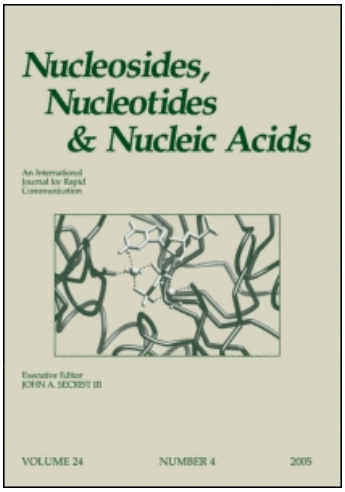

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Inhibitors of Adenosine Deaminase: Continued Studies of Structure-Activity Relationships in Analogues of Coformycin

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Inhibitors of Adenosine Deaminase: Continued Studies of Structure-Activity Relationships in Analogues of Coformycin[†]

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ABSTRACT

Synthesis and adenosine deaminase (ADA) inhibitory activity of two analogues of coformycin, containing the imidazo[4,5-*e*][1,2,4]triazepine ring system, have been reported as part of the structure-activity relationship (SAR) studies to explore the factors responsible for the extremely tight-binding characteristics of coformycins to ADA.

Key Words: Adenosine deaminase; Inhibitors; Coformycin analogues; Ring-expanded (''fat'') purines; Imidazotriazepines.

[†]In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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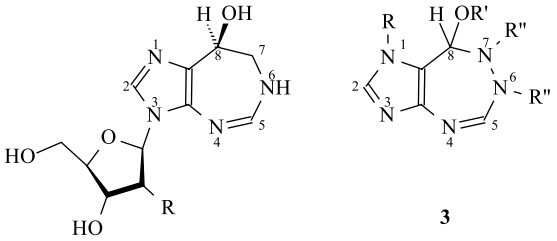
INTRODUCTION

Coformycin (**1**)[1–3] and Pentostatin (**2**)[4–7] are the two strongest known natural inhibitors of adenosine deaminase (ADA) with a K_i ranging 10^{-11}–10^{-13}.[8–13] The observed potent inhibition of ADA has been linked to the known potent antitumor properties of **1** and **2**, in that they mimic ADA deficiency and act as immunosuppressants to control several lymphoproliferative disorders, including lymphomas, leukemias, and other ailments associated with the hyperimmune system.[14,15] In addition, they exert synergistic effects upon co-administration with other antitumor compounds that are analogues of adenosine, such as Ara-A,[16] formycin,[17] or cordycepin,[18] which would otherwise be hydrolyzed by ADA into their inactive inosine counterparts. However, despite their promising therapeutic efficacy in cancer treatment, the severe toxicities of coformycin and pentostatin have greatly limited their clinical use todate.[19–21] These toxicities have now been attributed to their nearly irreversible, prolonged inhibition of intracellular ADA, thus requiring synthesis of a new enzyme molecule each time for recovery from the toxic effects.[22] Therefore, there exists a need for a reversible, somewhat less tight-binding analogues of **1** and **2** that are still potent inhibitors of ADA, but with a shorter duration of action and faster enzyme recovery, resulting in reduced toxicity. While some synthetic analogues of coformycin have been reported,[23–25] an ideal clinical candidate is yet to be discovered. To this end, we have been exploring[26,27] the molecular features of **1** and **2** that are responsible for their extremely tight-binding interaction with ADA, with an anticipation that our findings may eventually aid in rational structural modifications to render them less toxic and more suited for cancer chemotherapy.



3

1; R = OH (Coformycin)
2; R = H (Pentostatin)

a; R = CH_2Ph, R' = R" = CH_3
b; R = CH_2Ph, R' = CH_2CH_3, R" = CH_3

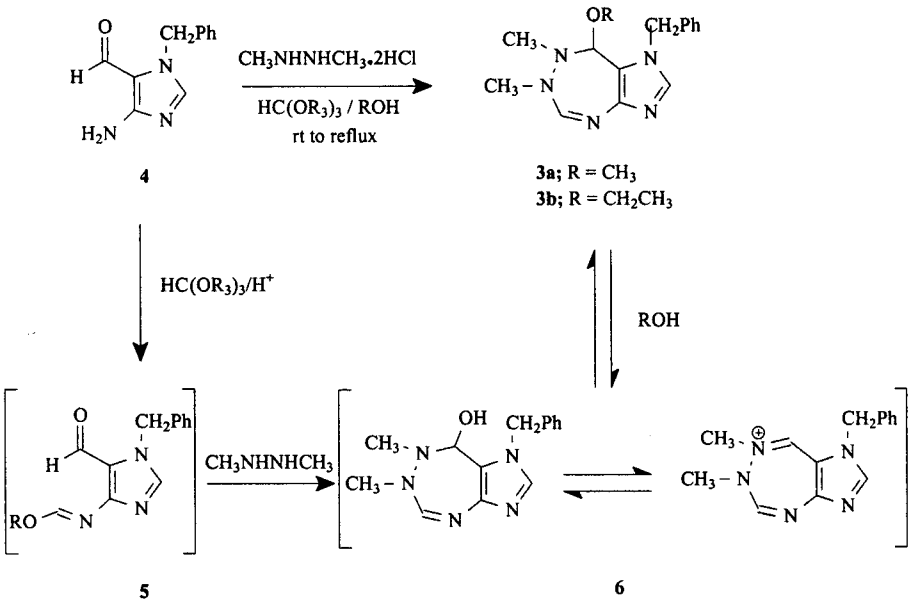
The reported single-crystal X-ray structure of ADA,[28,29] complexed with **2** in the active site of the protein, shows a surprising absence of any hydrogen bonding interactions between the protein and the heterocyclic ring of **2**. The only conspicuous interaction with the heterocycle is a coordination bond between the OH group at position 8 and the active site zinc atom.[28,29] By contrast, multiple H-bonds exist between the OH groups of the sugar moiety of **2** and the amino acid residues of ADA, in particular His 117 and Asp 19, in addition to interaction with an adventitious water molecule in the active site.[28,29] Therefore, the elimination of H-bonding interactions of the sugar hydroxyls, coupled with the weakening of the coordination bond between the 8-OH and zinc, is expected to considerably loosen the deleterious tight-binding

characteristics of **1** and **2** to ADA, alleviating their toxic effects. Compound **3**, the target of the present investigation, has been envisioned to meet both of these requirements because 1) it completely lacks the sugar moiety, and 2) the nucleophilicity of its 8-OH group is somewhat weakened by the electron-withdrawing inductive effect of an additional ring nitrogen atom at position-7. The addition of an aralkyl group at position-1 is based on our earlier structure-activity relationship (SAR) studies, which indicated that the complete removal of the sugar moiety of coformycin leads to inactivity against ADA, while the substitution of a benzyl group in place of the sugar retains a good part of the original activity. We also speculated that a benzyl group at position-1 may be preferred to position-3 as it would avoid the unfavorable hydrophobic interactions of the aromatic ring in a presumably hydrophilic sink where the original sugar hydroxyls of **1** or **2** lie in the active site of ADA. Furthermore, since the 5:7-fused imidazotriazepine ring of **3** is prone to facile opportunistic rearrangements to form a 5:6-fused system[30–34] unless substituted at either or both of the 6- and 7-positions of the triazepine ring, we chose to place a methyl group at each of those positions. We present here our preliminary SAR studies with two analogues **3a** and **3b** bearing the general structural features described above.

RESULTS AND DISCUSSION

Chemistry

Compounds **3a** and **3b** were both synthesized in 26–32% yield in a single-pot reaction (Scheme 1) of 4-amino-1-benzylimidazole-5-carbaldehyde[35] with 1,



Scheme 1.

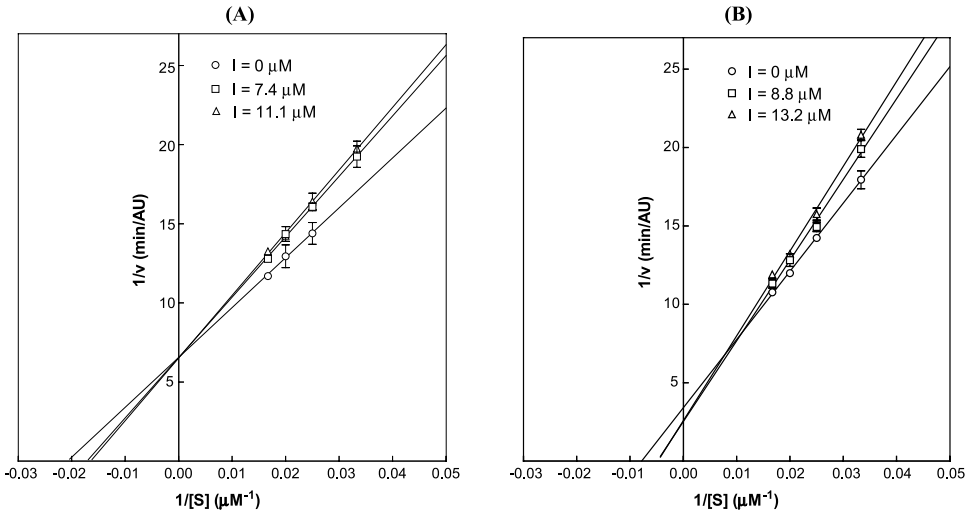


Figure 1. Lineweaver–Burk plots showing ADA inhibitory activity of (A) **3a** and (B) **3b**.

2-dimethylhydrazine dihydrochloride and trimethyl or triethyl orthoformate in the corresponding alcohol used as a solvent. The reaction pathway to the product is believed to involve the initial formation of the imidate **5**, which upon ring-closure would form an equilibrium mixture containing the aminol-iminium species (**6**). The latter upon reaction with the alcohol would form the target imidazotriazepinols **3a** and **3b**.

Both **3a** and **3b** were found to be somewhat unstable, especially when they were completely stripped of the adherent solvent molecules. However, the compounds are reasonably stable as a solution in dimethyl sulfoxide and/or ethanol, or when allowed to retain residual amounts of these solvents during the purification process. Therefore, satisfactory microanalytical data for these compounds could only be obtained by accounting for the adventitious solvent molecules. This observation is also consistent with the reported instability and elemental microanalytical data of the aglycon of pentostatin (**2**), which carried an adventitious molecule of DMSO. In any case, the ^{1}H NMR spectra, coupled with the high resolution mass spectral data, leave little doubt on the accuracy of the molecular structures of **3a** and **3b**. An interesting feature of the ^{1}H NMR spectra of both **3a** and **3b** is the resolution of the two methylene protons of the benzyl groups into two distinct doublets, separated by as much as 0.14 ppm, with a large (15 Hz) coupling constant. This appears to due to the restricted rotation of the methylene protons, imposed by steric interactions with the *peri*-alkoxy group at position-8. Our molecular modeling studies (Insight/Discover)[a] revealed that the alkoxy oxygen atom in **3a** and **3b** lies within 2.5 Å of the methylene protons of the benzyl group. In compound **3b**, restricted rotation could also be discerned in the two

[a]Molecular modeling studies were performed using Insight/DiscoverTM Software package, available from Molecular Simulations, Inc., San Diego, California.

methylene protons of the 8-ethoxy group, as they appeared as two distinct, broad multiplets separated by 0.28 ppm.

Biochemistry

Compounds **3a** and **3b** were screened for inhibition of ADA from bovine spleen (Sigma) in a 50 mM phosphate buffer (pH 7.0) at 25°C by spectrophotometrically monitoring the rate of hydrolysis of the substrate adenosine into product inosine at 265 nm (see Figure 1). The K_i's were computed from Lineweaver–Burk plots. Both **3a** and **3b** were determined to be competitive inhibitors of ADA with K_i's equal to 39 ± 4.9 and 18 ± 4.2 μM, respectively.

CONCLUSION

The observed K_i's of **3a** and **3b** suggest that these inhibitors bind about six to eight orders of magnitude less tightly to ADA than do coformycin and pentostatin ($K_i = 10^{-11}$–10^{-13}).[8–13] The considerably weaker inhibition of **3a** and **3b** compared to coformycin (**1**) or pentostatin (**2**) is consistent with the loss of the sugar hydroxyl-protein hydrogen bonds, which are responsible for part of the extremely tight enzyme binding of **1** and **2**. The slightly greater inhibitory activity of **3b** relative to **3a** may be due to the increased electron-donating inductive effect of the ethyl group compared to methyl, which can result in strengthening the coordination bond between the 8-OH and the active site zinc of ADA. However, it is also possible that the increased steric bulk of the ethyl group over the methyl will offset some of the gain made in bond strength by electronic means. In that context, groups much bulkier than the ethyl may well lessen the inhibitory activity via steric interference in the coordinate bond formation with zinc. It would also be interesting to explore how the hydrophilic groups attached to either the N-1 or the N-3 atom of **3** would affect the overall enzyme binding and inhibition. The preparation of a variety of additional analogues of **3** would thus be necessary to gain clear answers to such questions and to reach a more meaningful SAR on coformycin and its analogues. Such an endeavor is currently in progress in our laboratory.

EXPERIMENTAL SECTION

Organic Synthesis

The ^{1}H NMR spectra were recorded on a General Electric QE-300 NMR spectrometer, equipped with the NMR software Aquerius by Techmag™, and operating at 300 MHz. The data are reported in the following format: Chemical shift (all relative to Me_4Si), multiplicity (s = singlet, d = doublet, dt = doublet of triplet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet, b = broad, coupling constants, integration and assignment). Thin layer chromatography was performed on Merck Kieselgel 60 F_{254} (0.2 mm thickness). Flash column chromatography was performed using 32–63

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mesh silica gel. Elemental microanalyses were performed by Atlantic Microlab, Inc., Norcross, Georgia. Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Mass spectra were recorded either at the Mass Spectral Facility, Department of Biochemistry, Michigan State University, East Lansing, MI or the University of Maryland, College Park, MD. Anhydrous solvents DMF and acetonitrile were purchased and used without further drying. All alcohols and THF were dried over sodium metal, and stored over molecular sieves, type 3 Å and 4 Å, respectively.

1-Benzyl-7,8-dihydro-6*H*-8-methoxy-6,7-dimethylimidazo[4,5-*e*][1,2,4]-triazepine (3a). In a dry round bottom flask were added consecutively **4** (0.08 g, 0.39 mmol), 1,2-dimethylhydrazine dihydrochloride (0.08 g, 0.60 mmol), dry methanol (10–15 mL) and anhydrous trimethyl orthoformate (0.5 mL) (Aldrich). The flask was stirred at room temperature for 1.5 hours and then refluxed for 8 hours until all the starting material disappeared as monitored by TLC. The reaction flask was allowed to cool and then triethylamine (0.3 mL) was added to the flask and allowed to stir for 5 minutes at room temperature. A new spot moving lower than the starting material was observed by TLC [chloroform:methanol (10:1). The contents of the flask were rotary evaporated to near dryness, and a minimal amount of chloroform was added. The chloroform mixture was loaded onto a silica gel column, and eluted successively with chloroform and chloroform-methanol (10:1). The desired fractions were pooled and evaporated to obtain **3a** as a thick brown oil (0.036 g, 32 %). ^{1}H NMR (DMSO-d_6) δ 7.58 (s, 1H, H-2), 7.27–7.34 & 7.14–7.16 (m, 5H, Ar-H), 6.99 (s, 1H, H-5), 5.17 (d, J = 15 Hz, 1H from CH_2Ph), 5.03 (d, J = 15 Hz, 1H from CH_2Ph), 4.85 (s, 1H, H-8), 3.33 (s, 3H, OCH_3), 3.05 (s, 3H, N^6-CH_3), 2.03 (s, 3H, N^7-CH_3); HRMS (FAB) calcd. for $C_{15}H_{20}N_5O$ (MH^+) 286.1668, found m/z 286.1664. *Anal.* calcd. for $C_{15}H_{19}N_5O\cdot$ DMSO-$d_6\cdot$2/3 MeOH: C , 54.31; H, 7.09; N, 17.93. Found: C, 54.68; H, 6.91; N, 17.73.

1-Benzyl-7,8-dihydro-6*H*-8-ethoxy-6,7-dimethylimidazo[4,5-*e*][1,2,4]-triazepine (3b). The procedure and the quantities used are the same as in **3a** above, except that triethyl orthoformate (Lancaster) and dry ethanol replaced trimethyl orthoformate and dry methanol, respectively. Compound **3b** was obtained as a thick brown oil (0.030 g, 26%). ^{1}H NMR (DMSO-d_6) δ 7.58 (s, 1H, H-2), 7.27–7.36 & 7.11–7.13 (m, 5H, Ar-H), 6.99 (s, 1H, H-5), 5.20 (d, J = 15 Hz,1H from CH_2Ph), 5.06 (d, J = 15 Hz, 1H from CH_2Ph), 4.97 (s, 1H, 8-H), 3.75 & 3.47 (bm, 2H, OCH_2), 3.03 (s, 3H, N^6-CH_3), 2.04 (s, 3H, N^7-CH_3), 0.97 (t, 3H, J = 6.0 Hz, ether CH_3); HRMS (FAB) calcd. for $C_{16}H_{22}N_5O$: (MH^+) 300.1824, found m/z 300.1823. Anal. calcd. for $C_{16}H_{21}N_5O\cdot 1/5$ DMSO-$d_6\cdot$1/2 EtOH: C , 61.63; H, 7.44; N, 20.66. Found: C, 61.66; H, 7.14; N, 20.30.

Inhibition Studies

The target compounds were screened against bovine spleen ADA (Sigma) in vitro in 50 mM phosphate buffer (pH 7.0) at 25°C. The rate of hydrolysis of adenosine into inosine was monitored by following the absorbance decrease over 2 minutes at 265 nm using a thermostatted Varian Cary UV-Visible Spectrophotometer (50-BIO). All absorbances were below 1 absorbance unit in a 1 cm path length cuvette. The substrate concentration ranged from 20 to 60 μM, with up to five different concentrations for

each data set. The inhibitor concentration ranged from 7 to 15 μM. The enzyme concentration (0.022 unit) in assay was kept constant. In each assay, the inhibitors were used without pre-incubation with enzyme, and the reactions were initiated by addition of ADA. Assays were performed in duplicate or triplicate determinations. Lineweaver–Burk (1/V vs 1/[S]) plots were constructed for each dataset and the K_i was calculated using GraphPad Prism Software.

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