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How Important Is the *N*-3 Sugar Moiety in the Tight-Binding Interaction of Coformycin with Adenosine Deaminase?

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Preliminary findings on the possible important role of the N-3 sugar moiety of coformycin in its tight-binding interaction with adenosine deaminase (ADA) are reported. The compound 3-β-D-Ribofuranosyl-5,6,7,8-tetrahydro-4H-imidazo[4,5-*d*][1,3]diazepin-5-one-8-ol (1), its 3-benzyl analogue (6), and the aglycon (7) served as probes. The first two were both found to be competitive inhibitors of ADA with K_i's in the range of 10^{-5} *M*, while the last one was inactive. © 1997 Academic Press

Coformycin[1] (CF) and 2′-deoxycoformycin (dCF, pentostatin)[2] are two naturally occurring antitumor antibiotics, known for their nearly irreversible, strongest inhibition of the enzyme adenosine deaminase (ADA) ($K_i = 10^{-11} - 10^{-13}$ *M*).[3] The observed strong inhibition is attributed to the extremely tight-binding interaction of coformycins with ADA, mimicking the transition state structure that occurs during the ADA-catalyzed hydrolysis of adenosine to inosine.[3] However, the recently reported crystal structure of ADA with 2′-deoxycoformycin (dCF) bound in the active site,[4] schematically represented in **Figure 1,** surprisingly revealed little participation of the heterocyclic ring nitrogen atoms of coformycin in its hydrogen bond interactions with the protein. As a matter of fact, the only conspicuous interaction of the heterocycle was a coordination bond between the 8-OH of dCF and the active site zinc of ADA. This is in sharp contrast to the earlier reported crystal structure of ADA complexed with another ADA inhibitor 6(R)-hydroxy-1,6-dihydropurine ribonucleoside (HDPR), wherein three of the four nitrogen atoms of the heterocyclic ring played key roles in H-bond interactions with the enzyme.[5] These observations made us wonder how coformycins, with little involvement of their heterocyclic ring nitrogen atoms, manage to bind so tightly to the enzyme. Could the crystallographer have grossly erred, which is seemingly not so rare given the limitations of resolution in protein structure analysis? The crystal structure of ADA-dCF complex (see **Fig. 1**), however, did reveal several hydrogen bonds between the sugar hydroxyl groups and the amino acid residues of ADA. But the question still remained as to whether these sugar H-bonds alone could be sufficient to render the observed extremely tight-binding characteristics of coformycins. This is especially because the sugar H-bonds would be highly dependent upon a particular sugar conformation in solution, which can be any of a number of conformations in equilibrium, ranging from the highly *syn* to the highly *anti*. The predominantly *anti* conformation would apparently be necessary to maintain all the hydrogen bonds of dCF with ADA observed in the crystal structure, if the same were to be maintained in solution.

How would one confirm the implicated major role of the sugar H-bonds in the tight-binding interaction of coformycin with ADA? One approach is to cause changes in the structure such that the preferred sugar conformation would be *syn* (the 5′-OH oriented towards the 7-membered ring) instead of *anti* as shown in Fig. 1. This would, in principle, eliminate the H-bonding interactions of the sugar with the protein, provided no new interactions will arise from the *syn* orientation. Second, replace the sugar with other non-sugar groups such as the benzyl, and third, remove the sugar moiety from N-3 altogether.

Nucleoside **1** was considered a good candidate for the first approach described above. Compound **1** has an NH-C=O functionality in place of the N=CH group of coformycin at position 4-5. The choice of **1** was based upon anticipation that the conversion of a hydrogen bond acceptor N-4 of coformycin into a hydrogen bond donor NH-4 of **1** would result in a highly *syn* sugar conformation, dictated by a strong intramolecular H-bond between the 5′-O and HN-4. This reasoning was

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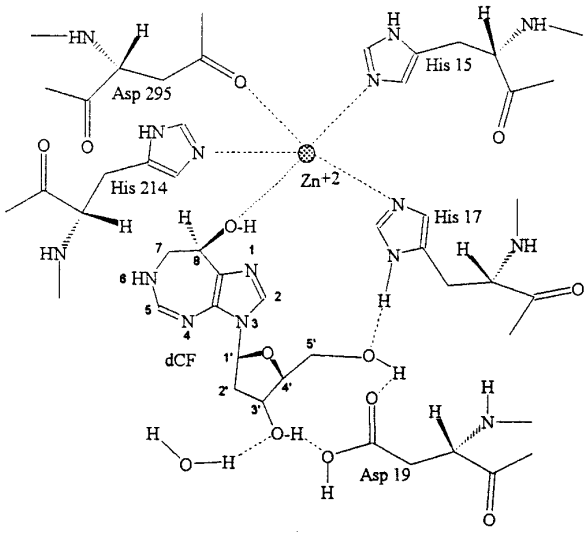


FIG. 1. Schematic representation of important protein–ligand interactions in the crystal structure of ADA–dCF complex.[4]

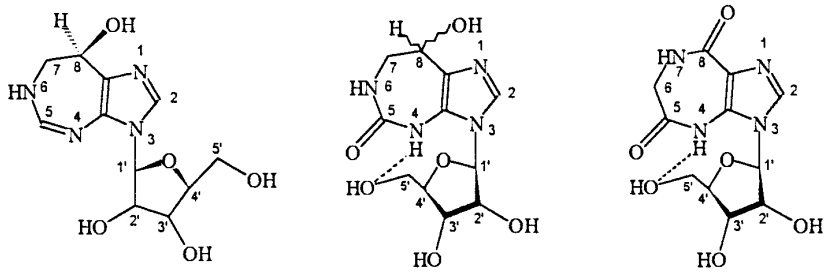
based on our earlier observations with a close structural analogue **2** that exhibited an unusually high *syn* conformation both in the solid state (X-ray)[6] as well as in solution (NOE, CD).[7] The molecular modeling studies[8] with **1** corroborated this notion. The least energy *syn* conformation of **1** was one in which there existed three intramolecular H-bonds between the heterocyclic ring and the sugar moiety: N4-H . . . O-ring (sugar) (2.33 Å), 5′-O . . . H-N4 (2.03 Å), and 5′-OH . . . O=C5 (2.25 Å). The energy-minimized ADA-**1** complex (see **Figure 2**) revealed even further-shortened, intramolecular H-bond between 5′-O. . . . H-N4 (1.96 Å). A femtosecond molecular dynamics simulation study (300 K, 5000 iterations) of the energy-minimized ADA-**1** complex, soaked with an aqueous layer of 5Å thickness all around the active site, suggested that the positioning of the ligand at the protein active site differed considerably from that of either CF or dCF, resulting in the loss of all intermolecular sugar H-bonds observed in the crystal structure of ADA-dCF complex. Furthermore, the distance between the heterocyclic NH-4 and the sugar 5′-O or ring-O, as computed from the graphs derived from dynamics trajectories, ranged from 1.73→2.27 Å or 2.29→2.94 Å, respectively, indicating that the molecule maintained a highly *syn* conformation throughout the simulations. This was further corroborated from the trajectory graph of the dihedral angle, C2-N3-C1′-O-ring (sugar), which remained essentially constant at −180° throughout the duration of simulations. To address the role of the sugar moiety by the second and third approaches described above, compounds **6** and **7** were considered as good probes.

MATERIALS AND METHODS

(A). Organic Synthesis

Compounds **1** and **4-8** were synthesized using procedures analogous to the ones reported for the synthesis of coformycins.[1c,2c] Detailed experimental procedures for the synthesis of all intermediates, byproducts and end products will be published at a later date. Listed herebelow are the physical, spectroscopic, and microanalytical data of only those new compounds that are pertinent to this paper.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on an IBM NR/80 (80 MHz), a General Electric QE-300 (300 MHz), or a General Electric GN-500 (500 MHz) spectrometer. The data are reported in the following format: chemical shift (all relative to Me_4Si), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constants, exchangeability after D_2O addition, and assignment of resonances. ^{13}C nuclear magnetic resonance spectra were recorded on a General Electric QE-300 (75 MHz) spectrometer in DMSO-d_6; chemical shifts are reported relative to Me_4Si. Electron impact (EI) or chemical ionization (CI) mass spectra were recorded at 70 eV on a Hewlett Packard 5988A mass spectrometer. Other mass spectra including high resolution FAB were recorded at the Mass Spectral Facility, Department of Biochemistry, Michigan State University or University of Maryland College Park. Infrared spectra were recorded on a Perkin-Elmer 1420 ratio recording instrument. Elemental microanalyses were performed by Atlantic Microlab, Inc., Norcross, Georgia. UV spectra were recorded on a Gilford Response UV/Vis spectrometer. Thin layer chromatography was performed on Merck Kieselgel 60 GF_{254} (0.2 mm thickness). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. X-ray crystal structure analyses were performed at the Department of Chemistry, Southern Methodist University, Dallas, Texas. Anhydrous solvents were prepared as follows: MeOH was distilled from CaH_2 and was stored over molecular sieves (type 3A); acetonitrile was distilled from P_2O_5 and was stored over molecular sieves (type 3A); triethylamine was distilled from CaH_2; tetrahydro-



SCHEME 1.

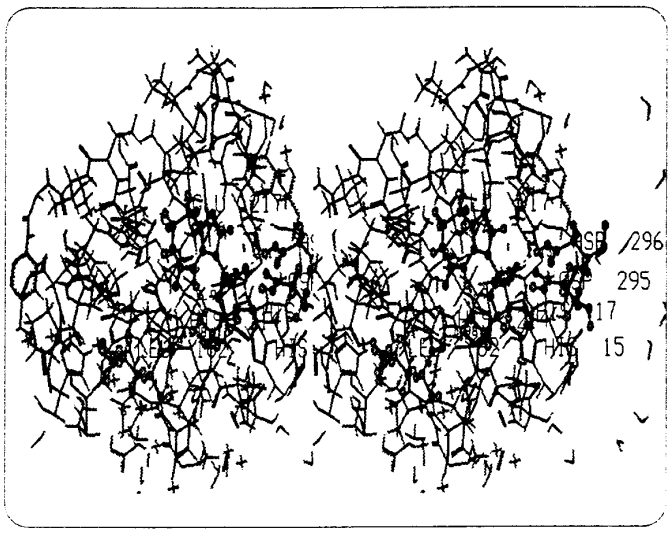


FIG. 2. Stereoview of the energy-minimized ADA-**1** complex. Only those residues that lie within a 12 Å radius from the active site are shown.

furan was distilled from Na (s) and benzophenone and was stored over molecular sieves (type 4A).

*3-Benzyl-5,6,7,8-tetrahydro-4H-imidazo[4,5-d][1,3]diazepine-5,8-dione (**4**).* It was prepared by ring-closure of **3**[2c] via condensation with *p*-nitrophenyl chloroformate in the presence of triethylamine. Colorless crystals from EtOH, 59% yield, mp 214 °C dec.: ^{1}H NMR (Me_2SO-d_6) δ 9.79 (s, 1H, H-4, exchangeable with D_2), 7.63 (s, 1H, H-2), 7.3 (m, 6H, Ar-H + H-6, exchangeable with D_2O), 5.36 (s, 2H, benzyl CH_2), 3.65 (d, J = 4.9 Hz, 2H, H-7, changing to a singlet upon D_2O exchange); IR (KBr) 3400, 3100, 3000, 1700, 1650 cm^{-1}; mass spectrum (70 eV) m/z 256 (M^+), 200, 91; UV (MeOH) 284.5 nm, (pH 13) 333.5; *Anal.* Calcd. for $C_{13}H_{12}N_4O_2$· 0.25 H_2O: C, 59.88; H, 4.79; N, 21.49. Found: C, 60.01; H, 4.84; N, 21.59.

*5,6,7,8-Tetrahydro-4H-imidazo[4,5-d][1,3]diazepine-5,8-dione (**5**).* It was prepared by debenzylation of **4** with $Pd(OH)_2$ on (20%) carbon. Colorless crystals from water, 83% yield, mp > 300 °C: ^{1}H NMR (Me_2SO-d_6) δ 12.88 (br s, 1H, H-1, exchangeable with D_2O), 9.75 (s, 1H, H-4, exchangeable with D_2O), 7.74 (s, 1H, H-2), 7.14 (br s, 1H, H-6, exchangeable with D_2O), 3.65 (d, J = 4.5 Hz, 2H, H-7, changing to a singlet upon D_2O); IR (KBr) 3350-2950, 1750-1650 cm^{-1}; mass spectrum (70 eV) m/z 166 (M^+), 138, 110, 83; UV (H_2O) 278.5 nm, (pH 13-14) 304.0; *Anal.* calcd. for $C_6H_6N_4O_2$: C, 43.38; H, 3.64; N, 33.72. Found: C, 43.29; H, 3.65; N, 33.66.

*3-Benzyl-5,6,7,8-tetrahydro-4H-imidazo[4,5-d][1,3]diazepin-5-one-8-ol (**6**).* It was prepared by sodium borohydride reduction of **4** using a mixture of MeOH-H_2O (1:4) as solvent. White solid from MeOH, 62 % yield, mp 222 °C: ^{1}H NMR (Me_2SO-d_6) d 8.52 (s, 1H, H-4, exchangeable with D_2O), 7.34-7.13 (m, 6H, Ar-H + H-2), 6.82 (br s, 1H, H-6, exchangeable with D_2O), 5.23 (s, 2H, benzyl CH_2), 4.90-4.89 (d, J = 3.0 Hz, 1H, OH, exchangeable with D_2O), 4.72 (s, 1H,H-8), 3.16-3.06 (m, 2H, H-7); HRMS (EI) calcd for $C_{13}H_{14}O_2N_4$ (M^+) 258.1116, found m/z 258.1117.

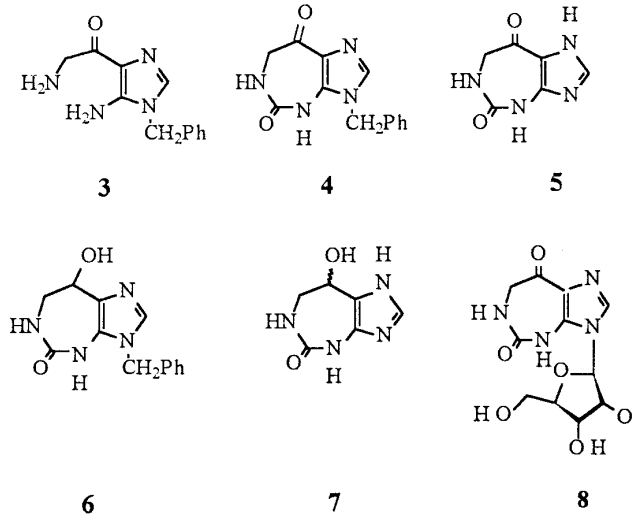
*5,6,7,8-Tetrahydro-4H-imidazo[4,5-d][1,3]deazepin-5-one-8-ol (**7**).* It was prepared by sodium borohydride reduction of **5** in MeOH-H_2O (1:2). White solid from MeOH, 70 % yield, mp 220 °C: ^{1}H NMR (Me_2SO-d_6) d 11.81 (br s, 1H, H-1, exchangeable with D_2O), 8.33 (br s, 1H, H-4, exchangeable with D_2O), 7.27 (s, 1H, H-2), 6.62 (br s, 1H, H-6, exchangeable with D_2O), 5.2 (d, J = 6.6 Hz, 1H, OH, exchangeable with D_2O), 4.53 (t, 1H, H-8), 3.14-3.06 (m, 2H, H-7); *Anal.* calcd. for $C_6H_8N_4O_2$: C, 42.86; H, 4.80; N, 33.32. Found: C, 42.70; H, 4.65; N, 33.27.

*3-β-D-Ribofuranosyl-5,6,7,8-tetrahydro-4H-imidazo[4,5-d][1,3]diazepine-5,8-dione (**8**).* It was prepared by ribosylation of **5,** using the Vorbrüggen procedure,[9] with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, followed by deprotection of the sugar hydroxyl groups with sodium methoxide in methanol. Colorless crystals from water, 69% yield, mp > 250 °C; ^{1}H NMR (Me_2SO-d_6) δ 9.89 (br s, 1H, H-4, exchangeable with D_2O), 7.8 (s, 1H, H-2), 7.58 (br s, 1H, H-6, exchangeable with D_2O), 5.80 (d, J = 7.0 Hz, 1H, H-1′), 4.3-3.48 (m, ribose-H + ribose-OH), 3.65 (d, J = 4.0 Hz, H-7, singlet upon D_2O exchange); UV (H_2O) 249.5, 290.0 nM, (pH 13) 294.5, 340.5, (pH 2) 289.5; *Anal.* Calcd. for $C_{11}H_{14}N_4O_6$: C, 44.30; H, 4.73; N, 18.78. Found: C, 44.56; H, 4.89; N, 18.71.

*3-β-D-Ribofuranosyl-5,6,7,8-tetrahydro-4H-imidazo[4,5-d][1,3]diazepin-5-one-8-ol (**1**).* It was prepared by sodium borohydride reduction of **8** in MeOH-H_2O (1:4). Colorless solid from EtOH, mp 188-190 °C; ^{1}H NMR (Me_2SO-d_6) d 8.45 (s, 1H, H-4, exchangeable with D_2O), 7.46 (s, 1H, H-2), 6.90 (br s, 1H, H-6, exchangeable with D_2O), 5.56 (d, J = 6.0 Hz, 1H, H-1′), 5.4 (br s, 1H, OH, exchangeable with D_2O), 5.22 (br s, 1H, ribose-OH, exchangeable with D_2O), 4.98 (br s, 1H, ribose-OH, exchangeable with D_2O), 4.45 (br t, 1H, H-2′), 4.18 (br t, 1H, H-3′), 4.02 (br t, 1H, H-8), 3.92 (br t, 1H, H-4′), 3.56 (s, 2H, H-5′), 3.19-3.09 (m, 2H, H-7); mass spectrum (FAB) m/z 301 (MH^+), 277, 207, 115, 93; *Anal.* Calcd for $C_{11}H_{16}N_4O_6$: C, 43.98; H, 5.37; N, 18.66. Found: C, 44.02; H, 5.28; N, 18.51.

(B) Molecular Modeling Studies

Molecular modeling was performed on a Silicon Graphics workstation, using the software INSIGHT/DISCOVER (Molecular Simulations, Inc., San Diego, California). The reported X-ray coordinates of adenosine deaminase (ADA), complexed with the inhibitor 6(R)-hydroxy-1,6-dihydropurine ribonucleoside (HDPR),[5] were imported from the Brookhaven national Laboratory, Upton, New York. Nucleoside **1** was energy-minimized, and docked into the HDPR pocket of ADA, followed by energy minimization of the ADA-**1** complex. All atoms that were 12 Å or farther from the ligand were fixed with a temperature constant of 300 K. No constraints were applied to the remaining residues in and around the ligand site. The complex was minimized to convergence using consecutive Steepest Descent and Conjugate Gradient (VAO9A) energy minimization protocols, with a final Insight energy = −775.8786 kcal, average absolute derivative



SCHEME 2.

= 1.29×10^{-5}, std. dev. of absolute derivative = 1.14×10^{-5}, and RMS derivative = 1.72×10^{-5}. In order to simulate the natural environment even further, a femtosecond molecular dynamics simulation (5000 iterations) at 300 K was performed on the above energy-minimized protein-ligand complex by soaking the latter in an aqueous layer of 5 Å thickness all around the active site of complex. There were no morse or cross terms. The distance ranges of the heterocyclic NH-4 from the sugar 5′-O or ring 4′-O were computed from graphs derived from the dynamics trajectories. The information on variations of the dihedral angle C2-N3-C1′-O(sugar ring) during simulations was obtained the trajectory graph of the said dihedral angle (X-axis) *vs* the frame number (Y-axis).

(C) Biochemical Studies: Inhibition of Adenosine Deaminase

Adenosine deaminase from calf intestinal mucosa, obtained from Sigma, was employed in the described biochemical studies. All studies were carried out at 25 °C by spectrophotometric measurements of the rate of hydrolysis of the substrate adenosine at λ_{max} 265 nm. Compounds **1** and **4-8** were screened for inhibitory activity against ADA.

1. Principle.

$$\text{Adenosine} + H_2O \rightarrow \text{Inosine} + NH_3$$

The change in optical density at 265 nm per unit time is a measure of the adenosine deaminase activity. Michaelis constant (K_m) of adenosine is 25-31 μM.

2. Solutions. (a) Phosphate buffer, pH = 7.0, 50 mM (1.5 g $NaH_2PO_4 \cdot H_2O$ and 10.4 g $Na_2HPO_4 \cdot 7H_2O$ were dissolved and then diluted to 1000 mL using deionized distilled water). (b) Adenosine Solution, 2.8×10^{-4} M (7.5 mg adenosine was dissolved in 50 mL of 50 mM phosphate buffer). (c) Substrate concentration in each assay was in the range of 10-40 mM (taking calcd. amount of above adenosine solution, then using the phosphate buffer to dilute to 1 mL). (d) Inhibitor solution, 1.3×10^{-5} (*e.g.* 3.3 mg of inhibitor **6** was dissolved in 50 mL of 50 mM phosphate buffer). (e) Inhibitor concentration in each assay was 20 or 25 mM (taking calcd. amount of above inhibitor solution, then using the phosphate buffer to dilute to 1 mL). (f) Enzyme solution, 2.2 units/mL (1.2×10^{-2} mg (10 mL) was diluted with 1 mL cold phosphate buffer). Adenosine deaminase from calf intestinal mucosa (type VI) is available from SIGMA as a solution in 50 % glycerol/0.01 mM potassium phosphate, pH 6.0, activity = 1.2 mg protein/mL; 180 units/mg. (g) The amount of enzyme in each assay is 0.022 unit (taking 10 mL of the above enzyme, then using the phosphate buffer to dilute to 1 mL).

3. Spectrophotometric measurements. Wavelength: 265 nm; final volume: 1.00 mL; light path: 1 cm; temperature: 25 °C; read against air.

4. Procedure. A mixture of calcd. amount adenosine solution (7 different substrate concentrations in the range of 10-40 mM) and calcd. amount of inhibitor solution (the concentration of inhibitor was constant for each series) was added into a 1 mL cuvette followed by the addition of the phosphate buffer to make up to a volume of 990 mL. The phosphate buffer was used as reference. In each case the concentration of enzyme was kept constant. After adding a 10 mL of enzyme solution to the above cuvette, the solution was quickly mixed by stirring with a plastic rod. The reaction was followed at 25 °C by measuring the decrease in absorbance at 265 nm using a Gilford UV instrument. The Lineweaver-Burk plots were used to calculate K_m, V_{max}, and K_i.

RESULTS

Compound **1** was synthesized using procedures analogous to the ones reported for the synthesis of coformycins.[1c,2c] The synthesis was accomplished in five steps, commencing from an aminoimidazole derivative **3,** which in turn was synthesized in seven steps from 4(5)-methylimidazole.[2c] Compounds **4-8** are either the intermediates or the products derived therefrom during the synthesis of nucleoside **1.** All products and byproducts, including regio- and stereoisomers, were characterized by spectroscopic and microanalytical data. In view of the documented propensity of 5:7-fused heterocyclic systems to undergo opportunistic rearrangements to form ring-contracted 5:6- or 5:5-fused systems,[10] the structure of the parent heterocycle **5** was also confirmed by single-crystal X-ray diffraction analyses.[11]

Compounds **1** and **4 - 8** were screened *in vitro* against ADA from calf intestinal mucosa (Sigma) in a 50 mM phosphate buffer (pH 7) at 25 °C, by spectroscopically monitoring the rate of hydrolysis of substrate adenosine at 265 nm. A total of seven different concentrations of the substrate, ranging 10-40 μM, was employed for each inhibitor concentration that ranged 20-25 μM, while the amount of enzyme in each assay was 0.022 unit. The K_i's were computed from Lineweaver-Burk plots (see **Fig. 3**). Compounds **1** and **6** were both found to be competitive inhibitors of ADA with K_i's of $2.02 \pm 0.5 \times 10^{-5}$ M and $3.79 \pm 0.3 \times 10^{-5}$ M, respectively, while **4, 5, 7,** and **8** were inactive.

CONCLUSIONS

The above results lend support for the following conclusions to be made, although, in the absence of an X-ray structure or an extensive NMR study of the ADA-**1** complex, they may not at this time constitute any more than a mere working hypothesis on the structure-activity relationships of coformycins: (a) The hydrogen bonds of the N-3 sugar moiety may be playing a major role in ascribing the tight-binding characteristics to coformycin in its interaction with ADA, (b) the *anti* conformational orientation of the sugar group is necessary for formation of these H-bonds, (c) the observed significantly lowered ADA inhibitory activity of **1,** as compared with coformycin, is consistent with the possible loss of as many as four hydrogen bonds when **1** binds to ADA, (d) the importance of the sugar H-bonds of coformycin is further corroborated by the N-3 benzyl analogue **6** that lacks these H-bonds, but is nearly as good an inhibitor as **1,** (e) the presence of a benzyl or sugar group at position-3, however, is necessary for activity as the unsubstituted **7** is completely inactive, (f) the hydroxyl group at C-8 is also crucial for activity, since **5** and **8** which contain a carbonyl group at the same position are devoid of activity. Nevertheless, (g) the hydroxyl at C-8 alone does not confer the tight-binding characteristics since compounds **1, 6,** and **7,** which all contain such a functionality, were found to be either reversible, competitive inhibitors of ADA (**1** and **6**) or completely inactive (**7**).

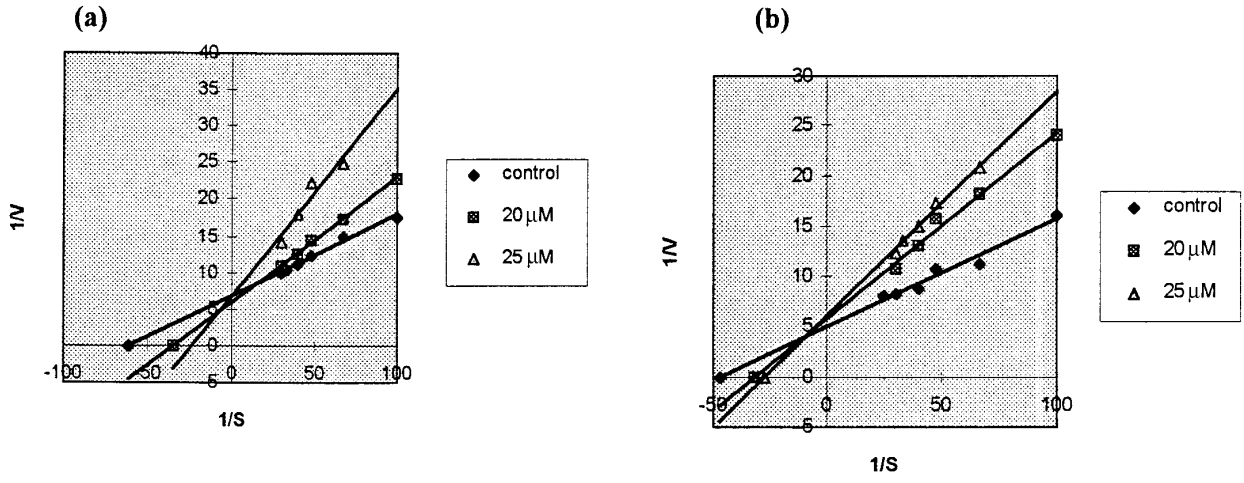


FIG. 3. Lineweaver–Burk plots showing ADA activity of (a) compound **1,** and (b) compound **6.**

IMPLICATIONS

The above research findings may have some important implications in the cancer chemotherapy of coformycins. The antitumor activity of coformycins is known to arise from two different pathways, both of which are dependent upon the ability of coformycins to strongly inhibit ADA: one, coformycins exert synergistic effects upon co-administration with potent antitumor adenosine analogues such as Ara-A,[12a] formycin A,[12b] or cordycepin,[12c] which would otherwise be hydrolyzed by ADA into their inactive inosine counterparts. In the second pathway, coformycins act as immunosuppressants,[13] by mimicking ADA deficiency, to control several lymphoproliferative disorders, including lymphomas, leukemias, and other diseases associated with the hyperimmune system. The ADA deficiency is known to be the principal cause of the severe combined immunodeficiency (SCID) syndrome in children.[14] However, despite their promising therapeutic efficacy in cancer treatment, the severe toxicities of coformycins, including nausea, vomiting, impaired hepatic and renal functions, confusion, emotional instability, seizures, coma, conjunctivitis, hemolysis, hyperuricemia, and vasodilation, have greatly limited their clinical use to-date.[15] These toxicities have now been traced to the nearly irreversible, prolonged inhibition of intracellular ADA, thus requiring synthesis of a new enzyme molecule each time for recovery from toxic effects.[16] Therefore, the search continues for a reversible, less tight-binding, but still a potent inhibitor of ADA, which could permit a shorter duration of action with faster enzyme recovery, and hence, minimized toxicity. To this end, it is necessary to explore the structural parameters of coformycin that give its extremely tight-binding characteristics during interaction with ADA. In this regard, our initial research findings on this subject may pave way for eventually rational structural modifications of coformycins so as to render them less toxic and more suitable for cancer chemotherapy.

ACKNOWLEDGMENTS

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