Defined Dimensional Changes in Enzyme Substrates and Cofactors. Synthesis of *lin*-Benzoadenosine and Enzymatic Evaluation of Derivatives of the Benzopurines

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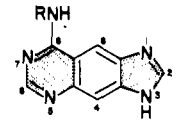
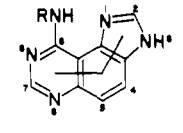
Abstract: A biochemical evaluation of derivatives of 8-aminoimidazo[4,5-*g*]quinazoline (**1**), 9-aminoimidazo[4,5-*f*]quinazoline (**2**), and 6-aminoimidazo[4,5-*h*]quinazoline (**3**), "stretched-out" versions of adenine which are given the descriptive names *lin*-, *prox*-, and *dist*-benzoadenine, respectively, is reported, along with the synthesis of *lin*-benzoadenosine (**10**), the ribonucleoside of **1**. The synthesis of **10** involves the reaction of tri-*O*-acetyl-D-ribofuranosyl bromide with 8-methylthioimidazo[4,5-*g*]quinazoline (**11**) in the presence of mercuric cyanide to afford two methylthio ribofuranosides, **12** and **13**, which, when treated with ethanolic ammonia, are converted to **10** and an isomeric compound, 1-β-D-ribofuranosyl-*lin*-benzoadenine (**14**). Ribonucleoside **10** and the active cytokinin analogues, N^8-benzyl-*lin*-benzoadenine (**4**) and N^8-(Δ^2-isopentenyl)-*lin*-benzoadenine (**7**), exhibit potentially useful fluorescence properties. *lin*-Benzoadenosine is hydrolyzed to *lin*-benzoinosine (**17**) by adenosine deaminase at a relative rate comparable to that for the conversion of adenosine to inosine. Surprisingly, adenosine deaminase also promotes conversion of *lin*-benzoadenine to *lin*-benzohypoxanthine (**16**); the isomeric nonlinear benzoadenines **2** and **3** are refractory. Xanthine oxidase converts *lin*-benzohypoxanthine (**16**) to *lin*-benzoxanthine (**20**) and *lin*-benzouric acid (**24**). *lin*-Benzoinosine (**17**) is oxidized to the corresponding ribonucleosides, namely *lin*-benzoxanthosine (**21**) and 3-(β-D-ribofuranosyl)-*lin*-benzouric acid (**25**). *prox*-Benzohypoxanthine (**18**) reacts with xanthine oxidase at a slow relative rate to afford *prox*-benzoxanthine (**22**) and *prox*-benzouric acid (**26**). *dist*-Benzohypoxanthine (**19**) is oxidized to the first stage, *dist*-benzoxanthine (**23**). Nucleoside phosphorylase does not promote glycosidic cleavage of *lin*-benzoadenosine or of *lin*-benzoinosine, and adenine phosphoribosyltransferase does not accept the benzoadenines **1–3** as substrates. The activity—or lack of activity—of the benzopurine derivatives with the selected enzymes demonstrates the successful application of the concept of testing the dimensional restrictions of enzyme active sites by lateral "stretching" (by 2.4 Å in the case of the *lin*-benzopurines) of the normal substrates.

In terms of describing enzyme specificity for substrates and cofactors,[1] it is now possible and desirable to proceed to define the dimensions of molecules that can be accommodated in an active site. With the purine nucleotides, this position has not been reached although (1) triphosphate alternates and nonalternates for ATP have been examined, particularly with the kinases; (2) elongation-site substitutes for ATP and GTP with bacterial DNA-dependent RNA polymerase have been partially delineated; and (3) triphosphates modified by substitution of NH, CH_2, and S for pyrophosphate O have proven to be useful analogues for studying enzyme activation and inhibition.[1] In earlier research in this Laboratory, we examined the biological effects of formally shifting the ribonucleoside or ribonucleotide group laterally from the 9 to the 3 position of the adenine nucleus, as in 3-isoadenosine, 3-iso-AMP, 3-iso-ADP, 3-iso-ATP, NMN-3-iso-AMP, etc.[2] More recently, we have found that nicotinamide 3,N^4-ethenocytosine dinucleotide, in which there are similarities in spatial outline and potential binding areas for the ϵ-cytidine moiety compared with adenosine, not only mimics the structural features of the natural coenzyme NAD^+ but also shows equivalent activity with at least three dehydrogenases: malate, horse liver alcohol, and lactate.[3]

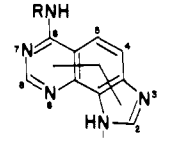
We have now implemented the decision to provide a more defined dimensional change in various purine substrates and cofactors, namely, by the formal insertion of a benzene ring (actually four carbons) into the center of the purine ring system. The opportunity has come about with the synthesis of "stretched-out" adenine analogues: 8-aminoimidazo[4,5-*g*]quinazoline (**1**), 9-aminoimidazo[4,5-*f*]quinazoline (**2**), and 6-aminoimidazo[4,5-*h*]quinazoline (**3**),[4] which we call *lin*-, *prox*-, and *dist*-benzoadenine, respectively.[5] *lin*-Benzoadenine (**1**) is approximately 2.4 Å wider than adenine, while the terminal ring characteristics are preserved and the potential for π interaction is increased. This well-defined 2.4 Å increase in the lateral dimension or—in the case of the *prox*- and *dist*-benzoadenines—defined changes in both dimensions should help to set limitations on the size and flexibility of enzyme binding sites specific for purine substrates or cofactors.



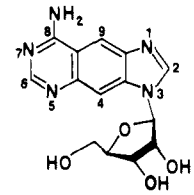
1, R = H
4, R = $C_6H_5CH_2$
7, R = $(CH_3)_2C{=}CHCH_2$



2, R = H
5, R = $C_6H_5CH_2$
8, R = $(CH_3)_2C{=}CHCH_2$



3, R = H
6, R = $C_6H_5CH_2$
9, R = $(CH_3)_2C{=}CHCH_2$



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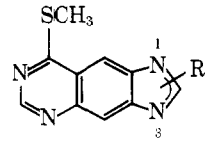
The first encouragement that the stretched-out analogues could exhibit biological activity came with the preparation of the cytokinin[6] analogues of **1–3**, namely, the benzylamino derivatives **4–6** and Δ^2-isopentenylamino derivatives **7–9**,[7] which were evaluated in the tobacco callus bioassay.[8] The *lin*-benzoadenine derivatives **4** and **7** were found to promote the same levels of tobacco callus growth as their respective adenine counterparts, N^6-benzyladenine and N^6-(Δ^2-isopentenyl)adenine, although they were less active as evidenced by the greater concentrations of **4** or **7** needed to effect identical growth levels. The angular *N*-isopentenyl compounds **8** and **9** retained some activity although they were less active than the corresponding linear compound **7**, while the angular *N*-benzyl compounds **5** and **6** were essentially inactive at the concentrations tested. The findings that some of the tricyclic

Table I. Principal Absorption Bands for Derivatives of 8-Methylthioimidazo[4,5-*g*]quinazoline



R					
	λ_{max} 95% EtOH, nm				
3-$PhCH_2$	359	342	329	284	243
3-$RibAc_3$	356	340	329	286	241
1-$PhCH_2$	366	348	323	284	243
1-$RibAc_3$	366	348	330	286	244
	λ_{max} 0.1 N HCl (95% EtOH), nm				
3-$PhCH_2$	352		298	289	252
3-$RibAc_3$	356		302	280	248
1-$PhCH_2$	348		298		248
1-$RibAc_3$	350		302	291	251
	λ_{max} 0.1 N NaOH (95% EtOH), nm				
3-$PhCH_2$	359	338 (sh)	329	284	243
3-$RibAc_3$	360	342	330	286	245
1-$PhCH_2$	366	348	327	298	243
1-$RibAc_3$	366	348		286	244

compounds possessed cytokinin activity and that those with the linear array of the three rings gave the best response encouraged us in the belief that other benzoadenine derivatives would possess biological activity.

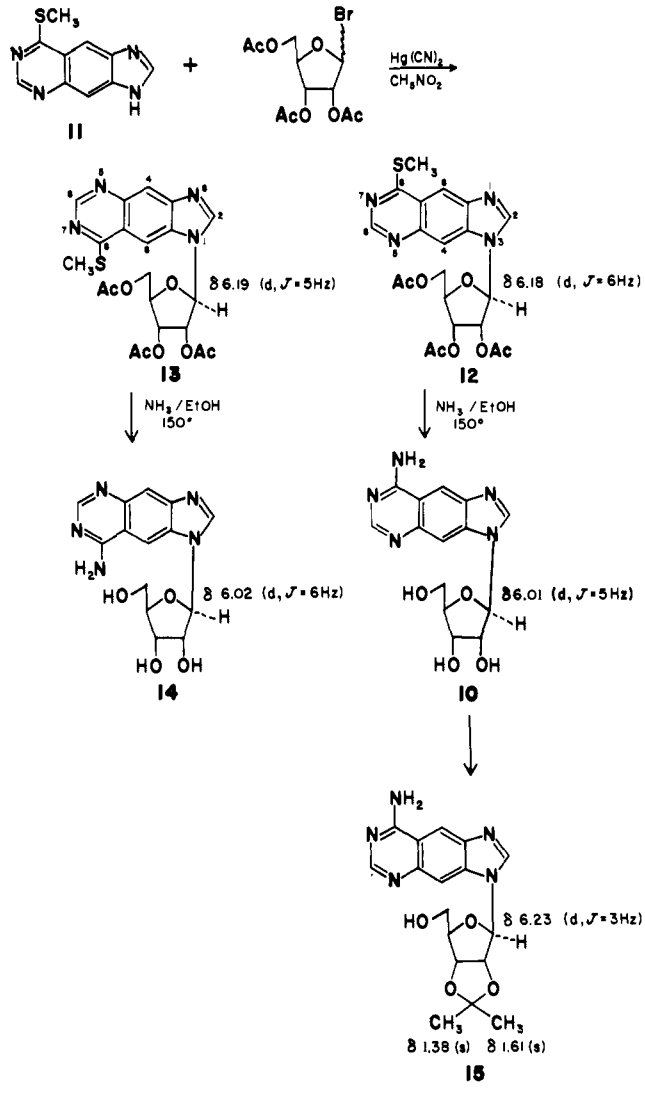
Since, of the three isomeric benzoadenines, the linear compound **1** appeared most similar to adenine in structural morphology, we had earlier synthesized a series of substituted *lin*-benzoadenines to provide uv models for predicting the N position of substituent attachment.[4a] Thus, we had a sound basis for assigning a structure to the benzologue of adenosine, *lin*-benzoadenosine (**10**), the preparation and enzyme activity of which we describe in this paper, along with the enzyme activities of the *lin*-, *prox*-, and *dist*-hypoxanthines previously prepared.[4]

Results and Discussion

Chemistry. The preparation of *lin*-benzoadenosine (**10**) was effected via ribosidation of 8-methylthioimidazo[4,5-g]quinazoline (**11**), a compound previously described and shown to be readily convertible to *lin*-benzoadenine (**1**).[4a] By analogy with benzylation of **11** in the presence of potassium carbonate, the preferred sites of ribosidation were expected to be N-1 and N-3 (see **1** and **10** for the numbering system). The N-3 position is the desired location of the ribofuranosyl group for correspondence with adenosine. A further isomeric complication during synthesis was anticipated in the form of C-1′ anomers, since it was not guaranteed that precedents established for the stereochemical control of ribosidation of purines[9] and benzimidazoles[10] would necessarily hold for this system. Some of the standard ribosidation procedures[11-13] were either unsuccessful or yielded a mixture of 1- and 3-substituted products of both α and β configuration at C-1′. Chromatographic separation of the product mixtures and identification of the components by field desorption mass spectrometry[14] restored some value to the pilot syntheses. The NMR chemical shifts observed for the separate anomeric protons in the products permitted the assignment of the respective β and α diastereomers comprising the position-isomer pairs.[15]

The successful ribosidation route was based upon the use of a mercuri salt[9a] and the convenient procedure developed by Yamaoka et al.[16] The methylthio compound **11**, 8-methylthioimidazo[4,5-*g*]quinazoline,[4a] was treated with 2′,3′,5′-tri-*O*-acetylribofuranosyl bromide in the presence of mercuric cyanide using anhydrous nitromethane as the solvent. After chromatographic separation of the product mixture, two

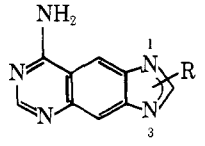
Scheme I



compounds were obtained, **12** and **13**, identified as tri-*O*-acetyl ribonucleosides by field desorption mass spectrometry. The respective structural assignments of **12** and **13** were made by comparison of their uv absorption spectra with those of 1- and 3-benzyl-8-methylthioimidazo[4,5-*g*]quinazolines[4a] (see Table I).[17] The NMR spectra of **12** and **13** each showed one doublet corresponding to the anomeric proton at δ 6.18 (J = 6 Hz) in the case of **12** and at δ 6.19 (J = 5 Hz) in the case of **13**. From the NMR data accumulated for α and β anomer mixtures resulting from the initial ribosidations, the β C-1′ configuration was indicated for both isomers.[15] Since the acyl proton magnetic resonance signals of both isomers, **12** and **13**, fell within the range δ 2.02-2.22, this NMR feature was additionally indicative of the β configuration for both.[18]

Treatment of **12** and **13** with ethanolic ammonia at 150° effected concomitant deblocking of the sugar groups and displacement of methylthio by amino to afford *lin*-benzoadenosine (3-β-D-ribofuranosyl-*lin*-benzoadenine, **10**) and 1-β-D-ribofuranosyl-*lin*-benzoadenine (**14**) in 25 and 9% overall yield, respectively. The isolated yields of products varied somewhat from reaction to reaction. The assignments for the position of attachment of the ribofuranosyl group for compounds **10** and **14** were further supported by comparison of the principal uv absorption bands for **10** and **14** with those for a series of substituted *lin*-benzoadenine model compounds, previously described (Table II).[4a] The similar NMR chemical shifts for the anomeric protons in **10** and **14** were consistent

Table II. Principal Absorption Bands for Derivatives of *lin*-Benzoadenine



R						
	λ_{max} 95% EtOH, nm					
3-$PhCH_2$	349	333	306 (sh)	266	260	242
3-C_6H_{11}	351	334	307 (sh)	266	260	242
3-Rib	348	332	305 (sh)	265	260	241 (sh)
1-$PhCH_2$	359	343	306	293	264	242
1-Rib	356	340	305	295	265	240
	λ_{max} 0.1 N HCl (95% EtOH), nm					
3-$PhCH_2$	351	335	321	271	263	236
3-C_6H_{11}	352	336	322	266	260	233
3-Rib	350	335	321	271	262	237
1-$PhCH_2$[a]	359	343	326	313	275	237
					266	
1-Rib[a]	358 (sh)	340	323 (sh)	310	272	237
					263	
	λ_{max} 0.1 N NaOH (95% EtOH), nm					
3-$PhCH_2$	349	338	318	265	260	242
3-C_6H_{11}	351	334	319	266	260	242
3-Rib	349	334	319	266	260	242
1-$PhCH_2$	358	342	327	306	264	237
1-Rib	357	341	327	306	262	238 (sh)

[a] These spectra recorded in 0.01 N HCl (95% EtOH).

with same-ring substitution and with β configuration. To obtain final confirmation of the β configurational assignment for *lin*-benzoadenosine (**10**), the corresponding isopropylidene derivative **15** was prepared by the method of Hampton.[19] The observed coupling constant $J_{H_1'H_2'}$ for the proton at C-1′ was 3 Hz and therefore not sufficiently low[15,20] to make the assignment unequivocally. However, it was possible to apply the correlations of Imbach and his co-workers,[21] who found for a variety of isopropylidene ribosides that if the chemical shift difference observed for the isopropylidene methyl groups is less than 0.10 ppm, the α configuration can be assigned; if $\geq$0.18 ppm, the β configuration can be assigned. For compound **15** $\Delta\delta$ was 0.22–0.23 ppm, allowing the assignment of the β configuration to this product and accordingly to its precursors **12** and **10**, consistent with all of the accumulated NMR data.

Optical Properties. The ultraviolet and fluorescence spectra of *lin*-benzoadenosine (**10**) are shown in Figure 1. The positions of the principal electronic absorption bands do not change appreciably in neutral, basic, or acidic solution, just as for the spectroscopic models 8-amino-3-benzylimidazo[4,5-*g*]quinazoline and 8-amino-3-cyclohexylimidazo[4,5-*g*]quinazoline.[4a] Thus, the favored site of first protonation of *lin*-benzoadenosine (**10**) may be assumed to be the pyrimidine ring, at N-5 or N-7 (see Table III for pK_a values), as in the models (cf. 4-aminoquinazoline, pK_a = 5.8 in H_2O, and 1-glycosylbenzimidazoles, pK_a av ~ 4.0 in H_2O[10]). The similar pK_a values for **10** and **14** (in 66% DMF) reinforce the assignment of the same-ring substitution of these two isomers. The pK_a of *lin*-benzoadenosine of 5.6 in H_2O, compared with 3.5 for adenosine, suggests that since **10** is a stronger base than adenosine, differences in enzyme behavior from adenosine may also be related to this feature as well as to the 2.4-Å stretch in linear dimension. Nevertheless, at pH 7 compound **10** will be mainly unprotonated according to the titration curve.

The fluorescence emission spectrum of *lin*-benzoadenosine in aqueous buffer (pH 7.0) includes a well-defined maximum at 372 nm and subordinate peaks at 358 and 385 nm. The positions of the maxima shift to longer wavelength, but only to a small extent, as the polarity of the solvent decreases (Table IV). The fluorescence excitation and emission maxima for the

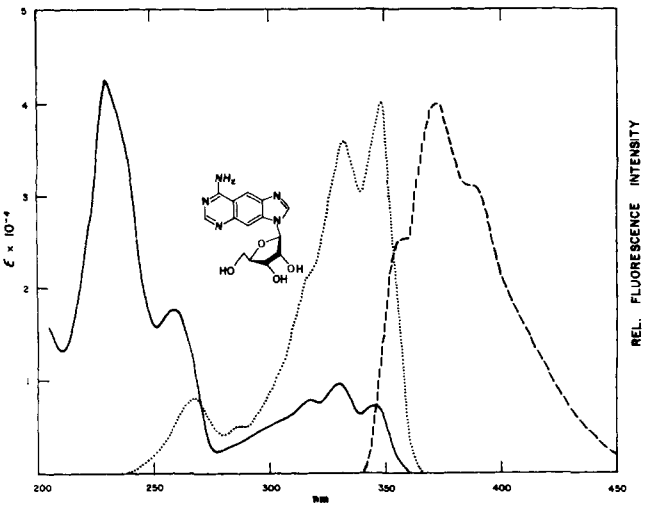


Figure 1. The fluorescence and ultraviolet spectra of *lin*-benzoadenosine (**10**): (—) ultraviolet absorption, (· · · ·) technical fluorescence excitation, (- - - - -) technical fluorescence emission (pH 7.0).

Table III. Acidity Constants for Benzoadenines

Compound	pK_a[a]
lin-Benzoadenine (**1**)	5.6, 11.7
prox-Benzoadenine (**2**)	5.2, 11.4
dist-Benzoadenine (**3**)	4.9, 12.2
lin-Benzoadenosine (**10**)	5.3 (5.6)[b]
1-β-D-Ribofuranosyl-*lin*-benzoadenine (**14**)	5.3
Adenosine	3.0 (3.5)[b]

[a] Determined in 66% DMF. [b] In H_2O at 20°.

angular benzoadenine derivatives **5, 8, 6,** and **9** appear at shorter wavelengths than those for the linear compounds **4, 7, 10,** and **14.** This is mainly a consequence of the linear types, which can be related to the carbocyclic analogue anthracene, exhibiting lower energy electronic transitions than isomers of the angular type, which can be related to phenanthrene.[22]

lin-Benzoadenosine (**10**) and the substituted *lin*-benzoadenine compounds **4** and **7** are strongly fluorescent, as indicated by their relatively high quantum yields (Table IV). The proximal derivatives **5** and **8** are less fluorescent, and the distal derivatives **6** and **9** show very weak fluorescence, probably due to quenching via hydrogen transfer between N-1 and N-9 in the last two cases.[23]

Fluorescence techniques have shown general applicability in elucidating structural and dynamic properties of proteins, nucleic acids, and coenzymes.[24] The fluorescence properties of the benzoadenine series, if coupled with possible biological activity, make the series attractive as potential probes of binding and for investigation of the relationship between molecular structure and biological function. Selective ultraviolet excitation of all the benzoadenine derivatives is possible in the presence of enzymes and nucleic acids, and, in the case of the *lin*- and *prox*-benzoadenine derivatives, excitation is possible at relatively long wavelength, 340–350 nm. For *N*-benzyl- and *N*-(Δ^2-isopentenyl)-*lin*-benzoadenine (**4, 7**) which are active cytokinins,[7] the fluorescence lifetimes and quantum yields increase with decreasing solvent polarity, suggesting that their fluorescence would be intensified when either compound would be bound to a hydrophobic region of an enzyme or membrane.[25] Moreover, the fluorescence lifetimes of compounds **4** and **7,** although relatively short, may lengthen appreciably in a sufficiently hydrophobic region to enable the study of dynamic features of binding by fluorescence depolarization.[26] While the lifetime and quantum yield of the ribonucleoside

Table IV. Fluorescence Data

Compound	Solvent	Excitation >300, nm (uncor)[a]	Emission, nm (uncor)[a]	τ,[b] ns	Φ[c]
lin-Benzoadenosine (10)	H_2O	320 (sh), 332, 348	358, 372, 385	3.7	0.44[d]
	EtOH	322 (sh), 334, 350	358, 374, 390 (sh)	3.7	0.44[e]
	Dioxane	322 (sh), 336, 352	360, 376, 392	3.7	0.44[e]
1-β-D-Ribofuranosyl-*lin*-benzoadenine (14)	H_2O	328 (sh), 340, 356	365, 382, 396	3.0	0.18[e]
N^8-Benzyl-*lin*-benzoadenine (4)	H_2O	320 (sh), 335, 350	365 (sh), 380, 395 (sh)	1.3	0.14[e]
	EtOH	320 (sh), 335, 350	365 (sh), 380, 395 (sh)	1.8	0.26[e]
	Dioxane	320 (sh), 335, 350	365, 380, 395 (sh)	2.2	0.29[e]
N^8-(Δ^2-Isopentenyl)-*lin*-benzoadenine (7)	H_2O	320 (sh), 335, 350	365 (sh), 380, 395 (sh)	0.9	0.07[e]
	EtOH	320 (sh), 335, 350	365 (sh), 380, 395 (sh)	1.7	0.29[e]
	Dioxane	320 (sh), 335, 350	365, 380, 395 (sh)	2.0	0.33[e]
N^9-Benzyl-*prox*-benzoadenine (5)	H_2O	325, 338	350, 362, 378 (sh)	2.0	0.12[e]
N^9-(Δ^2-Isopentenyl)-*prox*-benzoadenine (8)	H_2O	325, 340	350, 362, 378 (sh)	1.0	0.09[e]
N^6-Benzyl-*dist*-benzoadenine (6)	H_2O	302 (sh), 312	328 (sh), 340, 355 (sh)	1.7	0.007[e]
N^6-(Δ^2-Isopentenyl)-*dist*-benzoadenine (9)	H_2O	302 (sh), 312	330 (sh), 340, 355 (sh)	1.5	0.003[e]

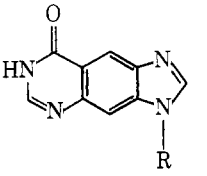
[a] ±2 nm. [b] For fluorescence lifetime determinations, see Experimental Section. [c] Absolute quantum yields (corrected). [d] Determined relative to quinine in 1 N H_2SO_4 (Φ = 0.70). [e] Absolute quantum yield determined relative to **10**.

Table V. Kinetic Data for Adenosine Deaminase

Compound	λ,[a] nm	ΔE_M[b] $\times 10^{-3}$	K_M $\times 10^5$ M	V_{max}[c] $\times 10^{-6}$
Adenosine[e]	265	8.1	2.2[d]	2.0[d]
lin-Benzoadenosine (10)		7.5	5.3	1.7
1-β-D-Ribofuranosyl-*lin*-benzoadenine (14)			No reaction[f]	
lin-Benzoadenine (1)	346	6.6	6.2	1.7
prox-Benzoadenine (2)			No reaction[f]	
dist-Benzoadenine (3)			No reaction[f]	

[a] Wavelength monitored. [b] Change in molar extinction coefficient. [c] Expressed as mol min^{-1} per 1 unit of enzyme. [d] Experimentally determined values. [e] Reported values for K_M and V_{max}, respectively: 4.4×10^{-5} M and 0.51×10^6 mol min^{-1} per μg of enzyme [J. L. York and G. A. LePage, *Can. J. Biochem.*, 44, 331 (1966)]; 5.0×10^{-5} M (no absolute V_{max} reported) [B. M. Chassy and R. J. Suhadolnick, *J. Biol. Chem.*, **242**, 2655 (1967)]; 3.3×10^{-5} M and 3.0×10^6 mol min^{-1} per unit of enzyme [S. Fredericksen, *Arch. Biochem. Biophys.*, 113, 383 (1966)]; 2.9×10^{-5} M and 1.4×10^6 mol min^{-1} per unit of enzyme [H. P. Baer, G. I. Drummond, and J. Gillis, *ibid*, 123, 172 (1968)]. [f] No change was observed in the uv spectrum over an 18-h period.

Table VI. Principal Ultraviolet Absorption Bands for 3-Substituted *lin*-Benzohypoxanthine Derivatives



R	λ_{max}, nm					
$C_6H_5CH_2$[a,b]	326	312	300	258	251	245
C_6H_{11}[a,b]	327	313	302	259	251	245
Ribofuranosyl (17)[c]	327	313	298	259	247	243

[a] For preparation, see ref 4a. [b] Determined in 95% EtOH. [c] Determined in 0.05 M phosphate buffer (pH 7.5).

lin-benzoadenosine (**10**) do not change in the three solvents tested (Table IV), these values are high enough to indicate that the corresponding nucleotide and coenzyme analogues will also be highly fluorescent. Such a series, in which the binding sites of the pyrimidine and imidazole are unchanged from adenosine except by a 2.4-Å lateral stretching of the purine system, should prove to be complementary to the fluorescent 1,N^6-ethenoadenosine nucleotide and coenzyme series,[24a,27] in which the normal pyrimidine-ring binding site is blocked by the third ring while the remainder of the structure is unchanged. The benzoadenines have significantly greater spatial requirements than those for adenine and bicyclic analogues[28] that have been previously synthesized and biochemically evaluated as surrogates for adenine and its congeners.

Enzymatic Activity. For the benzoadenine derivatives to be useful as fluorescent probes for the study of biological activity, it is important that they be accepted as substrates or cofactors by a variety of enzymes. The possibility of activity along with existing information concerning substrate specificity with particular enzymes should shed additional light on the steric requirements and size restrictions imposed by active sites. For this study, we selected enzymes that act on free bases and on the heteroaromatic portion of ribonucleosides, as well as enzymes that are involved in ribosyl or phosphoribosyl transfer. For reactions involving free bases, potential substrates related to the linear and angular benzopurines were evaluated. Transformations involving ribonucleosides were limited to those nucleosides described earlier in the Chemistry section. No attempts were made to optimize reaction conditions, but in all cases the conditions employed were consistent with those previously described for the natural substrates.

Adenosine deaminase (calf intestinal mucosa)[29] converted *lin*-benzoadenosine (**10**) rapidly to the corresponding *lin*-benzoinosine (**17**). The reaction was assayed spectrophotometrically by following the decrease in absorption at 345 nm. The kinetic parameters apparent K_M and V_{max} (Table V) were calculated by an adaptation of Kalckar's method.[30] The structure of the product was assigned as **17** by comparison with the uv spectra of 3-substituted *lin*-benzohypoxanthine derivatives synthesized previously (Table VI).[4a] Adenosine deaminase had no detectable action on the isomeric 1-β-D-ribofuranosyl-*lin*-benzoadenine (**14**), the "stretched-out" analogue of 7-β-D-ribofuranosyladenine. The calf enzyme, in our hands, also effected no detectable conversion of adenine to hypoxanthine, although a very slow conversion of this type has been observed by Wolfenden and co-workers[31] when catalyzed by adenosine deaminase from calf duodenum. It was surprising, therefore, to find that the free base *lin*-benzoadenine (**1**) was deaminated to *lin*-benzohypoxanthine (**16**) by adenosine deaminase at a rate comparable to that of the conversion of **10** to **17**. The angular benzoadenines **2** and **3** did not react under comparable conditions. The kinetic parameters in Table V indicate that the linear base **1** and ribonucleoside **10** react at about 85% the V_{max} of the natural substrate adenosine, even though the K_M values are 2.5–3 times greater than that measured for adenosine.

The facile deamination of *lin*-benzoadenosine (**10**) by adenosine deaminase is an indication that the enzyme can accept a substrate of larger size, i.e., 2.4 Å laterally, than the normal substrate adenosine. The conformation of the ribose

Table VII. Relative Reaction Rates with Xanthine Oxidase

Compound	λ, nm[a]	OD_i[b]	OD_f[c]	$\epsilon \times 10^{-3}$[d]	Half-rxn time, min[e]	Rel rate
Hypoxanthine	292	0.050	0.622	10.5	2.10	100
Inosine					No reaction	
lin-Benzohypoxanthine (16)	300	0.252	0.190	4.96	0.83	250
lin-Benzoinosine (17)[f]	310	0.585	0.265	11.7[g]	1.75	120
prox-Benzohypoxanthine (18)	318	0.020	0.190	8.08	144.5	1.5
dist-Benzohypoxanthine (19)	296	0.263	0.153	5.26	0.85	250

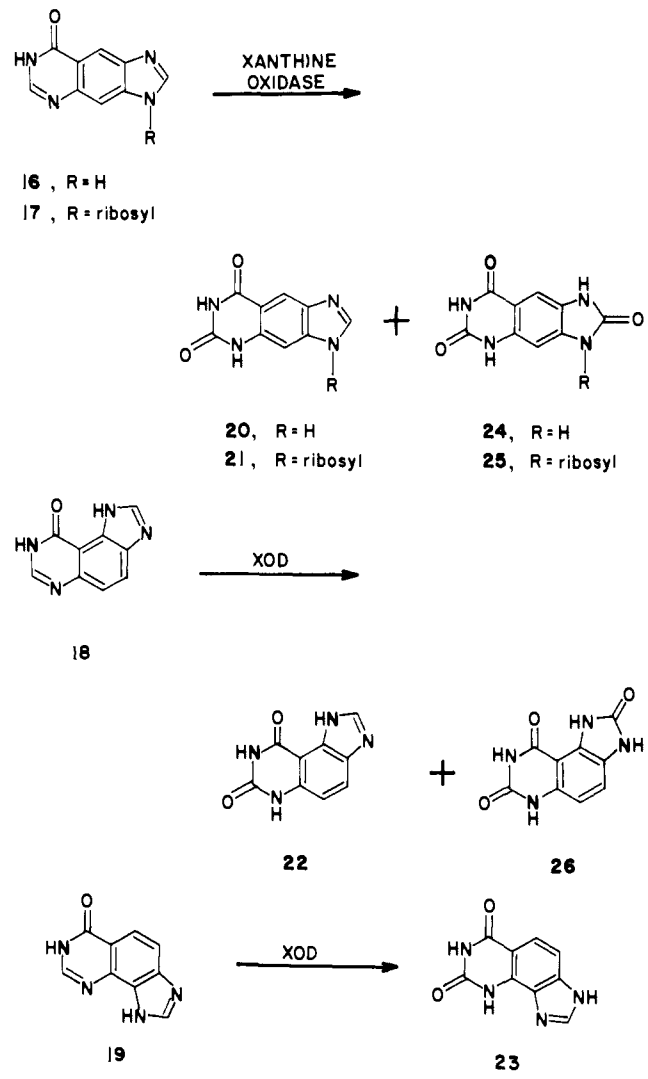
[a] Wavelength monitored. [b] Initial OD reading (all substrates at 5×10^{-5} M). [c] Final OD reading. [d] Extinction coefficient of substrate at given wavelength. [e] 0.15 unit of enzyme (Sigma Grade I) added. [f] Prepared by prior treatment of 10 with adenosine deaminase. [g] Calculated from ε value for 10.

attachment is probably favored anti as in the case of adenosine, so that other features remain unaltered. The equivalent of an unsubstituted N-7 in the adenine system, an important feature for acceptance by adenosine deaminase,[32] is the N-1 of *lin*-benzoadenosine. Thus, the stretched model is not simply related to bulkier molecules such as 8-substituted adenosine derivatives that offer steric hindrance[32] and at the same time possibly altered conformations around the N-9 to C-1′ bond. The adenosine deaminase from calf intestinal mucosa is known to possess broad substrate specificity, promoting hydrolysis of a variety of 6-substituted (halo, methoxy, hydroxyamino, etc.) and 2,6-substituted (e.g., 2-amino-6-halo) purine ribonucleosides as well as several glyconucleosides.[33] It has also been found that potential substrates must be 9-glycosyl derivatives in order to be hydrolyzed by adenosine deaminase at rates comparable to adenosine[34] and that they must have a free 5′-OH for hydrolysis to occur.[35,36] Therefore, it is a surprising and unusual finding that the base *lin*-benzoadenine (**1**) is hydrolyzed to *lin*-benzohypoxanthine (**16**) at the same rate that *lin*-benzoadenosine (**10**) is hydrolyzed to *lin*-benzoinosine (**17**). By contrast, the isomeric 1-β-D-ribofuranosyl-*lin*-benzoadenine (**14**), in which the locus of attachment is equivalent to N-7 on adenine, is not a substrate for adenosine deaminase.

Xanthine oxidase from buttermilk was used to catalyze the air oxidation of the hypoxanthine analogues **16, 18,** and **19** and the inosine analogue **17.** The oxidation was followed by monitoring changes in the uv spectra which were striking, involving the appearance of new maxima and minima as the reaction progressed.

Attempts at measuring V_{max} and K_M at concentrations between 10^{-6} and 10^{-5} M resulted in recorded reaction rates that decreased as substrate concentration increased. This is not unusual since xanthine oxidase is a complex enzyme that exhibits substrate inhibition at unusually low substrate concentrations and obeys complex kinetics.[37] For this reason, relative rates were measured by means of half-reaction times, as shown in Table VII. The *lin*- and *dist*-benzohypoxanthines **16** and **19** reacted at an apparently *faster* rate than hypoxanthine, and the ribonucleoside *lin*-benzoinosine (**17**) was oxidized at approximately the same rate as hypoxanthine. *prox*-Benzohypoxanthine (**18**) reacted two orders of magnitude slower than hypoxanthine. The relative rate of reaction of *lin*-benzoinosine (**17**), about half that of the corresponding base *lin*-benzohypoxanthine (**16**) but at least as fast as hypoxanthine, is particularly unusual since inosine was not a substrate for our preparation of xanthine oxidase. The structures of the products arising from xanthine oxidase catalyzed oxidation of **16–19** were determined by NMR spectra and by mass spectrometry. The reactions were run on a large enough scale (7–10 mg) that the products could be separated, purified, and characterized conveniently (see Experimental Section).

High-resolution mass spectrometry indicated that the oxidation of *lin*-benzohypoxanthine (**16**) with xanthine oxidase afforded two compounds, a monooxidized and a dioxidized product. Corresponding products were obtained from *lin*-



benzoinosine (**17**) and from *prox*-benzohypoxanthine (**18**). The oxidation of *dist*-benzohypoxanthine (**19**) yielded a single monooxidized product, for which the structure could be assigned as *dist*-benzoxanthine (**23**) on the basis of its NMR spectrum. The presence of AB doublets at δ 7.15 and 7.55 (J = 8.5 Hz) indicated that the aromatic protons of the benzene ring were intact. The other aromatic proton in the product, observed as a singlet at δ 8.18, was exchanged by deuterium when the sample was heated with NaOD, indicating that the hydrogen (nonoxidized position) was in the imidazole ring at C-2,[38] consistent with the formulation *dist*-benzoxanthine (**23**). The occurrence of oxidation at C-8 of **19** is equivalent to oxidation at the C-2 position of hypoxanthine. In the case of the oxidation of **16–18,** it was evident from the uv spectra of the products that oxidation did not occur in the central benzene ring, since the absorption spectra were generally shifted to

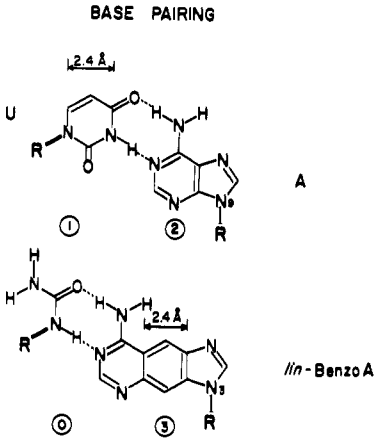


Figure 2. Complementary base pairing between adenosine and uridine compared with that between *lin*-benzoadenosine and *N*-ribosylurea.

shorter wavelength with respect to starting material. The oxidation of the linear and proximal models proceeded further than the xanthine stage, producing monooxidized products **20–22** and dioxygenated products consistent with structures **24–26.** The course of reaction is analogous to the known sequence of xanthine oxidase oxidation of hypoxanthine (→xanthine → uric acid). The relatively slow oxidation of the proximal hypoxanthine benzologue **18** is a probable consequence of the shielding of the carbonyl by the adjacent imidazole ring or by hydrogen bonding through the N-1 hydrogen.

The reaction of the benzohypoxanthine analogues **16, 18,** and **19** with xanthine oxidase was not completely unexpected due to the broad substrate specificity attributed to this enzyme, but the relative rates of oxidation and degree of oxidation of these tricyclic substrates in comparison with hypoxanthine do provide additional definition of the geometrical adaptivity of the active site(s). What is unusual is the enzymatic oxidation of the nucleoside *lin*-benzoinosine (**17**), since there are no previous reports of nucleoside oxidation using xanthine oxidase. In fact, 7- and 9-substituted hypoxanthine derivatives are inactive toward xanthine oxidase.[39] The oxidation of the distal benzologue **19** is interrupted at the xanthine stage, suggesting that structure **23** cannot be accommodated at an oxidation site either because of steric interference of the imidazole ring or hydrogen bonding between N-1 and the newly formed N-H at the 9 position.

Enzymes that promote purine transfer failed to utilize the purine benzologues as substrates. Thus, *lin*-benzoadenosine (**10**) and *lin*-benzoinosine (**17**) were unchanged upon treatment with nucleoside phosphorylase, an enzyme that normally promotes cleavage of the glycosidic bond in ribofuranosides and deoxyribofuranosides of xanthine, hypoxanthine, and guanine, accompanied by phosphorylation of the departing ribosyl moiety.[40] Samples were incubated for prolonged periods with a large excess of enzyme in phosphate buffer with no change in uv spectra.[41] The three isomeric benzoadenines **1–3** failed to react with adenine phosphoribosyltransferase (*E. coli*), an enzyme that promotes the reaction of adenine and 5-phosphoribosyl-1-pyrophosphate (PRPP) to yield 5′-adenylic acid.[42] These reactions were monitored by TLC on cellulose using 1.0 M ammonium acetate for development. Only starting materials were observed (R_f 0.05).[43] The failure of these purine transfer enzymes provides further indication of the highly discriminating nature of each enzyme studied[44] and specific information that a substrate analogue stretched by 2.4 Å or stretched and bent (**2, 3**) cannot be accommodated in an active site. The benzopurine analogues can thus provide positive information even in those cases where they do not serve as substrates. Where these unnatural substrates show high activity, e.g., with adenosine deaminase and xanthine oxidase, the concept of testing the dimensional restrictions of enzyme active sites by lateral extension of the molecular periphery has proved successful. The concept of defined variations in dimensions can be applied to the construction and study of inhibitors as well.

The enzymatic evaluation of the *lin*-benzoadenine nucleotides will be additionally informative, especially since there is a variety of readily available enzymes that utilize adenine nucleotides as substrates, coenzymes, or allosteric effectors. It is interesting to consider base complementarity to *lin*-benzoadenosine (**10**). It could hydrogen bond to uridine or thymidine, but a double helix made up of stretched cross sections consisting of tricyclic and monocyclic bases would be distorted from the normal. By contrast, a perfectly proportioned double helix can be constructed (Figure 2) with an *N*-ribosylurea or *N*-ribosylamide derivative as the complementary partner to *lin*-benzoadenosine. In this combination of tricyclic and acyclic units, the distance and angle between N-$C_{1'}$ bonds are identical with those in the normal complementary pair of bicyclic adenosine and monocyclic uridine. In like manner, *lin*-benzoinosine (**17**) could theoretically hydrogen bond with acyclic *N*-ribosylguanidine or an *N*-ribosylamidine to form a correctly proportional double-helix cross section. Nucleotide incorporation experiments and polynucleotide binding experiments aimed at testing the new complementarity relationships appear feasible.

Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting apparatus and are corrected. The NMR spectra were recorded on Varian Associates A-60, A-56/60, or HA-100 spectrometers using tetramethylsilane (Me_4Si) as an internal standard. The ultraviolet spectra were obtained on either a Cary Model 15 or a Beckman Acta M VI spectrophotometer. Microanalyses were performed by Mr. Joseph Nemeth and his staff, who also weighed samples for the quantitative electronic absorption spectra. Mass spectra were obtained on a Varian-MAT CH-5 low-resolution or a Varian MAT-731 high-resolution spectrometer coupled with a 620i computer and Statos recorder by Mr. J. Carter Cook and his staff. Technical fluorescence excitation and emission spectra were obtained on a Perkin-Elmer Hitachi Model MPF-2A spectrofluorometer.

***lin*-Benzoadenosine (10) and 1-(β-D-Ribofuranosyl)-*lin*-benzoadenine (14).** 2′,3′,5′-Tri-*O*-acetylribofuranosyl bromide was prepared by passing hydrogen bromide through a cooled (ca. −20°) solution of tetra-*O*-acetyl-D-ribofuranose (4.5 g, 15 mmol) in 15 ml of sieve-dried methylene chloride under a stream of dry nitrogen for 30 min. The solution was allowed to warm to room temperature and reduced in vacuo to an oil. All manipulations and distillations were carried out under dry nitrogen with rigorous exclusion of moisture. Three portions of dry toluene were added and removed by distillation in vacuo, making certain that the bath temperature did not exceed 40°.

A slurry of 1.5 g (6.9 mmol) of anhydrous 8-methylthioimidazo-[4,5-*g*]quinazoline (**11**)[4a] and 1.8 g (7.05 mmol) of mercuric cyanide (dried at 100°, 0.5 Torr) in dry nitromethane (150 ml, azeotropically distilled, then distilled from $CaSO_4$) was stirred magnetically while heating at reflux under nitrogen for 1 h. A portion of the solvent (ca. 50 ml) was distilled from the flask to remove traces of moisture azeotropically. In a drybox purged with nitrogen, a methylene chloride solution of the bromo sugar (15 ml) was added to the slurry along with 4 g of Linde No. 4 molecular sieves (dried at 100°, 0.1 Torr). The stirring bar was removed and the slurry was heated at 45° under nitrogen for 3 h. At the end of this time, the solution had turned almost clear and the temperature was raised to 100° for a further 10 h. The solution was filtered and concentrated in vacuo to small volume. Chloroform was added (500 ml) and the solution was extracted three times with 30% aqueous potassium iodide, dried over sodium sulfate, and reduced in volume. The chloroform solution was applied to a silica gel column (300 g, packed in chloroform). Elution with chloroform afforded two fractions, the first uv-absorbing fraction yielding 8-methylthio-3-(β-D-tri-*O*-acetylribofuranosyl)imidazo[4,5-*g*]quinazoline (**12**) as a glass: NMR (CCl_4) δ 2.01 (s, $COCH_3$), 2.09 (s, $COCH_3$), 2.22 (s, $COCH_3$), 2.67 (s, SCH_3), 4.37 (s, CH_2), 4.06–4.55 (m), 5.30–5.55 (m), 5.56–5.75 (m), 6.08 (d, J = 6 Hz, 1′-H), 8.00 (s), 8.24 (s), 8.28 (s), 8.73 (s); $\lambda_{max}^{95\%\ EtOH}$ 240 nm, 278 (sh), 284, 329,

341, 356; field desorption mass spectrum *m/e* 474 (M^+).

Further elution with 99:1 (v:v) chloroform–ethanol afforded 8-methylthio-1-(β-D-tri-*O*-acetylribofuranosyl)imidazo[4,5-*g*]quinazoline (**13**) as a glass: NMR ($CDCl_3$) δ 2.03–2.20 (m, $COCH_3$), 2.70 (s, SCH_3), 4.46 (br s, CH_2), 5.35–5.70 (m), 6.19 (d, J = 5 Hz, 1′-H), 8.19 (s), 8.32 (s), 8.42 (s), 8.87 (s); $\lambda_{max}^{95\%\ EtOH}$ 244 nm, 286, 330, 348, 366; field desorption mass spectrum *m/e* 474 (M^+).

Each fraction was dried overnight under vacuum and taken up in 15 ml of ammonia-saturated ethanol. The resulting solutions were heated separately at 150° for 48 h in sealed tubes. Upon cooling, dark crystals of **10** and **14** were deposited respectively from treatment of **12** and **13.** Recrystallization of *lin*-benzoadenosine (**10**) from water using decolorizing charcoal yielded 540 mg (25%) of colorless needles: mp 294–296° dec; NMR [$(CD_3)_2SO$] δ 3.10 (br, 2, 5′-CH_2), 3.96–4.26 (m, 2, 2′-H and 3′-H), 4.35–4.55 (m, 1,4′-H), 5.12–5.83 (br, 3), 6.01 (d, 1, J = 5 Hz, 1′-H), 7.82 (br s, 2, NH_2), 8.04 (s, 1), 8.40 (s, 1), 8.71 (s, 1), 8.77 (s, 1); $\lambda_{max}^{pH\ 7}$ 231 nm (ε 42 700), 259 (17 600), 316 (7800), 330 (9700), 345 (7300); $\lambda_{max}^{pH\ 1}$ 226 (31 800), 234 (sh), 259 (13 800), 267 (11 600), 290 (sh), 308 (sh), 320 (sh), 332 (12 400), 347 (10 900); $\lambda_{max}^{pH\ 12}$ 231 (42 200), 258 (16 700), 306 (sh), 317 (7500), 330 (9800), 346 (7100); high-resolution mass spectrum *m/e* 317.11234 (M^+, calcd for $C_{14}H_{15}N_5O_4$, 317.11239). Anal. Calcd for $C_{14}H_{15}N_5O_4$: C 52.99; H, 4.77; N, 22.07. Found: C, 52.89; H, 4.69; N, 21.78.

1-(β-D-Ribofuranosyl)-*lin*-benzoadenine (**14**) was recrystallized from water using decolorizing charcoal, yielding 205 mg (9%) of colorless needles: mp 277–280° dec; NMR [$(CD_3)_2SO$] δ 3.64 (d, 2, J = 5 Hz, 5′-CH_2), 3.90–4.26 (m, 2, H-2′ and H-3′), 4.56 (t, 1, J = 5 Hz, H-4′), 5.2–5.7 (br s), 6.02 (d, 1, J = 6 Hz), 7.64 (br s, 2, NH_2), 7.96 (s, 1), 8.33 (s, 1), 8.64 (s, 1), 8.82 (s, 1); $\lambda_{max}^{pH\ 7}$ 223 nm (ε 28 100), 237 (31 200), 263 (17 100), 292 (5600), 304 (sh), 324 (6300), 339 (8700), 354 (6900); $\lambda_{max}^{pH\ 1}$ 221 (32 100), 232 (sh), 259 (11 900), 267 (11 400), 300 (6300), 320 (sh), 332 (11 100), 347 (10 200); $\lambda_{max}^{pH\ 12}$ 239 (33 300), 260 (17 300), 292 (5600), 305 (4700), 326 (6000), 339 (8600), 346 (6800); mass spectrum *m/e* 317 (M^+). Anal. Calcd for $C_{14}H_{15}N_5O_4$: C, 52.99; H, 4.77; N, 22.07. Found: C, 53.34; H, 4.79; N, 21.80.

2′,3′-Isopropylidene-*lin*-benzoadenosine (15). Di-*p*-nitrophenyl hydrogen phosphate (160 mg, 0.47 mmol) was added to a slurry of *lin*-benzoadenosine (127 mg, 0.4 mmol) in 5 ml of acetone containing 0.4 ml of dimethoxypropane. Upon stirring at 25°, the solution cleared, and after 5 h, light purple crystals were deposited (90 mg, 63%). Recrystallization from DMF–methanol yielded colorless crystals of **15:** mp 242–245° dec; NMR [$(CD_3)_2SO$] δ 1.38 (s, 3, CH_3), 1.60 (s, 3, CH_3), 3.50–3.62 (m, 2, 5′-CH_2), 4.92–5.28 (m, 3, H-2′, H-3′, and H-4′), 6.23 (d, 1, J = 3 Hz), 7.75 (br s, 2, NH_2), 7.90 (s, 1), 8.36 (s, 1), 8.66 (s, 1), 8.69 (s, 1); $\lambda_{max}^{95\%\ EtOH}$ 232 nm, 260, 300 (sh), 318, 332, 347; mass spectrum *m/e* (rel intensity) (70 eV) 357 (16.73), 185 (100); MS (10 eV) 357 (86), 185 (100); high resolution *m/e* 357.14300 (M^+, calcd for $C_{17}H_{19}N_5O_4$, 357.14293).

Fluorescence. The technical fluorescence emission and excitation spectra obtained at 25° were not corrected for monochromator efficiency and photomultiplier response. The relative quantum yield of each compound was calculated from the observed absorbance (at 338 nm for **4, 5, 7, 8,** 10, and 14, and 310 nm for **7** and **9**) and the area of the emission spectra. The absolute quantum yield of **10** was determined by comparison with the fluorescence emission of quinine sulfate (quantum yield 0.70 in 0.1 N H_2SO_4).[45] Corrected fluorescence emission of quinine sulfate and compound **10** were determined on a photon counting scanning spectrofluorometer,[46] interfaced to a Nuclear Data ND 4410 data acquisition system. The absolute quantum yields of **4–9** and **14** were calculated from the absolute quantum yield of **10.**

Fluorescence lifetimes were determined at 25° using the cross-correlation spectrofluorometer described by Spencer and Weber.[47] The exciting light was modulated at 14.2 MHz and was filtered through a monochromator and a CS-7-54 Corning filter. The emission was observed through a CS-0-52 Corning filter. Fluorescence lifetime determinations by both phase and modulation were identical to within 0.3 ns for compounds with lifetimes greater than 1 ns. Below 1 ns, only phase determinations are reliable.

Enzyme Studies. For the enzyme studies, adenosine deaminase (calf intestinal mucosa) (E.H. 3.5.4.4), xanthine oxidase (buttermilk) (E.H. 1.2.3.2), and nucleoside phosphorylase (calf spleen) (E.H. 2.4.2.1) were purchased from Sigma Chemical Co. Adenine phosphoribosyltransferase (*E. coli*) (E.H. 2.4.2.7) was a gift from R. L. Switzer. Enzyme assays were performed with a Beckman Acta M VI spectrophotometer at 25°.

Adenosine Deaminase. Solutions of adenosine, *lin*-benzoadenosine (**10**), and *lin*-benzoadenine (1) were made in 0.05 M phosphate buffer (pH 7.5). The concentrations were determined from molar extinction values and optical densities in the uv spectra. To a given amount of substrate, 1 unit of enzyme and sufficient buffer were added to make 3.0 ml. The reaction was monitored by following the change in OD per minute for 10–20% reaction completion (265 nm for adenosine, 346 nm for **10,** and 345 nm for 1). Values of K_M and V_{max} were calculated from the experimental data by the Lineweaver–Burk method,[48] using a Digital PDP 8/e computer to fit a least-squares program. Compounds which did not react (e.g., **2, 3,** and **14**) showed no change in OD reading over an 18-h period.

Xanthine Oxidase. Solutions of hypoxanthine, **16, 17, 18,** and **19** were made 5×10^{-5} M in 0.05 M phosphate buffer (pH 7.5). The solution of **17** was prepared by treating a 5×10^{-5} M solution of *lin*-benzoadenosine (**10**) with adenosine deaminase. Enzyme (0.125 unit) was added to 3.0 ml of each solution, and the change in OD was recorded as a function of time (300 nm for **16,** 310 nm for **17,** 318 nm for **18,** 296 nm for **19,** and 293 nm for hypoxanthine). The reactions were allowed to stand 4 h to determine the final OD reading so that accurate half-reaction times could be measured.

For preparative runs, 7–10 mg of **16, 18,** and **19** was dissolved in 1 ml of hot DMF and then poured into 100 ml of hot buffer (0.1 N NH_4OAc, pH 8.0). The solutions were cooled to 37° and an excess of enzyme was added. The solutions were then incubated at 37° for 18 h. The products precipitated and were collected by filtration or centrifugation.

The oxidation products of **16,** namely **20** and **24,** were analyzed by mass spectrometry: *m/e* 202.04924 (M^+, calcd for $C_9H_6N_4O_2$, 202.04924); *m/e* 218.04412 (M^+, calcd for $C_9H_6N_4O_3$, 218.04411).

The products isolated from **18,** namely, **22** and **26,** showed the following molecular ions in the mass spectra: *m/e* 202.04912 (M^+, calcd for $C_9H_6N_4O_2$, 202.04924); *m/e* 218.04408, (M^+, calcd for $C_9H_6N_4O_3$, 218.04411).

The reaction of **19** yielded **23:** *m/e* 202.04912 (M^+, calcd for $C_9H_6N_4O_2$, 202.04924); NMR [$(CD_3)_2SO$] δ 7.30 and 7.72 (AB doublets, 2, J = 8 Hz), 8.32 (s, 1). Four drops of 1.0 N NaOD was added and the sample was heated at 100° for 48 h. The peak corresponding to δ 8.32 shifted to δ 7.85 and lost 80% of its intensity.

lin-Benzoadenosine (7 mg) was treated with xanthine oxidase as described above. After digestion, the resulting solution was reduced in volume to 2 ml and applied to a column of Aminex A-5.[49] Elution with 0.4 M ammonium formate afforded two fractions (monitored by absorption at 260 nm) which were passed through a column of −400 mesh P-2 gel (20% ethanol).[50] The resulting fractions were analyzed by field desorption mass spectrometry:[14] *m/e* 334 for **21,** 350 for **25** (M^+ in each case).

Nucleoside Phosphorylase. To separate solutions of **10** and **17** (made 5×10^{-5} M in 0.1 M phosphate buffer, pH 7.5) was added 1 unit of enzyme. No change in uv spectra occurred over a 4-h period (spectra taken at acidic, neutral, and basic pH).

Adenine Phosphoribosyltransferase. These assays were performed in a medium containing 0.15 mM analogue, 0.05 M Tris (pH 7.5), 1 mM EDTA, 0.01 M NaF, 0.01 M $MgCl_2$, 4 mM PRPP, and an excess of enzyme. The substrates were incubated at 37° for 40 min and quenched by adding excess EDTA. The reactions were monitored by TLC on neutral cellulose (Eastman, with fluorescent indicator) using 1 M NH_4OAc as the eluent. For 1, **2,** and **3,** the only spots observed corresponded to starting materials (R_f ~0.05).

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(5) The prefix lin refers to the linear disposition of the three rings in compound **1**; prox for proximal and dist for distal refer to the relationship of the amino group in compounds **2** and **3**, respectively, with respect to the imidazole ring. The numbering is as shown. We feel justified in using the term "benzo" in the trivial names of these compounds because *only when the additional ring is central* does it contain no nitrogens and is accordingly "benzo". This terminology is capable of easy adaptation to derivatives related to adenosine, adenylic acid, adenosine diphosphate, nicotinamide adenine dinucleotide, etc., and also can be extended to benzologues of other purinoid compounds, i.e., benzoguanine, benzohypoxanthine, benzoinosine, etc., where the prefix prox or dist relates the position of the imidazole ring to the 6-substituent.

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