of **38** (25 g, 0.134 mol), H_2O (210 mL), and concentrated HCl (27 mL) was added $NaNO_2$ (9.8 g, 0.142 mol) in H_2O (27 mL) at 0–5 °C. After completion of dropping, the reaction mixture was neutralized with Na_2CO_3. The mixture was added to the suspension of CuCN obtained above at 0–5 °C. The resulting mixture was stirred at room temperature for 2 h and warmed to 50 °C. The organic layer was separated after cooling, dried (Na_2SO_4), and sistilled to yield the cyanide **39** (6.51 g, 23%): bp 78–82 °C (0.3 mmHg). To a mixture of **39** (6.4 g, 33 mmol), toluene (60 mL), and CH_2Cl_2 (10 mL) was added DIBAL (25% in toluene, 28 mL, 50 mmol) at −50 to −60 °C, and the solution was stirred for 30 min at the same temperature. The reaction mixture was warmed to room temperature and stirred for 2 h. To the mixture was added MeOH (10 mL) at 0–10 °C, and the solution was stirred for 30 min. The white precipitate was filtered off. The organic layer was separated, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by column chromatography on silica gel (50% $CHCl_3$ in cyclohexane) to give the aldehyde **40** (5.34 g, 82%). A mixture of the aldehyde **40** (5.3 g, 26.6 mmol), $CH(OEt)_3$ (7.1 g, 48 mmol), EtOH (7 mL), and *p*-TsOH (0.2 g) was refluxed for 1 h. After the mixture was cooled, Et_3N (1 mL) was added to the mixture, which was concentrated in vacuo and distilled to give the acetal **41** (5.7 g, 80%): bp 102–104 °C (0.3 mmHg). To a solution of **41** (1.88 g, 6.9 mmol) in Et_2O (20 mL) was added *n*-BuLi (1.4 M in hexane, 5 mL, 6.9 mmol) at −70 °C. The mixture was warmed to 0 °C gradually and stirred for 1 h at the same temperature. Nicotinaldehyde (0.89 g, 7.5 mmol) was added to the mixture at −30 °C, and the resulting mixture was warmed to room temperature within 1 h. The solution was concentrated in vacuo. The residual oil was treated with 2 N HCl (10 mL) in THF (10 mL) at room temperature for 1 h. The mixture was basified with $NaHCO_3$ and extracted with Et_2O. The extracts were dried (Na_2SO_4) and concentrated in vacuo. The residue was chromatographed on a silica gel column (35% EtOAc in cyclohexane) to yield **42** (1.20 g, 77%) as white crystals: mp 97.5–99 °C (EtOAc–*i*-Pr_2O). A mixture of the aldehyde **42** (405 mg, 1.78 mmol), (ethoxycarbonyl)methylenetriphenylphosphorane (0.91 g, 2.5 mmol) and $CHCl_3$ (9 mL) was stirred at room temperature for 18 h. The mixture was purified by column chromatography on silica gel (3% EtOH in $CHCl_3$) to afford **43** (520 mg, 94%) as a colorless oil. A mixture of **43** (520 mg, 1.67 mmol) and $SOCl_2$ (2 mL, 28 mmol) was stirred at 60 °C for 1 h and concentrated in vacuo. The residue was basified with $NaHCO_3$ and extracted with Et_2O. The extracts were washed with brine, dried (Na_2SO_4), and concentrated in vacuo. To a solution of the residue in AcOH (4 mL) was added Zn dust (220 mg, 3.34 mg-atoms) at room temperature. The mixture was stirred for 2 h and concentrated in vacuo. The residue was purified by column chromatography on silica gel (35% cyclohexane in EtOAc) to give **44** (404 mg, 82%) as a pale yellow oil. The ester **44** (165 mg, 0.56 mmol) was treated with a mixture of 2 N NaOH (0.5 mL) and EtOH (2 mL) to give the title compound **10** (150 mg, 88%) as white crystals: mp 153–157 °C (EtOH–Et_2O; IR (KBr) 3420, 3050, 2760, 2710, 1690, 1630, 1550, 1394, 1370, 1210, 1120, 833, 690 cm^{-1}; NMR (D_2O) δ 2.02 (d, 3 H), 2.37 (s, 3 H), 4.43 (s, 2 H), 7.10–7.60 (m, 4 H), 8.23 (dd, 1 H), 8.55 (br d, 1 H), 8.89 (br s, 1 H), 8.99 (br d, 1 H); MS, *m/e* 267 (M^+). Anal. ($C_{17}H_{18}ClNO_2$) C, H, N.

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Inhibition of Inosinic Acid Dehydrogenase by 8-Substituted Purine Nucleotides[1]

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A series of 8-substituted derivatives of adenosine monophosphate (AMP) and inosine monophosphate (IMP) were synthesized and examined for their ability to inhibit *Escherichia coli* IMP dehydrogenase. All compounds studied were competitive inhibitors in IMP-dependent competition studies and lacked substrate activity. In oxidized nicotinamide adenine dinucleotide dependent studies, 8-(*p*-NO_2PhCH_2S)-IMP was noncompetitive and 8-(*p*-NO_2PhCH_2S)-AMP showed mixed inhibition. Multiple regression analysis showed that for the series of 8-(para-substituted-benzylthio)-AMPs and -IMPs, the electron-withdrawing ability of the para substituent on the benzylthio moiety correlated best with log K_i of the analogues.

The first of two reactions in the biochemical conversion of IMP to GMP is catalyzed by IMP dehydrogenase (EC 1.2.1.14, IMP-DH), which converts IMP to XMP. This enzyme is of vital importance to rapidly growing cells; the levels of IMP-DH in a series of rat hepatomas have been found to be markedly elevated over normal rat liver levels.[2] Furthermore, many inhibitors of IMP-DH have been shown to have anticancer activity; these include mycophenolic acid,[3] the ribonucleotides of 6-chloropurine and 6-mercaptopurine,[4-6] and a nucleotide derived from 2-amino-1,3,4-thiadiazole.[7] Studies carried out with IMP-DH from various sources indicate that removal of an OH from the phosphate is detrimental to binding,[8] the 5′-oxygen can be replaced by S or NH,[9] and a number of analogues of IMP with modifications in the heterocyclic ring bound somewhat less tightly than IMP itself.[10] Other nucleotide analogues found to bind better than IMP have been 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide 5′-phosphate[11] and 3-deaza-GMP.[12] We report here the effect of substitution of arylthio, aralkylthio, and alkylthio

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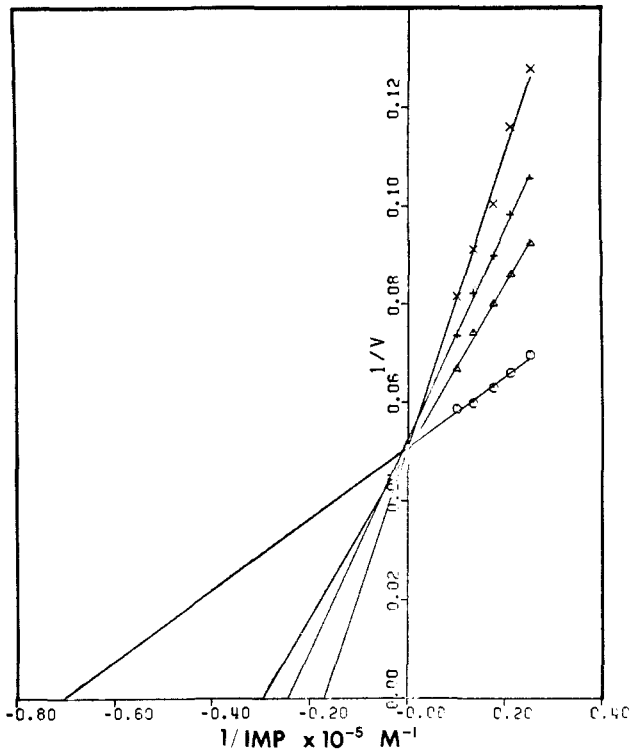

Figure 1. Inhibition of IMP-DH by 8-(p-O_2NPhCH_2S)-IMP at various IMP concentrations. Velocities are in nanomoles per second. Inhibitor concentrations are 0 (○), 27.7 (△), 55.5 (+), and 83.2 (×) μM. Details are given under Experimental Section. [NAD^+] is 1.7 mM. Each line in this and Figures 2–4 is individually calculated by a weighted fit of the data to $V = [S]. V_{max}/(K_m + [S])$.[18]

groups on the 8 position of IMP and AMP on the ability of those nucleotides to bind to and inhibit IMP-DH from *E. coli* B.

Results

Synthesis. The most versatile method for the investigation of the effects of a number of 8-substituents on the biological activity of purine nucleotides has proven to be via funtionalization of the 8 position by bromination of AMP[13] and then displacement of bromide by various nucleophilic reagents.[14-17] We found the most convenient method for preparation of the 8-RS-AMP analogues to be treatment of 8-Br-AMP with the anions of the aryl or alkyl mercaptans if those mercaptans were readily available. For some mercaptans not commercially available, the mercaptan was generated in situ by base-catalyzed decomposition of the *S*-alkylisothiouronium salt (prepared from the appropriate alkyl halide and thiourea). Alternatively, some of the other analogues were prepared by direct alkylation of 8-HS-AMP with alkyl halides. This method generally led to more side products (probably because of alkylation at other sites on the purine ring) than the former methods. These methods are diagrammed in Scheme I.

Treatment of 8-Br-AMP with nitrous acid gave 8-Br-IMP. Treatment of this intermediate with mercaptans in base was a general method for the preparation of the 8-RS-IMPs. Alternatively, the appropriate 8-RS-AMP could be converted to the corresponding 8-RS-IMP by nitrous acid treatment[16] when the reaction of mercaptides with 8-Br-IMP was sluggish or gave side products.

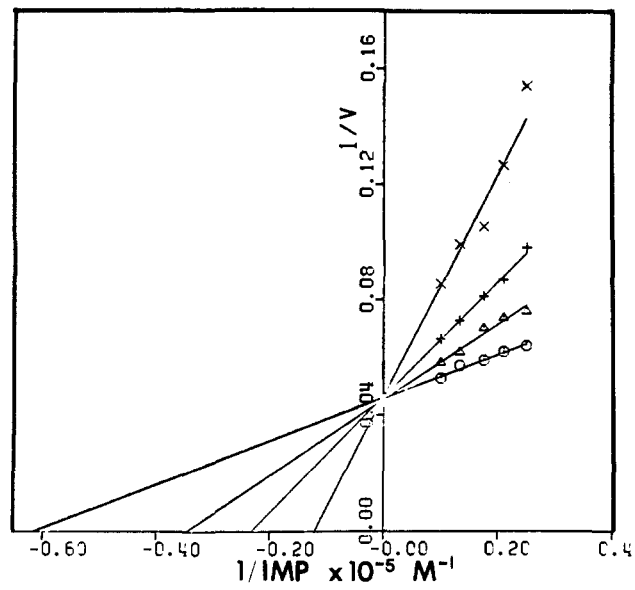

Figure 2. Inhibition of IMP-DH by 8-(p-O_2NPhCH_2S)-AMP. Velocities are in nanomoles per second. Inhibitor concentrations are 0 (○), 47.1 (△), 95.2 (+), and 186.6 (×) μM. [NAD^+] is 1.7 mM.

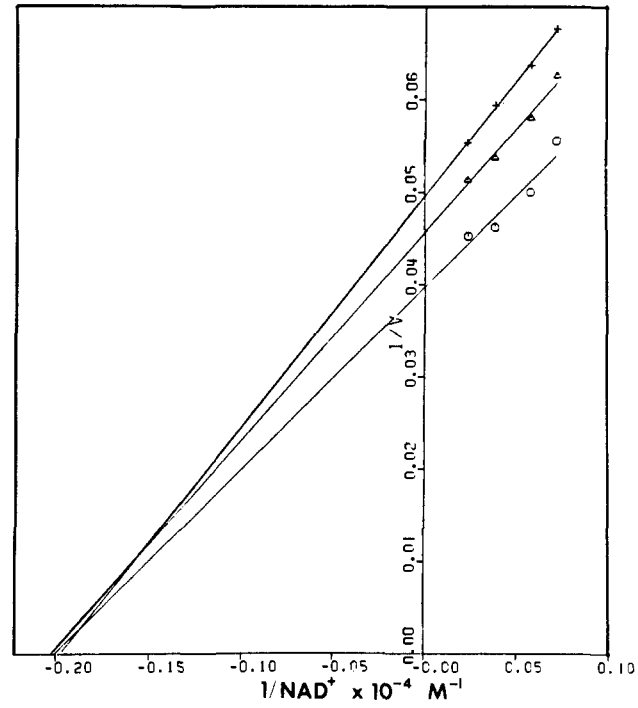

Figure 3. Inhibition of IMP-DH by 8-(p-O_2NPhCH_2S)-IMP. Velocities are in nanomoles per second and [IMP] = 0.5 mM. Inhibitor concentrations are 0 (○), 55 (△), and 166.5 (+) μM.

Inhibition of IMP-DH. All of the 8-substituted AMP and IMP analogues described in this report were tested as inhibitors of IMP-DH using at least two inhibitor concentrations, with at least five substrate concentrations for each inhibitor concentration. The K_i values, determined by a nonlinear fit[18] of the data to the linear competitive binding equation, are given in Table III. When IMP was the variable substrate (all values in Table III), 1/[IMP]

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Scheme I

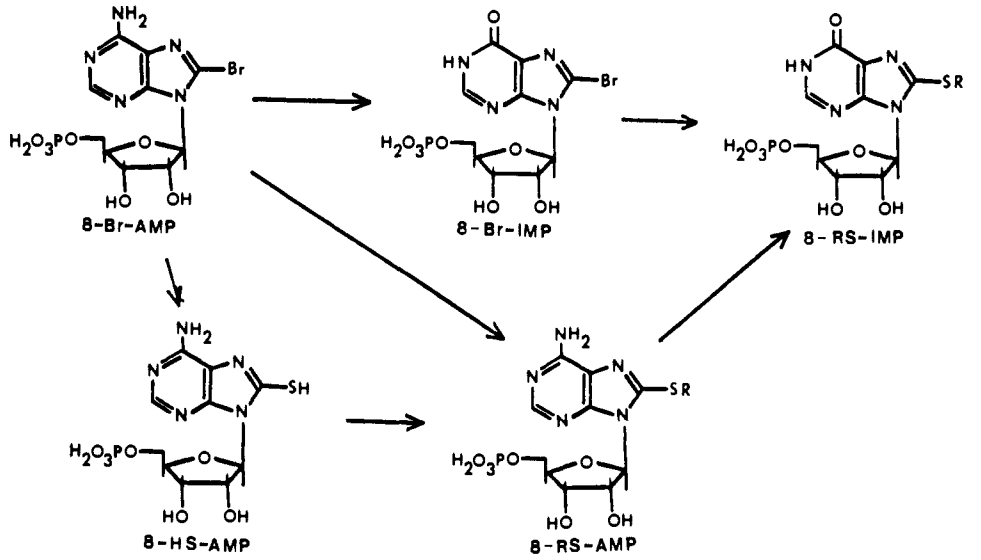


Table I. Preparation and Physical Properties of the 8-Substituted Analogues of AMP

R of 8-RS-AMP	method[a]	yield,[a] %	R_f[b] A	R_f[b] B	λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 1	pH 7.4	pH 13	formula[c]
n-C_5H_{11}	C	32	0.35	0.62	283 (20.9)	281 (20.0)	281 (20.2)	$C_{15}H_{22}Li_2N_5O_7PS\cdot2.25H_2O$
Ph	B	66	0.55	0.61	282 (18.3)	281 (16.2)	282 (16.6)	$C_{16}H_{18}N_5O_7PS\cdot2.5H_2O$
PhCl-*p*	B	4	0.52	0.64	282 (13.7)	282 (12.2)	282 (11.7)	$C_{16}H_{17}ClN_5O_7PS\cdot1.5H_2O$
CH_2Ph	B	44	0.40	0.56	285 (16.0)	282 (14.9)	283 (15.1)	$C_{17}H_{20}N_5O_7PS\cdot1.75H_2O$
$CH_2PhC(CH_3)_3$-*p*	A[d]	5	0.52	0.65	284 (17.1)	282 (15.2)	282 (15.9)	$C_{21}H_{28}N_5O_7PS\cdot0.25H_2O$
CH_2PhCOO^--*p*	A	37	0.29	0.56	282 (17.2)	282 (14.9)	282 (16.1)	$C_{18}H_{20}N_5O_9PS\cdot0.75H_2O$
CH_2PhF-*p*	A[d]	10	0.39	0.64	284 (20.4)	283 (17.5)	283 (19.2)	$C_{17}H_{19}FN_5O_7PS\cdot H_2O$
CH_2PhCl-*p*	B	44	0.44	0.66	284 (15.2)	283 (14.2)	283 (14.8)	$C_{17}H_{19}ClN_5O_7PS\cdot0.25H_2O$
CH_2PhOCH_3-*p*	B	11	0.31	0.52	281 (18.9)	282 (17.5)	282 (18.1)	$C_{18}H_{20}Li_2N_5O_8PS\cdot2H_2O$
CH_2PhCN-*p*	A	42	0.42	0.55	281 (18.4)	281 (15.4)	281 (15.7)	$C_{18}H_{19}N_6O_7PS$
CH_2PhNO_2-*p*	C	57	0.36	0.62	280 (27.8)	279 (25.2)	280 (26.0)	$C_{17}H_{19}N_6O_9PS$
CH_2PhNO_2-*m*	C[d]	45	0.41	0.60	280 (21.3)	279 (18.9)	280 (19.6)	$C_{17}H_{19}N_6O_9PS\cdot0.5H_2O$
CH_2Ph-2-Cl-4-NO_2	C[d]	24	0.50	0.60	278 (22.1)	277 (21.5)	279 (20.4)	$C_{17}H_{18}ClN_6O_9PS$[e]
CH_2Ph-3,5-$(NO_2)_2$	C[d]	54	0.41	0.59	248 (19.8) 275 (19.9)	245 (20.6) 275 (17.2)	245 (20.7) 275 (17.8)	$C_{17}H_{18}N_7O_{11}PS$
CH_2CH_2Ph	C	6	0.48	0.61	286 (17.8)	283 (16.9)	283 (17.5)	$C_{18}H_{22}N_5O_7PS\cdot0.75H_2O$
$CH_2CH_2PhNO_2$-*p*	A	31	0.57	0.65	278 (17.9)	278 (16.9)	278 (17.0)	$C_{18}H_{21}N_6O_9PS\cdot1.5H_2O$[f]
$CH_2CH_2CH_2Ph$	A	10	0.55	0.62	284 (17.7)	282 (16.7)	282 (16.6)	$C_{19}H_{24}N_5O_7PS\cdot H_2O$

[a] Methods are given under Experimental Section; yields are for purified product from the reaction described under each method. [b] TLC on EM Laboratories silica gel 60 F-254 plates using solvent system A [*i*-PrOH–concentrated NH_4OH–H_2O (7:1:2)] or B [*n*-BuOH–AcOH–H_2O (5:2:3)]. [c] All compounds gave correct analyses for C, H, and N within ±0.4% except where noted. [d] The benzyl halides (methods A and C) used for these compounds were the respective benzyl chlorides; all others were bromides. [e] N: calcd, 15.31; found, 14.26. [f] N: calcd, 15.13; found, 14.10.

vs. $1/V$ plots of the data gave lines intersecting on the $1/V$ axis. Replots of the slopes of these lines vs. [I] gave plots which were linear within experimental error. The results of the inhibition studies with the 8-[(*p*-nitrobenzyl)thio] derivatives of IMP and AMP are given in Figures 1 and 2, respectively.

The IMP analogue 8-(*p*-O_2NPhCH_2S)-IMP was examined for its inhibition of IMP-DH when NAD^+ was the variable substrate and the concentration of IMP was fixed. In this case, the family of lines on the $1/[NAD]$ vs. $1/V$ plot intersected on the x axis (see Figure 3), indicating noncompetitive inhibition. The plot of the results of in-

Table II. Preparation and Physical Properties of 8-Substituted IMP Analogues

R of 8-RS-IMP	method[a]	yield,[a] %	R_f[b] A	R_f[b] B	λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 1	pH 7.4	pH 13	formula[c]
Ph	D	30	0.22	0.44	271 (13.0)	271 (12.8)	281 (13.6)	$C_{16}H_{15}Li_2N_4O_8PS\cdot 3H_2O$
$PhCF_3$-*m*	D	20	0.32	0.56	273 (14.3)	273 (14.3)	282 (14.0)	$C_{17}H_{14}F_3Li_2N_4O_8PS\cdot 1.5H_2O$
CH_2Ph	D	19	0.33	0.60	275 (14.4)	274 (13.0)	280 (13.3)	$C_{17}H_{17}Li_2N_4O_8PS\cdot 4H_2O$
$CH_2PhC(CH_3)_3$-*p*	D	13	0.41	0.64	274 (13.6)	274 (14.4)	281 (14.9)	$C_{21}H_{25}Li_2N_4O_8PS\cdot 1.5H_2O$
CH_2PhCl-*p*	D	10	0.34	0.61	274 (11.9)	274 (12.4)	280 (12.4)	$C_{17}H_{16}ClLi_2N_4O_8PS\cdot 1.5H_2O$
CH_2PhOCH_3-*p*	E	9	0.37	0.60	277 (12.0)	275 (12.4)	280 (12.0)	$C_{18}H_{19}Li_2N_4O_9PS\cdot 2.5H_2O$
CH_2PhCN-*p*	E	17	0.24	0.55	274 (13.6)	274 (13.6)	278 (12.9)	$C_{18}H_{16}Li_2N_5O_8PS\cdot 2.5H_2O$[d]
CH_2PhNO_2-*p*	E	29	0.37	0.64	272 (18.3)	273 (18.1)	277 (20.5)	$C_{17}H_{16}Li_2N_5O_{10}PS\cdot 2.5H_2O$
CH_2Ph-2-Cl-4-NO_2	E	14	0.21	0.55	268 (16.4)	269 (16.9)	276 (16.3)	$C_{17}H_{15}ClLi_2N_5O_{10}PS\cdot 2.5H_2O$
$CH_2CH_2PhNO_2$-*p*	E	61	0.36	0.60	274 (17.3)	274 (16.4)	279 (16.3)	$C_{18}H_{18}Li_2N_5O_{10}PS\cdot 0.5H_2O$

[a-c] See corresponding footnotes in Table I. [d] N: calcd, 12.68; found, 11.44.

Table III. Inhibition of IMP Dehydrogenase by 8-Substituted AMPs and IMPs

8-substituent	K_i,[a] μM, of substituted nucleotide AMP	IMP
H	450	16 ± 3 (K_m)[b]
Br	161	271
SH	98	
SC_5H_{11}-*n*	84	
SPh	137	68
SPhCl-*p*	70	
$SPhCF_3$-*m*		105
SCH_2Ph	124	46
$SCH_2PhC(CH_3)_3$-*p*	130	129
SCH_2PhCOO^--*p*	196	
SCH_2PhF-*p*	74	
SCH_2PhCl-*p*	53	29
SCH_2PhOCH_3-*p*	84	41
SCH_2PhCN-*p*	58	19
SCH_2PhNO_2-*p*	52	26
SCH_2PhNO_2-*m*	47	
SCH_2Ph-2-Cl-4-NO_2	17	
SCH_2Ph-3,5-$(NO_2)_2$	21	
SCH_2CH_2Ph	91	
$SCH_2CH_2PhNO_2$-*p*	68	54
$SCH_2CH_2CH_2Ph$	64	
$NHCH_2Ph$	275	236

[a] Determined by fitting the inhibition data to the linear competitive inhibition binding equation as detailed under Experimental Section. All values are averages of two or more determinations; all determinations for a given compound were within 20% of the mean, and standard deviations were generally ± 20% or less. [b] Average of five determinations.

hibition by the corresponding AMP analogue [8-(*p*-O_2NPhCH_2S)-AMP] indicated mixed inhibition kinetics (Figure 4).

Since all of the IMP analogues were unsubstituted at the 2 position, it is possible that they could be substrates, giving 8-substituted XMP derivatives. It was found, however, that none of the analogues in this report were substrates for IMP-DH (using NADH formation as the criteria for substrate activity).

Discussion

The double-reciprocal plots (Figures 1 and 2) show that the analogues seem to be competing with IMP for its

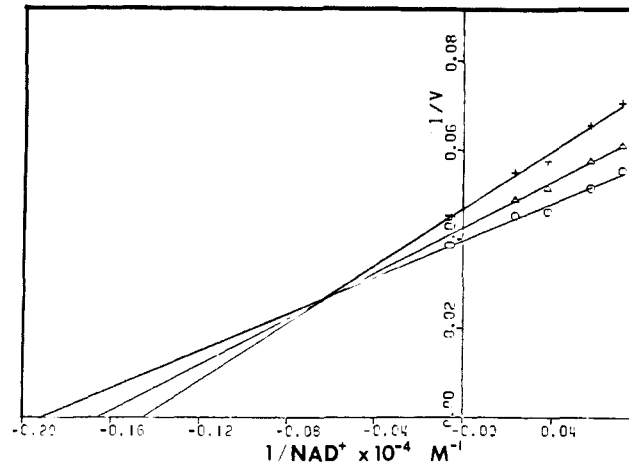


Figure 4. Inhibition of IMP-DH by 8-(*p*-O_2NPhCH_2S)-AMP. Velocities are in nanomoles per second and [IMP] = 0.5 mM. Inhibitor concentrations are 0 (○), 95 (△), and 190 (+) μM.

binding site; both AMP and IMP analogues give a family of lines intersecting at the $1/V$ axis. The inhibition by both types of compounds appears to be noncompetitive with NAD^+, however (Figures 3 and 4). This argues against the AMP analogues binding, for instance, in the AMP portion of the NAD^+ site.

An interesting pattern emerges in the increase in binding affinity as the nature of the 8 substituent in AMP and IMP is changed (see Table III). In the first place, AMP itself is a poor inhibitor, with a K_i of 0.45 mM (about 30 times higher than the K_m of IMP). Placement of *any* of the 8 substituents onto AMP increases the binding affinity. The 8-$PhCH_2S$ group, for instance, gives a 5-fold increase in affinity. When this same group is placed on IMP, however, a 3-fold decrease in binding occurs. As increasingly electron-withdrawing substituents are placed on the benzylthio group, however, the binding of both AMP and IMP analogues increases in a parallel manner, with a K_i of 19 μM being found for 8-(*p*-$NCPhCH_2S$)-IMP. It is striking that there is a constant factor of about 2 between the K_i values of the AMP and IMP analogues with identical 8-substituents.

Another feature which contributes to the elucidation of the mode of binding of these compounds is the fact that none of the IMP analogues show any substrate activity.

This could be explained by assuming that the analogues bind to the active site in such a way that the reactive center (the 2 position of the purine ring) is no longer oriented properly with respect to the catalytic groups in the enzyme or by the enzyme not being able to reach its catalytic conformation when the 8-substituent is present.

This accumulated evidence suggests one possible explanation for the data presented. It is known that purine nucleotides and nucleosides, particularly adenosine and AMP, exist in solution in predominantly the anti conformation. Furthermore, it is well established that placement of a large substituent, such as Br or MeS, in the 8 position of AMP or adenosine dictates that the purine nucleoside or nucleotide assume the syn conformation.[19,20] If IMP binds to the active site in the anti conformation and if the phosphoribose moiety of our analogues binds to the same site as the phosphoribose of IMP, then the purine ring binding points of the analogues, which are in the syn conformation, would not be available for binding. Two of our experimental results are then predicted by this model: the 8-substituted analogues should not be substrates and the large (30×) binding difference between IMP and AMP should diminish when the same 8-substituent is placed on each.

In addition to its influence on conformation, it seemed that the 8-substituent was also contributing to analogue binding. In order to gain a clearer picture of the nature of this interaction, we examined possible correlations between physiochemical parameters and our inhibition constants. The parameters evaluated were the hydrophobic constant π, the molecular refractivity MR, and the electronic terms σ_p and the $\mathcal{F}$ (field) and $\mathcal{R}$ (resonance) constants of Swain and Lupton.[21] All parameter values were taken from Hansch and Leo.[22] We used only the 8-(benzylthio)-substituted nucleotides with a para substituent on the phenyl ring in the correlation analysis to eliminate variation associated with highly specific steric interactions, and the constants used were for that para substituent. Both AMP and IMP analogues were included in the fit, with an indicator variable used to indicate the parent nucleotide. Using multiple regression analysis, only one equation was found to be clearly significant, and this is given by eq 1. In this equation, $\mathcal{F}$ is the field constant

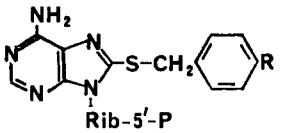
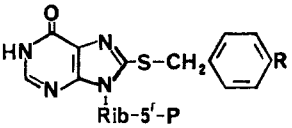
$$\log (1/K_i) = 3.87\ (\pm 0.11) + 0.75\ (\pm 0.25)\ \mathcal{F} + 0.32\ (\pm 0.14)\ I \quad (1)$$

$$n = 14,\ r = 0.933,\ s = 0.116,\ F_{2,11} = 36.8$$

of the para substituent on the 8-(benzylthio) moiety, I is the indicator variable using $I = 0$ for AMP analogues and $I = 1$ for IMP analogues, n is the number of compounds in the fit, r is the correlation coefficient, and s is the standard deviation. The data used in this derivation, as well as the calculated log $(1/K_i)$ values, are given in Table IV.

The large coefficient for $\mathcal{F}$ in the regression equation would seem to indicate that the effect of the substituent on the electron density of the phenyl ring of the benzylthio

Table IV. Field Constants for the Para Substituent on the 8-(Benzylthio) Nucleotides and the Observed and Predicted Activities

R	$\mathcal{F}$[a]	log $1/K_i$ exptl	log $1/K_i$ calcd[b]
			
H	0	3.91	3.82
F	0.43	4.13	4.37
Cl	0.41	4.27	4.34
OCH_3	0.26	4.07	4.15
CN	0.51	4.23	4.47
NO_2	0.67	4.28	4.68
$C(CH_3)_3$	−0.07	3.89	3.73
COO^-	−0.15	3.71	3.62
			
H	0	4.43	4.15
Cl	0.41	4.53	4.68
OCH_3	0.26	4.39	4.48
CN	0.41	4.72	4.81
NO_2	0.67	4.59	5.02
$C(CH_3)_3$	−0.07	3.89	4.06

[a] Taken from Hansch and Leo.[22] [b] Calculated from eq 1 using $I = 0$ for AMPs and $I = 1.0$ for IMPs.

substituent is the major determinant in the binding differences observed in this series. As noted above, there is a fairly constant factor of 2 (as reflected in the coefficient of the indicator variable I in eq 1) between the K_i values for the AMP and IMP analogues with identical 8-substituents. One possible explanation of these observations is that the purine ring has been "twisted" (about the glycosidic bond) away from its binding area and the 8-substituent has become the major binding determinant in the aglycon. The fact that only $\mathcal{F}$ gives a significant correlation with K_i could indicate that some type of charge transfer interaction with an electron-rich site in the active site is involved; no hydrophobic interactions were evident in the analysis. This latter fact would seem to also indicate that nonspecific interactions of the analogues with other proteins in our somewhat impure enzyme preparation are probably not significant.

The analysis is further strengthened if one examines the K_i values of 8-[3,5-$(O_2N)_2$-$PhCH_2S$]-AMP and 8-(2-Cl-4-O_2N-$PhCH_2S$)-AMP, which are the most potent inhibitors of the group. Using eq 1, the calculated K_i values of these compounds are 13.8 and 14.4 μM (experimental: 21 and 17 μM), respectively. The importance of correct distance between the purine and the substituted phenyl ring is also important: the addition of a p-NO_2 group to 8-($PhCH_2CH_2S$)-AMP does not give as much increase in binding as does the same addition to 8-($PhCH_2S$)-AMP.

Competition studies with 8-(2-Cl-4-O_2N-$PhCH_2S$)-IMP were anomalous. At low inhibitor concentrations in IMP-dependent competition studies, competitive inhibition was observed. Upon increasing inhibitor concentration, a decrease in V_{max} was found. Preliminary data indicate that this compound gives first-order irreversible inactivation of IMP-DH at 10 μM.[23] Further studies on

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the nature of this inactivation are in progress.

In summary, attachment of an aromatic ring by a short linkage to the 8 position of either AMP or IMP can give a good inhibitor of IMP-DH if the aromatic ring has strong electron-withdrawing substituents. It seems possible that the 8-substituents have twisted the purine ring in the analogues out of the normal binding conformation, since the difference seen in the binding of unsubstituted AMP and IMP are almost abolished and the nature of the 8-substituent has become the primary binding determinant. By exploitation of this binding area, it might be possible to design IMP-DH inhibitors which do not have the ionic phosphate.

Experimental Section

Isolation of IMP-DH. The enzyme was isolated from *E. coli* B (Grain Processing Inc., Muscutine, IA) frozen paste by the method of Streeter et al.[11] The preparation used for the assays was the 0–40% $(NH_4)_2SO_4$ precipitate. This fraction was found to have no NAD^+ reductase activity and no other activity which degraded IMP on prolonged incubation. A very small amount of NADH oxidase activity was present, but the initial velocities obtained by following either NADH or XMP formation were always within experimental error.

Assay of IMP-DH. The precipitate from the final ammonium sulfate fractionation was redissolved in a minimal amount of 0.05 M Tris buffer, pH 8.5. The standard assay, in 1 mL, consisted of 0.05 M Tris buffer containing 33.4 mM KCl, 1.5 mM glutathione, 1.6 mM NAD^+, 0.037 mg of enzyme preparation, and concentrations of 40, 47, 57, 75, and 100 μM IMP for each concentration of inhibitor. Reactions were started by addition of the enzyme. Initial velocities were measured at 25 °C on a Cary 118 spectrophotometer by following the increase in absorbance at 360 nm ($\Delta\epsilon = 5.27\ mM^{-1}\ cm^{-1}$).[10] Interfering absorbance from several of the inhibitors was present at 340 nm. In assays with no inhibitor, the increase in absorbance at 290 nm (formation of XMP) was used to verify that NADH destruction was not giving erroneously low velocities when NADH formation was being observed. At least two inhibitor concentrations, chosen to give a suitable amount of inhibition (about 1.5- to 3-fold), were used for each K_i determination. Each 8-substituted derivative was examined as a substrate for IMP-DH using the standard assay mixture (minus IMP) and ca. 1 mM analogue. No increase in absorbance at 360 nm was observed for any of the analogues, indicating no substrate activity.

When NAD^+ was used as the variable substrate, IMP concentration was 0.5 mM and NAD^+ concentration was 1.05, 1.40, 1.74, 2.6, and 4.28 mM. We found that our preparations gave a K_m for IMP of $1.59 \pm 0.32 \times 10^{-5}$ M and a V_{max} of 21.1 ± 1.0 nmol/s [or 570 nmol s^{-1} (mg of protein)$^{-1}$].

Data Analysis. The kinetic constants were determined by a weighted fit of the data to the linear competitive velocity equation using the nonlinear fitting routine of Cleland.[18] Additionally, a K_m (app) and V_{max} (app) were calculated for each set of velocities and substrate concentrations at constant inhibitor concentrations by using the weighted fit to $V = [S]V_{max}/(K_m + [S])$. These lines were then plotted on a graph of $1/V$ vs $1/[S]$. By this method, the type of inhibition could be determined according to the usual criteria.[24]

Synthetic Methods. All evaporations were carried out under high vacuum at 40 °C. UV spectra were determined on a Cary 118. TLC were run on EM Laboratories silica gel 60 F-254 plates using either solvent system A [*i*-PrOH–concentrated NH_4OH–H_2O (7:1:2)] or B [*n*-BuOH–AcOH–H_2O (5:2:3)]. Proton NMR spectra of all compounds were measured on a Varian FT-80 at 80 MHz in Me_2SO-d_6 or D_2O and were consistent with the assigned structures. Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. The spectral, chromatographic, and analytical data of all AMP derivatives prepared by methods A–C are given in Table I, and those IMP analogues prepared by methods D and E are given in Table II. No attempt was made to achieve optimal yields.

Synthesis of AMP Analogues. The method of Ikehara and Uesugi[13] was used to prepare 8-Br-AMP and the method of Ikehara et al.[14] was used to convert this to 8-HS-AMP. The following methods were then used to prepare the new analogues (Scheme I).

Method A. A mixture of the appropriate alkyl- or aralkyl halide (30 mmol) and thiourea (1.82 g, 24 mmol) in 50 mL of dioxane and 5 mL of EtOH was refluxed for 2 h and then cooled. If the S-substituted isothiouronium salt did not crystallize, the solution was evaporated to one-half volume and chilled. The *S*-alkyl- or *S*-aralkylisothiouronium salts were collected on a filter and washed with dioxane.

A solution of 8-Br-AMP (250 mg, 0.57 mmol) in 3.7 mL of 1 N NaOH and 15 mL MeOH was deoxygenated by bubbling in nitrogen. To this was added 2 mmol of the appropriate isothiouronium salt, and the solution was refluxed 2 h and then stirred at ambient temperature 12 h under nitrogen. Purification of the product is described below.

Method B. A deoxygenated solution of 8-Br-AMP (250 mg, 0.57 mmol) and 2 mmol of the appropriate phenyl or benzyl mercaptan in 1.7 mL 1 N NaOH and 15 mL of MeOH was refluxed under nitrogen for 2 h and then stirred at ambient temperature for 12 h. The product was purified as described below.

Method C. A solution of 8-HS-AMP (250 mg, 0.86 mmol) and 1.28 mmol of the appropriate benzyl halide (either chloride or bromide) in 2.58 mL of 1 N NaOH and 15 mL of MeOH was refluxed 2 h, stirred at ambient temperature 12 h, and purified as described next.

Purification of the 8-RS-AMP and IMP Derivatives. The reaction mixture was diluted with 500 mL of H_2O and the pH was adjusted to 7.0 with HOAc. After filtration through a pad of diatomaceous earth, this solution was passed through a column of Dowex 1 × 8 (Cl^-, 100–200 mesh). The column was washed with 500 mL of H_2O and eluted with a 1-L gradient of 0 to 0.5 N LiCl. The product was then eluted with 200 mL of 0.5 N LiCl in 50% aqueous EtOH. This last fraction was evaporated to dryness, and the residue was treated with 10 mL of EtOH followed by 300 mL of acetone. After the mixture was left standing overnight, the solid was removed by filtration, washed with acetone, and redissolved in ca. 25 mL of H_2O. For most of the AMP analogues, the pH of this solution was adjusted to 2 with concentrated HCl. Cooling overnight gave the crystalline free acid of the product, which was filtered, washed with acetone, and dried with high vacuum. For the IMP analogues and for 8-(*n*-$C_5H_{11}S$)-AMP and 8-(*p*-MeOPhCH_2S)-AMP, this aqueous solution was passed onto a column of 200 g of finely ground Amberlite XAD-4 (packed in H_2O), which was then washed with 1 L of H_2O. The product was removed with 200 mL of 50% aqueous MeOH. This solution was filtered through a fine sintered glass funnel and evaporated. The residue was dissolved in a minimal amount of H_2O, and the product was precipitated with acetone, filtered, and dried.

8-(Benzylamino)adenosine 5′-Phosphate. A mixture of 8-Br-AMP (500 mg, 1.14 mmol), 5 mL of benzylamine, and 100 mL of MeOH was heated and stirred in a stainless-steel bomb at 150 °C for 8 h. The contents were then evaporated to dryness, and the residue was washed several times with ether, then dissolved in H_2O, and applied to a column of Dowex 1 × 8 (formate, 100–200 mesh). After washing with 500 mL of H_2O, the product was eluted with a 1-L gradient of 0 to 0.5 N formic acid. Evaporation of the appropriate fractions and trituration of the residue with acetone gave a solid, which was recrystallized from water: yield 100 mg (18%); UV λ_{max} at pH 1, 266 nm (ϵ 14 800), 275 sh; at pH 7, 279 (18 900); at pH 13, 279 (17 400); TLC R_f (A) 0.49, R_f (B) 0.61. Anal. ($C_{17}H_{21}N_6O_7P \cdot H_2O$) C, H, N.

8-(Benzylamino)inosine 5′-Phosphate. To a solution of 8-(benzylamino)adenosine 5′-phosphate (1.0 g, 2.12 mmol) in 10 mL of 1 N NaOH were added 50 mL of H_2O and 4.0 g (73.2 mmol) of $NaNO_2$. After the solution was flushed with nitrogen, 10 mL of glacial AcOH was added, and the loosely covered solution was stirred for 2 days and then evaporated to dryness. The residue was dissolved in 100 mL of H_2O, the pH was adjusted to 7.0 with NaOH, and this solution was applied to a 100-mL Dowex 1 × 8 (formate, 100–200 mesh) column. The column was washed with

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water and then washed with 250 mL of 0.5 N formic acid, followed by 0.5 N formic acid in 50% ethanol. The product was removed with 300 mL of 50% formic acid. Evaporation yielded a residue, which was dissolved in 25 mL of methanol containing 5% water. A large volume of acetone, ca. 500 mL, was added to precipitate the product. This process was then repeated. The filtered material was then dried under vacuum: yield 0.7 g (67%); UV λ_{max} at pH 1, 257 nm (ϵ 15 800), 280 sh; at pH 7.4, 268 (14 500), 295 sh; at pH 13.0, 271 (15 300); TLC R_f (A) 0.15, R_f (B) 0.51. Anal. ($C_{17}H_{20}N_5O_8P \cdot H_2O$) C, H, N.

8-Bromoinosine 5'-Phosphate Dilithium Salt. The pH of a solution of 8-Br-AMP (1.0 g, 2.32 mmol) in 20 mL of H_2O was adjusted to 7.0 with 1 N NaOH, and 1.12 g (20.5 mmol) of $NaNO_2$ was added. After the solution was flushed with nitrogen, 2 mL of AcOH was added, and the loosely covered solution was stirred for 2 days and then evaporated to dryness. The residue was dissolved in 100 mL of H_2O, the pH was adjusted to 7.0 with NaOH, and this solution was applied to a column of Dowex 1 × 8 (Cl^-, 100–200 mesh). The column was washed with water and the product eluted with a 1-L gradient of 0 to 0.5 N LiCl. The appropriate fractions were concentrated to dryness in vacuo and triturated with acetone containing 5% ethanol, and then this residue was dissolved in water and the product was precipitated with acetone: yield 1.05 g (95%); UV λ_{max} at pH 1, 254 nm (ϵ 7030); at pH 7.4, 254 (7190); at pH 13, 259 (6820); TLC R_f (A) 0.12; R_f (B) 0.35. Anal. ($C_{10}H_{10}BrLi_2N_4O_8P \cdot 3.75H_2O$) C, N; H: calcd, 3.48; found, 2.98.

Synthesis of IMP Analogues. Method D. The appropriate aryl or aralkyl mercaptan was added to a deoxygenated solution of 8-Br-IMP·2.5H_2O dilithium salt (200 mg, 0.42 mmol) in 1.2 mL of 1 N NaOH and 15 mL of MeOH. The solution was refluxed for 2 h and stirred at ambient temperature overnight, and the product was purified as described above.

Method E. A solution of the appropriate 8-substituted AMP derivative and 0.40 g of $NaNO_2$ in 6 mL of 0.17 N NaOH was deoxygenated by bubbling with nitrogen, and 1 mL of HOAc was added. This solution was loosely covered, stirred for 2 days at ambient temperature, and then worked up as described above.

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Interaction of 1-(5-Phospho-β-D-arabinofuranosyl)-5-substituted-uracils with Thymidylate Synthetase: Mechanism-Based Inhibition by 1-(5-Phospho-β-D-arabinosyl)-5-fluorouracil[1]

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A number of 1-(5-phospho-β-D-arabinosyl)-5-substituted-uracils (*ara*-UMP's) have been examined as inhibitors of dTMP synthetase. As reversible inhibitors, all were substantially less potent than their 2'-deoxyribosyl counterparts. In the presence of 5,10-methylenetetrahydrofolate (CH_2-H_4folate), *ara*-FUMP caused a first-order, time-dependent inactivation of the enzyme. At 0 °C, kinetic studies indicated a reversible K_d of 3.6 μM for the *ara*-FUMP–CH_2-H_4folate complex, and $k = 0.22$ min^{-1} for the subsequent inactivation. Spectral studies of the complex and its behavior toward protein denaturants demonstrate that its structure and stoichiometry are directly analogous to those which have previously been described for FdUMP. The significance of this finding with regard to prodrugs of *ara*-FU and the potential of *ara*-FU as a chemotherapeutic agent are discussed.

Ara-C is one of the most effective drugs available for the treatment of acute myeloblastic leukemia in adults.[4-6] Its usefulness is, however, limited by development of resistance and a short half-life that in part is due to its deamination to the inactive *ara*-U.[7-9] Consequently, a large number of analogues of *ara*-C have been synthesized with the objective of surmounting these difficulties. One such analogue, *ara*-FC, is about as effective as is *ara*-C and appears to have a similar major mode of action (presumably on DNA synthesis); however, in contrast to *ara*-C, deamination provides the cytotoxic *ara*-FU[10] which has been suggested to have a mode of action similar to FUra.[11,12] Because 2,2'-anhydro-*ara*-C is hydrolyzed to *ara*-C, it was reasoned that the corresponding 2,2'-anhydro-*ara*-FC might serve as a "double-barreled" prodrug for both *ara*-FC and *ara*-FU, each of which might exert its effect by different mechanisms.[13] Recently, the

(1) Abbreviations used: *ara*-Py refers to 1-β-D-arabinofuranosylpyrimidines (Py) with cytosine (C), 5-fluorocytosine (FC), uracil (U), 5-fluorouracil (FU); *ara*-PyMP refers to 1-β-D-arabinofuranosylpyrimidine 5'-phosphates; *xylo*-FC, 1-β-D-xylofuranosyl-5-fluorocytosine; CH_2-H_4folate, 5,10-methylene-*dl*,L-tetrahydrofolate; NMM, *N*-methylmorpholine, NaDodSO$_4$, sodium dodecyl sulfate. All other abbreviations used are those recommended by IUPAC.

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