3.22 g, 10 mmol) (*29*) was dissolved in a solution of 250 ml of $CH_3OH$ containing 10 ml of 2 *M* HCl and heated under reflux for 24 hr. The $CH_3OH$ was removed *in vacuo* and the residue was taken up in 10 ml of $H_2O$. The aqueous solution was immediately neutralized with Dowex 1 ($HCO_3^-$ form). Filtration of the resin and evaporation of the filtrate gave **2** (2.65 g, 95%) as a white solid which was recrystallized from methanol, mp 180–181°C: uv $\lambda_{max}$ ($H_2O$) 261.5 nm (log ε 4.11); $^{13}C$ and $^1H$ nmr (see Tables 1 and 3).

*Anal.* Calcd for $C_{11}H_{15}N_5O_4 \cdot \frac{1}{2}H_2O$: C, 45.52; H, 5.56; N, 24.13. Found: C, 45.56; H, 5.48; N, 24.32.

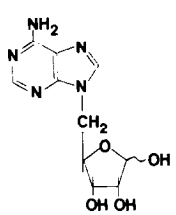
## RESULTS AND DISCUSSION

The reversed nucleosides **1** and **2** were synthesized by thermally induced coupling of the sodium salt of adenine and methyl 2,3-*O*-isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside followed by removal of protecting groups (*28–30*). The carbon-13 nmr spectra of **1** and **2** provided excellent confirmation of their structures (Table 1). The ratio of the two isomers α : β was 1 : 6 for **1** (*31*), and the stereochemistry at C1′ of the acetal **2** was inferred from the appearance of the H1′ resonance as a singlet. The nebularine analog **3** was synthesized by reaction of methyl 2,3-*O*-isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside with sodium purinide, subsequent separation of the mixture of 7-isomer (26%) and 9-isomer (52%) and finally removal of the protecting groups. Assignment of structure for the 7- and 9-isomers from the coupling reaction came from uv data and particularly from $^{13}C$ nmr spectral analysis (see Table 2). Photolysis (quartz tube, 254-nm irradiation) of **3** in dry oxygen-free methanol solution gave **4** as a pale yellow unstable oil in 81% yield. Its mass spectrum confirmed that a 1 : 1 photoadduct was formed and its $^{13}C$ nmr spectrum provided

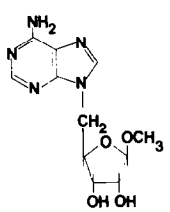
excellent evidence of gross structure. The nebularine photoadduct **6** was prepared in a similar manner from **5** and methanol (*25–27*).

The deaminations with adenosine deaminase were followed spectrophotometrically by monitoring of the uv absorption at 265 nm (*34*). The kinetic data were treated as described under Methods and Materials. The Lineweaver–Burk plots of adenosine, its reversed analog, and the β-methyl acetal of reversed adenosine are shown in Figs. 1, 2, and 3. Michaelis constants ($K_m$), maximal velocities ($V_{max}$), and correlation coefficients ($r$) are included with the plots. The results indicate that **1** and **2** exhibit much slower substrate activity with the enzyme than adenosine.

The contribution made to overall substrate activity by the 5′-hydroxyl group is apparent from these studies. The results presented are also consistent with the possibility that the anomeric β-hydroxyl group (86% β from $^1H$ and $^{13}C$ nmr data) may be capable of assuming the role of the 5′-hydroxyl group. This interpretation has some precedent for it had been suggested previously that the function of the 5′-hydroxyl group could be assumed effectively by a hydroxyl group on C3′ in a configuration *cis* to the adenine moiety (*5, 6, 12*). Thus, 5′-deoxyxylosyladenine is a substrate for adenosine deaminase whereas 5′-deoxyadenosine and 5′-deoxyarabinofuranosyladenine are not deaminated (*5, 12*). A possible explanation for this activity was given by Shah *et al.* (*12*) who suggested that the hydrogen of the β-hydroxyl group at C3′ of 5′-deoxyxylosyladenine can occupy an almost identical position as does the hydroxyl group at C5′ in one of the conformations of adenosine. The possible involvement of the β-hydroxyl group at C1′ in substrate activity in our studies is further supported by the observation that when the reversed nucleoside **1** was converted to its acetal **2**, $V_{max}$ diminished dramatically. In fact, the observed $V_{max}$ for adenosine in these studies was 26,500 times faster than that for **2**! The $K_m$ values are more difficult to interpret. If it is assumed that the $K_m$ values are an indication of enzyme–substrate affinities, then the acetal **2** ($K_m$ = 2.3




1

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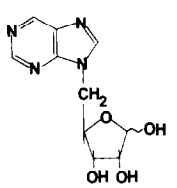


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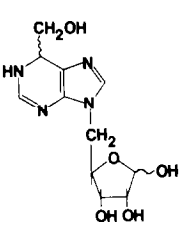


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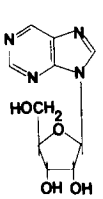


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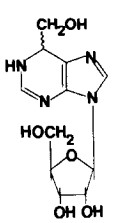


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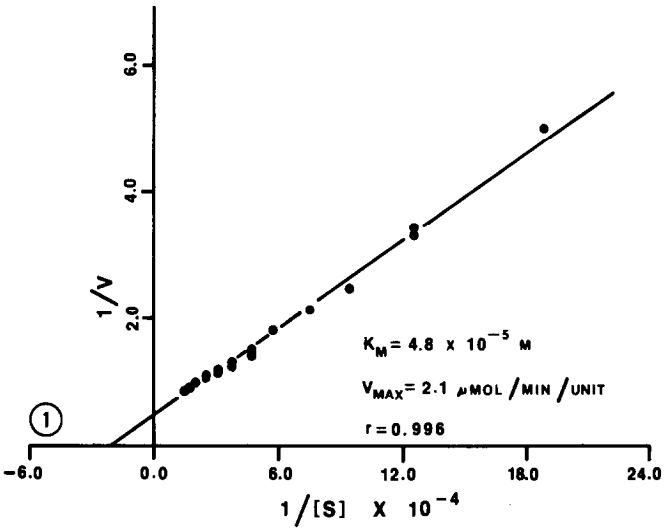
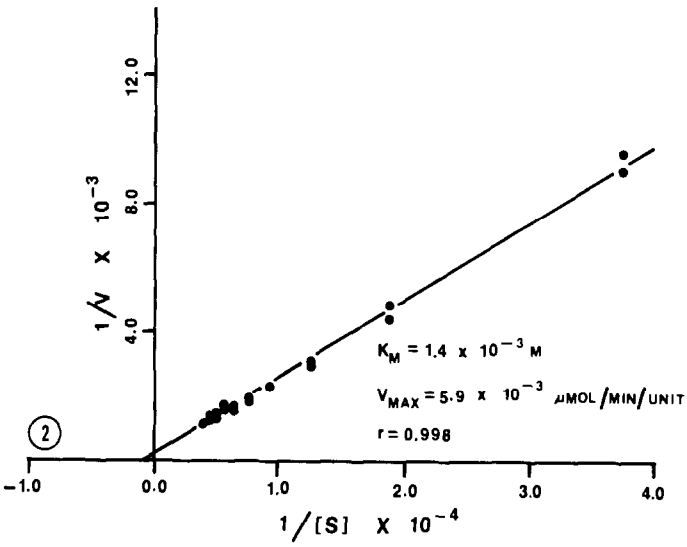
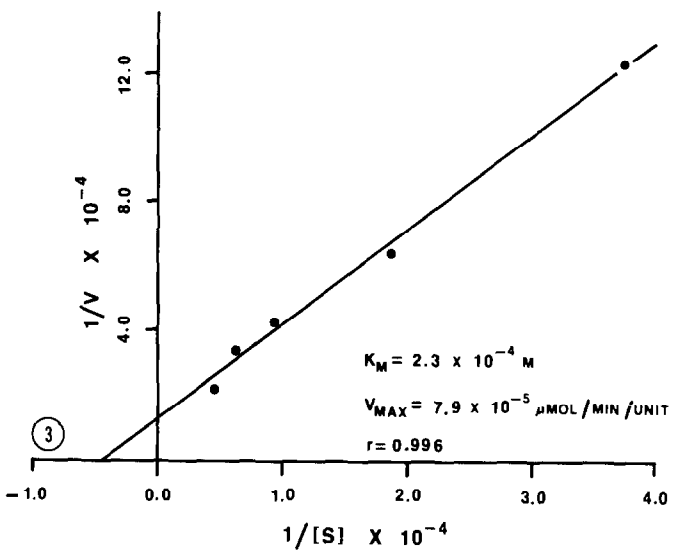



FIGS. 1–3. Lineweaver–Burk plots of activity of adenosine (Fig. 1), reversed adenosine 1 (Fig. 2), and reversed adenosine acetal 2 (Fig. 3). Substrate concentration [S] is expressed as mol/liter and initial velocity ($V$) as μmol/min/unit of ADA. A unit is defined as an amount of enzyme that will catalyze the conversion to product of 1 μmol of substrate/min. Final concentrations of ADA in the reaction mixture were $9.40 \times 10^{-3}$ U/ml for the adenosine analysis, 0.94 U/ml for the reversed adenosine analysis, and 18.8 U/ml for the reversed adenosine acetal analysis.

$\times 10^{-4}$ $M$) may be binding more effectively than **1** ($K_m = 1.4 \times 10^{-3}$ $M$). The observed differences in $V_{max}$ may then be attributed to the catalytic effectiveness of the enzyme–substrate complex formed.

Results of the inhibition studies with compounds **1** and **2** were compared with known inhibitors of ADA such as nebularine **5** and its methanol photoadduct **6**, a "transition-state" analog (*35*). Both **5** and **6** were found to be strongly inhibitory in our studies, and **2** showed weak inhibitory activity. Compound **1** showed no measurable inhibitor activity (Table 4). At relatively high concentrations of **1** (> 10:1 of **1**:adenosine), the apparent rate shows an increase above the control value due to the substrate activity of **1**. The difference in inhibitor activity between **1** and **2** is consistent with the relative magnitudes of $K_m$ for these compounds. For further comparison, we synthesized and examined the reversed analog of nebularine and its methanol photoadduct. Interestingly, compounds **3** and **4** do not appear to have any inhibitory effect.




FIGS. 2 AND 3

In summary, we suggest that although the β-hydroxyl group at C1′ may be contributing to the binding and activity of 1, this contribution is not significant enough in 1 (or the other reversed analog) for very effective substitution of the functional role of the 5′-hydroxyl group in either substrate or inhibitor activity.

TABLE 4

INHIBITION STUDIES WITH ADENOSINE DEAMINASE[a]

| Inhibitor | Inhibitor concn ($M$) | Initial velocity[b] |
|---|---|---|
| Control— No inhibitor added | — | 0.369 |
| 1 | $3.24 \times 10^{-5}$ | 0.379 |
| 1 | $5.40 \times 10^{-5}$ | 0.370 |
| 2 | $1.13 \times 10^{-4}$ | 0.293 |
| 2 | $2.26 \times 10^{-4}$ | 0.132 |
| 3 | $1.25 \times 10^{-4}$ | 0.358 |
| 3 | $2.50 \times 10^{-4}$ | 0.348 |
| 4 | $1.13 \times 10^{-4}$ | 0.358 |
| 4 | $2.25 \times 10^{-4}$ | 0.365 |
| 5 | $1.25 \times 10^{-4}$ | 0.022 |
| 6 | $1.13 \times 10^{-4}$ | —[c] |

[a] ADA concn = 9.4 units/liter; adenosine concn = $1.04 \times 10^{-5}$ $M$.

[b] $\mu$mol/min/unit of enzyme.

[c] No detectable substrate conversion.

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