Adenosine Deaminase Inhibitors. Synthesis and Biological Evaluation of Putative Metabolites of (+)-*erythro*-9-(2*S*-Hydroxy-3*R*-nonyl)adenine

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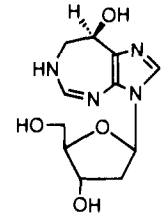
Received March 31, 1994®

The synthesis and biological evaluation of three chain-hydroxylated (+)-*erythro*-9-(2*S*-hydroxy-3*R*-nonyl)adenine [(+)-EHNA] derivatives are reported. Hydroxy groups at positions 9′, 8′, and 8′,9′ (**12**, **25**, and **16**) were introduced by either epoxidation or hydroboration of a terminal olefinic intermediate. Affinities for calf intestinal adenosine deaminase (ADA) were determined from the steady-state inhibition of adenosine deamination. K_i values of 0.82, 3.8, 6.4, and 15.8 nM were estimated for (+)-EHNA, 9′-hydroxy-(+)-EHNA (**12**), 8′-hydroxy-(+)-EHNA (**25**), and 8′,9′-dihydroxy-(+)-EHNA (**16**), respectively, by assuming a single class of binding sites. However, the data for all inhibitors conformed more closely to the kinetics of a heterogeneous system with different affinities for two or more binding sites. The fairly high potencies of **12** and **25** suggest that other substitutions at the terminal position of the nonyl chain could yield useful ADA inhibitors.

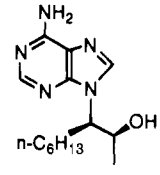
Introduction

Adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4) catalyzes the hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. Inhibition of ADA activity has significant physiological consequences due to accumulation of these substrates. ADA inhibition leads to adenosine receptor-mediated effects on neurological,[1] vascular,[2,3] and blood platelet functions.[4] It potentiates the cardio- and neuroprotective effects of adenosine against ischemia/reperfusion injury.[5–7] As a chemotherapeutic strategy, ADA inhibition potentiates 2′-deoxyadenosine-mediated lymphotoxicity in the treatment of some leukemias[8,9] and could block the inactivation of cytotoxic and antiviral adenosine analogs that are substrates of ADA.[10,11]

The potent ADA inhibitors fall into two classes: purine ribosides or 2′-deoxyribosides, in which the hydrated heterocycle resembles the putative transition state,[12] and compounds that have adenine or a modified congener attached to a hydrophobic substitutent at N-9.[13] 2′-Deoxycoformycin (**1**, dCF, pentostatin) belongs to the former class and *erythro*-9-(2-hydroxy-3-nonyl)-adenine (**2**, EHNA) is the most potent example of the latter. The long and nearly irreversible inhibition of intracellular ADA[14] offers a likely explanation for toxicities observed with dCF therapy. EHNA, on the other hand, is rapidly metabolized,[15,16] and its shorter duration of action allows faster recovery of the enzymic activity. A shorter-acting ADA inhibitor than dCF could permit a controlled duration of chemotherapeutic efficacy while avoiding toxicities associated with long-term inhibition of ADA. Attractive targets would be analogs that are 1–2 orders of magnitude more potent than (+)-EHNA.



1, dCF



2, (+)-EHNA

Racemic EHNA was designed by Schaeffer on the basis of extensive structure–activity relationship (SAR) studies.[17] Later work has shown that the ADA inhibitory activity resides mostly with the 2′*S*,3′*R* *erythro* enantiomer, (+)-EHNA.[18–21] The SAR studies suggested distinct binding pockets for the 1′-methyl and 2′-hydroxy groups and the hydrophobic alkyl chain.[22] The restrictive nature of these binding sites has been confirmed by the recent synthesis of C-1′ and nor-C-1′ derivatives of (+)-EHNA[23] and 3-deaza-(+)-EHNA.[24] On the other hand, little work has been done to study the requirements on the terminal end of the alkyl chain where metabolic hydroxylation is likely to take place. Earlier studies in monkeys[15] and mice[16] revealed the formation of several metabolites which were tentatively identified as hydroxylated derivatives. Herein we report the synthesis of such hydroxylated (+)-EHNA derivatives and their evaluation with calf intestinal ADA. Kinetic studies indicated potent inhibitory activity and heterogeneity of binding sites for (+)-EHNA as well as the title compounds. These hydroxylated compounds may help to identify *in vivo* EHNA metabolites and to assess their effects on adenosine metabolism in intact cells and animals. Furthermore, they may provide leads for the development of (+)-EHNA derivatives with improved pharmacokinetic properties.

Chemistry

The preparation of the two target compounds 9′-hydroxy- and 8′,9′-dihydroxy-(+)-EHNA is outlined in

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® Abstract published in *Advance ACS Abstracts*, October 1, 1994.

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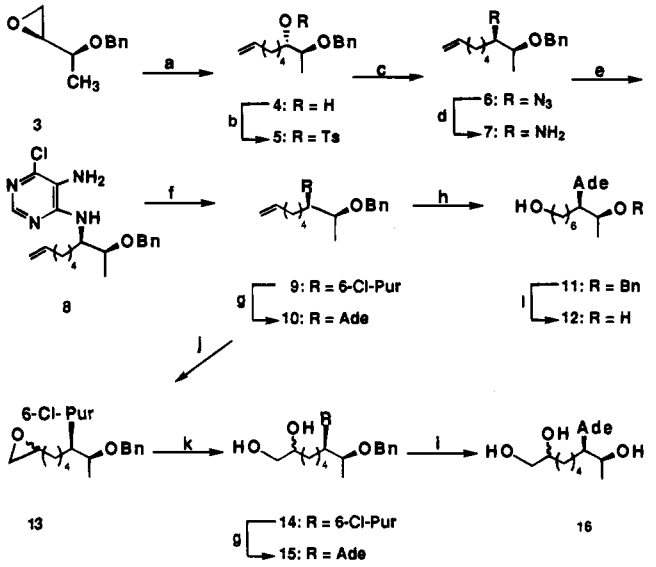
Scheme 1[a]



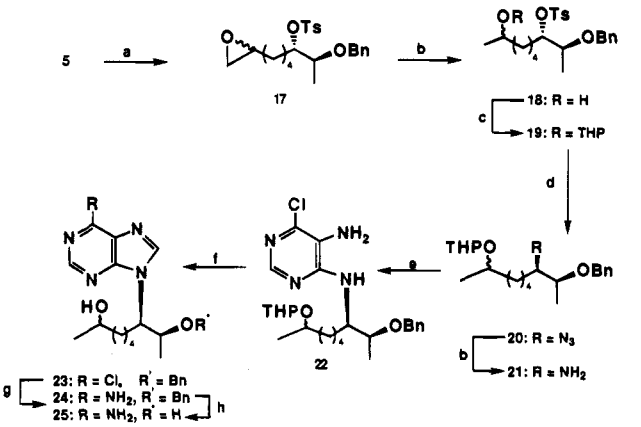
[a] (a) C_5H_9MgBr, Li_2Cl_4Cu, Et_2O, −78 °C, 96%; (b) TsCl, pyr, rt, 97%; (c) NaN_3, DMF, reflux, 96%; (d) LAH, Et_2O, reflux, 96%; (e) ADCP, *n*-Bu_3N, *n*-$C_5H_{11}OH$, reflux, 74%; (f) $HC(OEt)_3$, HCl, rt, 85%; (g) NH_3, 90 °C, 85%; (h) compd **10**, BH_3·THF, rt, NaOH, H_2O_2, 70%; (i) $Pd(OH)_2/C$, C_6H_{10}, EtOH, reflux, 90%; (j) compd **9**, *m*-CPBA, CH_2Cl_2, rt, 90%; (k) 5% $HClO_4$, CH_3CN, rt, 95%.

Scheme 1. Epoxide **3**, which is readily obtained from L-ascorbic acid,[25] was condensed with 4-pentenylmagnesium bromide to give alcohol **4**. The introduction of an azido group at C-3 in **4** by the Mitsunobu procedure proved to be a capricious reaction. Consequently, we resorted to a two-step sequence involving tosylation of **4** followed by azide displacement of the tosyl group in **5** to give azide **6** in excellent overall yield. Lithium aluminum hydride reduction of **6** furnished amine **7** which was condensed with 5-amino-4,6-dichloropyrimidine (ADCP) to give **8**. This, upon condensation with triethyl orthoformate, afforded chloropurine **9**, which, upon treatment with liquid ammonia, gave adenine **10**. 9′-Hydroxy-(+)-EHNA (**12**) was then obtained by oxidative hydroboration of **10** to give **11** and subsequent reductive debenzylation. It is worth mentioning that the successful conversion of **10** to **12** required a 3-fold mole excess of diborane, possibly due to complexation of the reagent with nitrogens in the molecule, and the formation of minor amounts (<10%) of the 8′-hydroxy isomer was also noted.

The synthesis of the 8′,9′-dihydroxy derivative **16** started with chloropurine **9**. Epoxidation with *m*-CPBA gave the diastereomeric epoxide **13**, whose hydration with perchloric acid furnished diol **14**. The final product was then obtained by successive amination and debenzylation, as was the case for **12**.

The final target compound, namely, the 8′-hydroxy derivative **23** (Scheme 2), could not be obtained using one of the suitable 8′-functionalized intermediates (**16**) discussed earlier. The successful approach (Scheme 2) required oxidation at the C-8 of the nonyl chain followed by construction of the purine ring. Thus, epoxidation of tosylate **5** with *m*-CPBA proceeded smoothly to give epoxide **17**. Selective reduction of the oxirane ring with LAH at 0 °C furnished alcohol **18**, which was protected as its tetrahydropyranyl derivative, **19**. Conversion of **19** to the final product followed a sequence identical to

Scheme 2[a]



[a] (a) *m*-CPBA, CH_2Cl_2, rt, 99%; (b) LAH, Et_2O, 0 °C, 95%; (c) DHP, CH_2Cl_2, cat. PPTS, rt, 99%; (d) NaN_3, DMF, reflux, 82%; (e) ADCP, *n*-Bu_3N, *n*-$C_5H_{11}OH$, reflux, 28%; (f) $HC(OEt)_3$, HCl, rt, 60%; (g) NH_3, 90 °C, 90%; (h) $Pd(OH)_2/C$, C_6H_{10}, EtOH, reflux, 90%.

Table 1. Inhibition Constants for Hydroxylated (+)-EHNA Analogs with Calf Intestinal ADA[a]

inhibitor	K_i[b] (nM)
(+)-EHNA	0.82
9′-hydroxy-(+)-EHNA	3.8 ± 0.4
8′-hydroxy-(+)-EHNA	6.4
8′,9′-dihydroxy-(+)-EHNA	15.8 ± 0.4

[a] Steady-state inhibition of adenosine deamination was measured at 30 °C after a 5 min preincubation with the inhibitor. [b] K_i values (means of two or means ± SD of three experiments) were estimated by fitting the data to eq 1 and may represent composites of two or more distinct inhibition constants. See Figure 1 and text for explanation. The ranges of the K_i values for (+)-EHNA and 8′-hydroxy-(+)-EHNA were 0.81−0.83 and 6.4−6.5 nM, respectively.

that outlined earlier in Scheme 1 for the conversion of **5** to the target compounds.

Results and Discussion

The hydroxylated (+)-EHNA analogs were tested as inhibitors of calf intestinal mucosa ADA by employing a wide range of inhibitor concentrations and a fixed concentration of adenosine as substrate. Analysis of the data according to eq 1 (see the Experimental Section), which assumes a single class of inhibitor binding sites, yielded the K_i values listed in Table 1. The results indicate that hydroxylation at the terminal carbon of (+)-EHNA causes decreased affinity and confirm the hydrophobic nature of the binding pocket for the aliphatic chain. The 9′-hydroxylated analog displays a ~5-fold increase in K_i, relative to (+)-EHNA. When the hydroxyl group is shifted one carbon closer to the purine, to the 8′-position, a further 1.7-fold loss in affinity occurs, for an overall 8-fold increase in K_i. The 8′,9′-dihydroxylated compound shows a 2.5-fold loss in affinity relative to 8′-hydroxy-EHNA, for an overall 20-fold increase in K_i. The modest differences in affinity among the three hydroxylated analogs resemble those reported by Schaeffer's laboratory for 9-*n*-alkyl- or 9-(*n*-hydroxyalkyl)adenines.[26] In their studies, truncation of the alkyl chain caused at most a 2.8-fold decresae in affinity per carbon unit. This suggests the loss of hydrophobic interactions at the chain terminus but little interference with overall inhibitor binding.

The inhibition data used to calculate the K_i values in Table 1 did not fit simple hyperbolic curves. This is

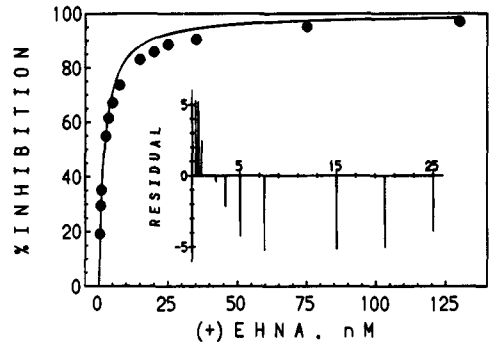


Figure 1. Complex kinetics of ADA inhibition by (+)-EHNA. Steady-state inhibition of adenosine deamination was determined at 30 °C after a 5 min preincubation of calf intestinal mucosa ADA with the inhibitor. The curve represents the best fit to eq 1, which yielded a K_i value of 0.81 nM. The inset is a plot of residuals from nonlinear regression analysis vs (+)-EHNA concentration. Positive deviations from the curve are seen at (+)-EHNA concentrations below 1.5 nM; negative deviations occur at all higher concentrations (only the 0.4–25 nM range is shown). Equation 2 gave a smooth fit, with randomly deviating data points, and yielded K_{i1} = 0.45 nM and K_{i2} = 4.7 nM. For this curve, A = 0.75 and may represent the fraction of inhibition due to high-affinity binding. The R^2 values for this curve were 0.989 and 1.000 for eqs 1 and 2, respectively.

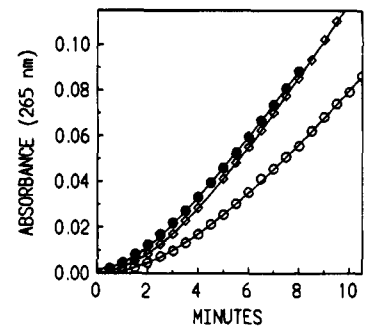


Figure 2. Dissociation of ADA from its complex with (+)-EHNA or 9′-hydroxy-(+)-EHNA. ADA (100 nM) was preincubated with 200 nM (+)-EHNA or 9′-hydroxy-(+)-EHNA for 5 min at the assay temperature before it was diluted 500-fold into a cuvette containing 70 μM 2′-deoxyadenosine. Dissociation of ADA from (+)-EHNA at 25 °C (○) and 30 °C (●) or from 9′-hydroxy-(+)-EHNA at 25 °C (◇) was revealed by the time-dependent increase in enzymic activity until a steady-state rate of deamination was reached (linear portion of the curve). Data analysis and fitting to eq 3, as described in the Experimental Section, generated the curves shown and yielded the following dissociation rate constants: (+)-EHNA, 0.0033 s^{-1} (25 °C) and 0.0045 s^{-1} (30 °C); 9′-hydroxy-(+)-EHNA, 0.007 s^{-1} (25 °C).

illustrated for (+)-EHNA in Figure 1, where the data points deviate systematically from the theoretical curve generated by eq 1. As shown in the Figure 1 inset, the observed inhibition was higher than the calculated values at (+)-EHNA concentrations that caused less than 50% inhibition, and the reverse was true at higher concentrations. Likewise, use of the equation for a partially competitive inhibitor[27] resulted in systematic deviations of the data points (not shown). On a linearized Dixon plot ($1/v$ vs I), the points yielded a curved line (not shown). In contrast, eq 2 (see the Experimental Section), which assumes two binding sites with different affinities, provided a good fit. More than 70% of the inhibition appeared to be due to high-affinity binding sites with a K_{i1} value of 0.44 nM. The K_{i2} value estimated for the low-affinity sites was 4.3 nM. The mean K_i value of 0.82 nM (Table 1), which was determined with single-site analysis, may reflect the relative contributions of two (or more) classes of binding interactions. The K_{i2} value is closer to the K_i value of 3.2 nM determined previously for calf intestinal ADA from the slopes of double-reciprocal plots of rates obtained in the presence of 2–8 nM (+)-EHNA.[21] The failure to notice biphasic inhibition kinetics in earlier studies may have resulted from the small number and limited range of inhibitor concentrations used in those experiments.

The inhibition kinetics observed for the hydroxylated (+)-EHNA analogs were even more markedly biphasic (curves not shown). Equation 2 again provided a good fit for the data, and the estimated K_i values for 9′-hydroxy-, 8′-hydroxy-, and 8′,9′-dihydroxy-EHNA at high-affinity sites were respectively 2.5 ± 0.6, 1.6 (mean of 1.22 and 1.69), and 8.7 ± 3.2 nM and the percent inhibitions due to high-affinity binding were 83 ± 7, 39 (mean of 32 and 45), and 66 ± 22. The estimated parameters for binding at low-affinity sites varied greatly from one experiment to the next with 9′-hydroxy- and 8′,9′-dihydroxy-EHNA. For 8′-hydroxy-EHNA, low-affinity K_i values of 12.2 and 14.8 nM were estimated in the two separate experiments. However, the estimated K_i values for binding at low-affinity sites varied greatly for each compound from one experiment to the next. Since hydroxylation of the 8′-position results in a diasteriomeric mixture, it could be argued that the biphasic inhibition kinetics of 8′-hydroxy-EHNA and 8′,9′-dihydroxy-EHNA reflect the binding of the two diasteriomers. This argument does not apply to (+)-EHNA and 9′-hydroxy-(+)-EHNA, which are optically pure. Although the 9′-hydroxy-(+)-EHNA preparation contained 8′-hydroxy-(+)-EHNA as a contaminant (<10%), this had little influence on the kinetics since "spiking" of the 9′-hydroxy-(+)-EHNA with an additional 10% of the contaminant did not significantly alter the appearance of the curve or the estimated K_i values.

These results suggest that there are two (or more) distinct calf ADA proteins or that a single ADA molecule can bind flexible molecules like (+)-EHNA and some of its derivatives in two (or more) orientations. In order to determine whether the apparent high- and low-affinity binding sites are distinguishable by their dissociation rate constants, the dissociation of (+)-EHNA from calf intestinal ADA was measured at 25 and 30 °C, employing 2′-deoxyadenosine as substrate. Figure 2 shows that the data points closely follow a monophasic dissociation curve (eq 3, Experimental Section). The dissociation rate constants were estimated to be 0.0033 and 0.0045 s^{-1}, respectively. In comparison, k_{-1} for 9′-hydroxy-(+)-EHNA at 25 °C was 0.007 s^{-1}. Thus, the dissociation kinetics do not support the presence of two or more classes of binding sites, although both high- and low-affinity sites should be occupied since 100 nM ADA was preincubated with 200 nM (+)-EHNA. As regards the inhibitor association rates, it was recently shown that the pseudo-first-order rate constant increases linearly with (+)-EHNA concentrations. Association of 5–30 μM (+)-EHNA with 1.8 μM calf intestinal ADA (Boehringer Mannheim) was monitored by an increase in absorbance at 280 nM.[28] That experimental protocol precluded the use of nanomolar inhibitor concentrations.

The steady-state kinetic analysis employed in the present study suggests that two or more EHNA-binding sites exist. A mixture of enzyme–inhibitor complexes could be due to heterogeneity of the enzyme preparation or to the ability of different EHNA conformations to

interact with different binding energies. Other experimental approaches are needed to confirm or refute multiple binding sites and to determine whether association kinetics or some other factor(s) are responsible for the biphasic inhibition pattern. The X-ray structure of calf intestinal ADA complexed to a transition-state inhibitor has been determined.[29] To date, however, there has been no report on the structure of an ADA–(+)-EHNA complex.

Experimental Section

(2S,3S)-2-(Benzyloxy)non-8-en-3-ol (4). A solution of epoxide **3** (2 g, 11.24 mmol) in ether (50 mL) was added to a cold (−78 °C) ether solution containing 1-pentenylmagnesium bromide [22.5 mmol, prepared by reacting magnesium (0.66 g, 22.5 mmol) and 5-bromopentene (4.11 g, 22.5 mmol)] and 0.1 mmol of lithium tetrachlorocuprate, while stirring was continued for 1 h. The reaction was quenched with a saturated solution of NH_4Cl (100 mL) and the mixture extracted with ether. Pure **4** (2.67 g, 96%) was obtained by silica gel column chromatography eluting with EtOAc–hexanes (5:95): $[\alpha]^{25}_D$ + 2.09° (c 1.65, EtOH); ^1H NMR ($CDCl_3$) δ 1.13 (d, J = 6 Hz, 3H), 1.13–1.28 (m, 6H), 1.81–2.28 (m, 2H), 2.45–2.68 (bs, 1H, D_2O exchangeable), 3.05–3.65 (m, 2H), 4.25–4.75 (q_{AB}, J = 12 Hz, 2H), 4.75–5.05 (m, 2H), 5.45–5.98 (m, 1H), 7.25 (s, 5H). Anal. ($C_{16}H_{24}O_2$) C, H.

[(2S,3S)-2-(Benzyloxy)non-8-en-3-yl]-*p*-toluenesulfonate (5). To a stirred solution of the alcohol **4** (5.4 g, 21.7 mmol) in pyridine (10 mL) was added *p*-toluenesulfonyl chloride (4.5 g, 23.9 mmol), and stirring was continued for 12 h at room temperature. The mixture was poured into water (100 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic solutions were then washed with cold 1 N HCl (2 × 50 mL) and water (2 × 100 mL), dried ($MgSO_4$), and filtered. Removal of solvent left an oil, which was purified by silica gel chromatography eluting with EtOAc–hexane (1:50) to afford **5** (8.35 g, 97%): $[\alpha]^{25}_D$ +11.46° (c 2.62, EtOH); ^1H NMR ($CDCl_3$) δ 1.13 (d, J = 6 Hz, 3H), 1.13–2.1 (m, 8H), 2.43 (s, 3H), 3.5–3.83 (m, 1H), 4.23–4.63 (q_{AB}, J = 9 Hz, 2H), 4.46–4.66 (m, 1H), 4.76–5.03 (m, 2H), 5.4–5.93 (m, 1H), 7.18 (d, J = 7.5 Hz, 2H), 7.23 (s, 5H), 7.73 (d, J = 7.5 Hz, 2H). Anal. ($C_{23}H_{30}O_4S$) C, H, S.

(2S,3R)-3-Azido-2-(benzyloxy)non-8-ene (6). Sodium azide (1.027 g, 15.8 mmol) was added to a stirred solution of **5** (4.0 g, 13.2 mmol) in anhydrous DMF (20 mL). After the solution had refluxed for 45 min, DMF was removed and pure **6** (2.61 g, 96%) was obtained by silica gel column chromatography eluting with EtOAc–hexanes (5:95): $[\alpha]^{25}_D$ +12.45° (*c* 2.5, EtOH); ^1H NMR ($CDCl_3$) δ 1.2 (d, J = 6 Hz, 3H), 1.3–1.8 (m, 6H), 1.9–2.23 (m, 2H), 3.24–3.7 (m, 2H), 4.5 (q_{AB}, J = 7.5 Hz, 2H), 4.76–5.13 (m, 2H), 5.46–6.03 (m, 1H), 7.23 (s, 5H). Anal. ($C_{16}H_{23}N_3O$) C, H, N.

[(2S,3R)-2-(Benzyloxy)non-8-en-3-yl]amine (7). To a stirred solution of lithium aluminum hydride (LAH; 0.25 g, 5.2 mmol) in anhydrous ether (50 mL) was added, dropwise, a solution of azide **6** (1 g, 3.66 mmol) in anhydrous ether (50 mL). The reaction mixture was then heated at reflux for 2 h and cooled to room temperature, and excess LAH was decomposed by the careful successive dropwise addition of water (0.25 mL), 15% NaOH (0.24 mL), and water (0.5 mL). Filtration, drying, and evaporation of the solvent gave a pure colorless liquid, **7** (0.87 g, 96%): $[\alpha]^{25}_D$ +17.66° (*c* 5.37, EtOH); ^1H NMR ($CDCl_3$) δ 1.3 (d, J = 6 Hz, 3H), 0.96–1.66 (m, 8H, 2 D_2O exchangeable), 1.86–2.26 (m, 2H), 2.76–3.03 (m, 1H), 3.23–3.56 (m, 1H), 4.5 (q_{AB}, J = 7.5 Hz, 2H), 4.76–5.1 (m, 2H), 5.5–6.03 (m, 1H), 7.26 (s, 5H). Anal. ($C_{16}H_{25}NO$) C, H, N.

(2S,3R)-(5-Amino-6-chloropyrimidin-4-yl)[2-(benzyloxy)non-8-en-3-yl]amine (8). 5-Amino-4,5-dichloropyrimidine (ADCP; 0.39 g, 2.336 mmol), *n*-Bu_3N (0.433 g, 2.336 mmol), and **7** (0.5907 g, 2.336 mmol) in anhydrous pentanol (10 mL) were heated at reflux for 48 h under an N_2 atmosphere. Pentanol and *n*-Bu_3N were removed, and the residue was chromatographed over silica gel (EtOAc–hexanes, 1:10) to give **8** (0.65 g, 74%): $[\alpha]^{25}_D$ +38.4° (c 1.775, EtOH); ^1H NMR ($CDCl_3$) δ 1.2 (d, J = 6 Hz, 3H), 1.1–2.23 (m, 8H), 3.3–3.9 (m, 3H, 2 D_2O exchangeable), 4.43 (q_{AB}, J = 13.5 Hz, 2H), 4.00–4.40 (bs, 1H, D_2O exchangeable), 4.66–5.36 (m, 3H), 5.40–5.96 (m, 1H), 7.23 (s, 5H), 7.90 (s, 1H). Anal. ($C_{20}H_{27}ClN_4O$) C, H, N, Cl.

(2S,3R)-3-(6-Chloropurin-9-yl)-2-(benzyloxy)non-8-ene (9). An acidified (concentrated HCl, 0.3 mL) solution of **8** (0.58 g, 1.55 mmol) in triethyl orthoformate (15 mL) was stirred at room temperature for 24 h. The yellow oil obtained after removal of TEOF was purified by silica gel column chromatography eluting with EtOAc–hexanes (1:10) to provide **9** (0.5 g, 85%): $[\alpha]^{25}_D$ +54.49° (c 3.74, EtOH); ^1H NMR ($CDCl_3$) δ 1.26 (d, J = 6 Hz, 3H), 0.73–1.60 (m, 4H), 1.60–2.40 (m, 4H), 3.53–4.00 (m, 1H), 4.4 (q_{AB}, J = 13.5 Hz, 2H), 4.5–5.03 (m, 3H), 5.33–5.9 (m, 1H), 7.13 (s, 5H), 8.23 (s, 1H), 8.63 (s, 1H). Anal. ($C_{21}H_{25}ClN_4O$) C, H, N, Cl.

(2S,3R)-3-(6-Aminopurin-9-yl)-2-(benzyloxy)non-8-ene (10). Compound **9** (0.3 g, 0.78 mmol) was dissolved in liquid ammonia (15 mL) and heated at 90 °C in a steel bomb for 24 h. After cooling, excess ammonia was allowed to evaporate. The residue was taken up in CH_2Cl_2 (25 mL) and washed with water (10 mL). The organic layer was dried, and pure **10** (0.245 g, 85%) was obtained as a white solid: $[\alpha]^{25}_D$ +68.01° (c 1.375, EtOH); ^1H NMR ($CDCl_3$) δ 1.20 (d, J = 6 Hz, 3H), 0.65–1.60 (m, 4H), 1.66–2.26 (m, 4H), 3.56–4.00 (m, 1H), 4.4 (q_{AB}, J = 6 Hz, 2H), 4.43–5.03 (m, 3H), 5.33–5.93 (m, 1H), 6.03–6.40 (bs, 2H, D_2O exchangeable), 7.16 (s, 5H), 7.9 (s, 1H), 8.26 (s, 1H). Anal. ($C_{21}H_{27}N_5O$) C, H, N.

(2S,3R)-3-(6-Aminopurin-9-yl)-2-(benzyloxy)nonan-9-ol (11). To a solution of the olefin **10** (0.365 g, 1 mmol) in dry THF (1 mL), placed in a three-necked flask fitted with a condenser and a septum, was added a 1 M solution of BH_3·THF (3 mL, 3 mmol) at 0 °C. The reaction was done under nitrogen atmosphere. The mixture was permitted to stir for additional hours at room temperature to continue the completionof the reaction. Water (0.05 mL) was added slowly, and the mixture was allowed to stir at room temperature until hydrogen no longer evolved. The flask was immersed in an ice bath, and 3 M NaOH (1 mL) was rapidly added to the reaction mixture. The organoboronic acid intermediate was oxidized by the slow addition of 30% hydrogen peroxide (1 mL). The reaction mixture was then allowed to stir for 3 h at 50 °C to ensure completion of the oxidation. The mixture was brought to room temperature, and NaCl was added to saturate the lower aqueous phase. The THF phase was separated and dried ($MgSO_4$). The crude compound obtained after solvent removal was purified by silica gel column chromatography eluting with EtOAc to provide 11 (0.268 g, 70%): $[\alpha]^{25}_D$ +59.04° (c 0.965, EtOH); ^1H NMR ($CDCl_3$) δ 0.76–1.80 (m, 11H), 1.8–2.36 (m, 2H), 3.30–4.80 (m, 7H, 1 D_2O exchangeable), 6.26 (bs, 2H, D_2O exchangeable), 7.30 (s, 5H), 7.86 (s, 1H), 8.26 (s, 1H). Anal. ($C_{21}H_{29}N_5O_2$) C, H, N.

(2S,3R)-3-(6-Aminopurin-9-yl)nonane-2,9-diol (12). A solution of compound 11 (0.227 g, 0.593 mmol) in EtOH (25 mL) and cyclohexene (10 mL) was treated with 20% palladium hydroxide on carbon [$Pd(OH)_2$/C, 0.05 g]. The resulting suspension was stirred at reflux for 12 h. After cooling to room temperature, the mixture was filtered and the filtrate was concentrated at reduced pressure. The residue was chromatographed over silica gel (EtOAc–MeOH, 9:1) to give pure **12** (0.156 g, 90%): $[\alpha]^{25}_D$ +32.2° (c 0.145, EtOH); ^1H NMR (D_2O) δ 0.79–1.22 (m, 11H), 1.77–1.89 (m, 2H), 3.26–3.31 (m, 2H), 3.93–4.01 (m, 1H), 4.22–4.29 (m, 1H), 8.04 (s, 1H), 8.06 (s, 1H). Anal. ($C_{14}H_{23}N_5O_2$) C, H, N.

(2S,3R)-2-(Benzyloxy)-3-(6-chloropurin-9-yl)-8,9-epoxynonane (13). To an ice cold solution of the olefin **9** (0.769 g, 2 mmol) in CH_2Cl_2 (15 mL) was added 85% *m*-chloroperbenzoic acid (0.488 g, 2.4 mmol). After stirring the reaction mixture at room temperature overnight, it was diluted with ether (50 mL), washed successively with saturated $NaHCO_3$ (15 mL), 10% $NaHSO_3$ (15 mL), saturated $NaHCO_3$ (15 mL), and brine, and dried ($MgSO_4$). The residue obtained after evaporation of ether was purified by silica gel column chromatography eluting with EtOAc–hexanes (1:10) to afford pure epoxide **13** (0.721 g, 90%): ^1H NMR ($CDCl_3$) δ 1.30 (d, J = 6 Hz, 3H), 0.76–1.76 (m, 6H), 1.83–2.5 (m, 3H), 2.56–2.96 (m, 2H), 3.66–4.06 (m, 1H), 4.63 (q_{AB}, J = 13.5 Hz, 2H), 4.63–4.86 (m, 1H), 7.19 (s, 5H), 8.26 (s, 1H), 8.66 (s, 1H). Anal. ($C_{21}H_{25}ClN_4O_2$) C, H, N, Cl.

(2S,3R)-2-(Benzyloxy)-3-(6-chloropurin-9-yl)nonane-8,9-diol (14). A solution of compound **13** (0.3 g, 0.75 mmol) and 5% $HClO_4$ (2 mL) in acetonitrile (6 mL) was stirred at room temperature for 2 h. The reaction mixture was neutralized with solid $NaHCO_3$, and the mixture was filtered. The filtrate was diluted with CH_2Cl_2 (25 mL) and dried over $MgSO_4$. The residue obtained after solvent evaporation was purified by silica gel column chromatography using EtOAc–hexanes (1:1) to provide diol **14** (0.3 g, 95%): ^1H NMR ($CDCl_3$) δ 0.70–1.66 (m, 9H), 1.66–2.43 (m, 2H), 2.5–3.96 (m, 6H, 2 D_2O exchangeable), 4.36 (q_{AB}, J = 13.5 Hz, 2H), 4.4–4.7 (m, 1H), 7.12 (s, 5H), 8.2 (s, 1H), 8.53 (s, 1H). Anal. ($C_{21}H_{27}ClN_4O_3$) C, H, N, Cl.

(2S,3R)-3-(6-Aminopurin-9-yl)-2-(benzyloxy)nonane-8,9-diol (15). Compound **15** was obtained from **14** according to the procedure described for the preparation of **10** in 90% yield: ^1H NMR ($CDCl_3$) δ 0.60–1.60 (m, 9H), 1.60–2.30 (m, 2H), 2.93–4.86 (m, 9H), 6.43 (bs, 2H, D_2O exchangeable), 7.13 (s, 5H), 7.83 (s, 1H), 8.13 (s, 1H). Anal. ($C_{21}H_{29}N_5O_3$) C, H, N.

(2S,3R)-3-(6-Aminopurin-9-yl)nonane-2,8,9-triol (16). Triol **16** was prepared in 90% yield by debenzylating **15** using the procedure described for **12**: ^1H NMR (D_2O) δ 0.89–1.24 (m, 9H), 1.87–1.96 (m, 2H) 3.19–2.41 (m, 3H), 3.98–4.02 (m, 1H), 4.28–4.33 (m, 1H), 8.08 (s, 1H), 8.11 (s, 1H). Anal. ($C_{14}H_{23}N_5O_3 \cdot 1.1H_2O$) C, H, N.

[(2S,3S)-2-(Benzyloxy)-8,9-epoxynon-3-yl]-p-toluenesulfonate (17). Epoxidation of **5** was carried out as described for compound **13**. After workup, the residue was chromatographed using hexane–EtOAc (7:3) to give the epoxide **17** (99%) as an oil: ^1H NMR ($CDCl_3$) δ 1.06 (d, J = 6 Hz, 3H), 1.1–1.96 (m, 8H), 2.36 (s, 3H), 2.33–2.56 (m, 1H), 2.59–2.93 (m, 2H), 3.4–3.83 (m, 1H), 4.39 (q_{AB}, J = 9 Hz, 2H), 4.53–4.70 (m, 1H), 7.23 (s, 5H), 7.18 (d, J = 6 Hz, 2H), 7.70 (d, J = 6 Hz, 2H). Anal. ($C_{23}H_{30}O_5S$) C, H, S.

[(2S,3S)-2-(Benzyloxy)-8-hydroxynon-3-yl]-p-toluenesulfonate (18). A solution of lithium aluminum hydride (30 mL, 15 mmol, 1 M solution in THF) was added to epoxide **17** (3.143 g, 7.52 mmol) in dry ether (100 mL) at 0 °C. The mixture was stirred at room temperature for 1 h and decomposed slowly by the addition of water (25 mL), and the aqueous solution was extracted with ether (4 × 50 mL). The combined ether extracts were dried ($MgSO_4$) and concentrated to furnish **18** (3 g, 95%). An analytical sample was obtained by silica gel column chromatography with hexane–EtOAc (7:3) as eluent: ^1H NMR ($CDCl_3$) δ 0.80–1.93 (m, 15H, 1 D_2O exchangeable), 2.36 (s, 3H), 3.30–3.96 (m, 2H), 4.4 (q_{AB}, J = 7.5 Hz, 2H), 4.43–4.80 (m, 1H), 7.16 (d, J = 9 Hz, 2H), 7.23 (s, 5H), 7.7 (d, J = 9 Hz, 2H). Anal. ($C_{23}H_{32}O_5S$) C, H, S.

[(2S,3S)-2-(Benzyloxy)-8-[(2-tetrahydropyranyl)oxy]-non-3-yl]-p-toluenesulfonate (19). A solution of alcohol **18** (2.8 g, 6.6667 mmol) and dihydropyran (1.68 g, 13.33 mmol) in dry CH_2Cl_2 (50 mL) containing pyridinium p-toluenesulfonate (PPTS; 0.33 g, 1.33 mmol) was stirred at room temperature for 4 h. The reaction mixture was diluted with ether, washed once with half-saturated brine to remove the catalyst PPTS, and dried ($MgSO_4$). The residue obtained, after evaporation of the solvent, was purified by silica gel column chromatography using hexane–EtOAc (95:5) as eluent to afford **19** (3.3 g, 99%): ^1H NMR ($CDCl_3$) δ 0.65–2.03 (m, 20H), 2.38 (s, 3H), 3.18–4.11 (m, 4H), 4.40 (q_{AB}, J = 9 Hz, 2H), 4.5–5.78 (m, 2H), 7.18 (d, J = 9 Hz, 2H), 7.26 (s, 5H), 7.73 (d, J = 9 Hz, 2H). Anal. ($C_{28}H_{40}O_6S$) C, H, S.

(2S,3R)-3-Azido-2-(benzyloxy)-8-[(2-tetrahydropyranyl)oxy]nonane (20). Compound **20** was prepared from **19** following the procedure described for the formation of **6**. The crude product, after column chromatography (hexane–EtOAc, 95:5), gave pure **20** (82%): ^1H NMR ($CDCl_3$) δ 0.88–2.32 (m, 20H), 3.15–4.05 (m, 5H), 4.51 (q_{AB}, J = 10.5 Hz, 2H), 4.52–4.72 (m, 1H), 7.22 (s, 5H). Anal. ($C_{21}H_{33}N_3O_3$) C, H, N.

[(2S,3R)-2-(Benzyloxy)-8-[(2-tetrahydropyranyl)oxy]-nonan-3-yl]amine (21). The azide **20** was reduced by a procedure similar to that described for the preparation of **7** to afford the amine **21** quantitatively: ^1H NMR ($CDCl_3$) δ 0.76–1.23 (m, 22H, 2 D_2O exchangeable), 2.63–4.06 (m, 5H), 4.42 (q_{AB}, J = 7.5 Hz, 2H), 4.46–4.73 (m, 1H), 7.23 (s, 5H). Anal. ($C_{21}H_{35}NO_3$) C, H, N.

(2S,3R)-(5-Amino-6-chloropyrimidin-4-yl)[2-(benzyloxy)-8-[(2-tetrahydropyranyl)oxy]non-3-yl]amine (22). Compound **22** was prepared from **21**, by following the procedure described for the formation of **8**. The residue obtained after solvent removal was chromatographed over silica gel (EtOAc–hexanes, 1:5) to give **22** (28%): ^1H NMR ($CDCl_3$) δ 0.63–2.33 (m, 20H), 3.03–5.13 (m, 11H, 3 D_2O exchangeable), 7.13 (s, 5H), 7.83 (s, 1H). Anal. ($C_{25}H_{37}ClN_4O_3$) C, H, N, Cl.

(2S,3R)-2-(Benzyloxy)-3-(6-chloropurin-9-yl)nonan-8-ol (23). An acidified (concentrated HCl, 0.15 mL) solution of **22** (0.3 g, 0.63 mmol) in TEOF (15 mL) was stirred at room temperature for 24 h. The residue obtained, after removal of TEOF, was dissolved in absolute ethanol (10 mL), containing PPTS (0.025 g), and refluxed for 1 h. Solvent was then removed under reduced pressure, and the crude product was purified by silica gel column chromatography using EtOAc–hexanes (3:1) to give **23** (0.15 g, 60%): ^1H NMR ($CDCl_3$) δ 0.66–2.42 (m, 15H, 1 D_2O exchangeable), 3.36–3.96 (m, 2H), 4.4 (q_{AB}, J = 13.4 Hz, 2H), 4.46–4.79 (m, 1H), 7.19 (s, 5H), 8.26 (s, 1H), 8.62 (s, 1H). Anal. ($C_{21}H_{27}ClN_4O_2$) C, H, N, Cl.

(2S,3R)-3-(6-Aminopurin-9-yl)-2-(benzyloxy)nonan-8-ol (24). Amination of chloropurine derivative **23** was carried out as for the synthesis of **10** from **9**. Crude product, obtained after workup, was purified by silica gel column chromatography eluting with meOH–EtOAc (5:95) to give **24** (90%): ^1H NMR ($CDCl_3$) δ 0.70–1.60 (m, 12H), 1.63–2.33 (m, 2H), 2.80 (bs, 1H, D_2O exchangeable), 3.33–4.00 (m, 2H), 4.30 (q_{AB}, J = 13.5 Hz, 2H), 4.33–4.66 (m, 1H), 6.23 (bs, 2H, D_2O exchangeable), 7.13 (s, 5H), 7.83 (s, 1H), 8.20 (s, 1H). Anal. ($C_{21}H_{29}N_5O_2$) C, H, N.

(2S,3R)-3-(6-Aminopurin-9-yl)nonane-2,8-diol (25). Diol **25** was prepared in 90% yield by debenzylating **24** using the procedure described for **12**: ^1H NMR (D_2O) δ 0.71–1.10 (m, 12H), 1.77–1.88 (m, 2H), 3.38–3.54 (m, 1H), 3.90–3.98 (m, 1H), 4.19–4.26 (m, 1H), 8.02 (s, 1H), 8.08 (s, 1H). Anal. ($C_{14}H_{23}N_5O_2$) C, H, N.

Biological Evaluation. The hydroxylated analogs were tested only in the presence of 25 μM substrate. (+)-EHNA was tested in the presence of 25 and 50 μM adenosine and yielded the same kinetics at both substrate concentrations. For convenience, 50 μM adenosine was used with (+)-EHNA for all the results described above. Unless stated otherwise, kinetic studies were performed at 30 °C in a Gilford Model 240 spectrophotometer equipped with a water-jacketed cuvette chamber. Deamination of ADA substrates was monitored by the decrease in absorbance at 265 nm.[30,31] (+)-EHNA and its analogs were tested with calf intestinal mucosa ADA (type VI; Sigma). ADA stock solutions were made daily by direct dilution of the glycerol-stabilized commercial preparations into a stabilizing buffer of 50 mM potassium phosphate, pH 7.0, containing 2% poly(ethylene glycol) (PEG) 3350. Enzyme concentration was estimated from the stated protein content and a molecular weight of 34 000.

Steady-State Kinetics. Aliquots of ADA stock solutions were preincubated for 5 min at the assay temperature with different concentrations of analogs to permit steady-state association of enzyme with a semi-tight-binding competitive inhibitor.[13,21] Upon addition of 50 μL of substrate, the 1 mL reaction mixtures contained 25 or 50 μM adenosine, 50 mM phosphate, pH 7.0, and approximately 0.3 nM ADA. Linear reaction rates were achieved 1–2 min after substrate addition and fitted to the equation for a competitive inhibitor

$$1 - v/v_0 = I/(K_{app} + I) \quad (1)$$

wherein I is inhibitor concentration and v and v_0 are the observed rates in the presence and absence of inhibitor, respectively. Because the data deviated systematically from the curve generated by eq 1, they were analyzed by the equation for competitive inhibition at two binding sites

$$(1 - v/v_0) = A \cdot I/(K_{app1} + I) + B \cdot I/(K_{app2} + I) \quad (2)$$

wherein variables A and B are the fractional contribution of each site to the total inhibition. The K_i values for competitive inhibition at one or two binding sites were calculated from the relation $K_{app} = K_i(1 + S/K_m)$, wherein S is substrate concentration. The inhibition curve for (+)-EHNA was also determined

with calf spleen ADA (type X; Sigma) and calf intestinal ADA (Boehringer Mannheim) and repeated with type VI ADA at room temperature on a Hitachi 2000 spectrophotometer. Equation 2 provided the best fit in all experiments and yielded similar K_{i1} and K_{i2} values, although the type X and Boehringer enzymes displayed a lower fraction (~0.3) of inhibition due to high-affinity sites.

Adenosine kinetics were studied under identical conditions in the absence of inhibitor, and K_m values were estimated by fitting the initial velocities at seven substrate concentrations (10–75 μM) to the simple Michaelis–Menten equation, which gave a good fit. The K_m value for type VI calf intestinal ADA was 29.4 ± 1.5 μM. K_m and $K_{ap}3p$ values were determined by nonlinear regression analysis using the lnPlot computer program (GraphPAD Software Inc.). When the (+)-EHNA inhibition data shown in Figure 1 were analyzed by eq 2, the use of different initial estimates for A, ranging from 0.1 to 0.9, and for K_{app1}, ranging from 0.1 to 10 nM, yielded the same kinetic constants. The K_i determinations were carried out at least twice with 14 or more inhibitor concentrations per experiment.

Dissociation of ADA–Inhibitor Complexes. ADA (type VI; 100 nM) was preincubated for 5 min at the assay temperature in stabilizing buffer that contained 200 nM (+)-EHNA or 9′-hydroxy-(+)-EHNA. To initiate dissociation, 2 μL of the incubation mixture was diluted into 998 μL of 50 mM phosphate buffer, pH 7.0, containing 2% PEG 3350 and 70 μM 2′-deoxyadenosine. This substrate has a higher affinity than adenosine for calf intestinal ADA,[31] and its K_m value was 21 μM with the type VI enzyme. The reaction was recorded until a constant rate of decrease in 265 nm absorbance (a steady-state activity) was attained. These final rates were 80–97% of the rate observed for control reactions in which the inhibitor was omitted. The residual inhibition reflected the carry-over of 0.4 nM inhibitor into the cuvette. Product concentration, P, was plotted as the total decrease in absorbance at 0.5 min intervals. A first-order dissociation rate constant, k_{-1}, was estimated by fitting the data to the integrated rate equation

$$P = v_s t + (v_s/k_{-1})e^{-k_{-1}t} + C \quad (3)$$

wherein v_s is the final velocity at steady state, t is time, and C is a constant of integration. A v_s value about 5% higher than the observed steady-state rate gave the best fit and was used for nonlinear regression analysis employing the Enzfitter computer program (Elsevier-BIOSOFT).

Acknowledgment. J.D.S. gratefully acknowledges support from Robert E. Parks, Jr. This research was supported in part by Grant CA07340 and Roger Williams Cancer Grant CA13943 from the National Cancer Institute, NIH, DHHS.

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