Dimensional Probes of Enzyme–Coenzyme Binding Sites

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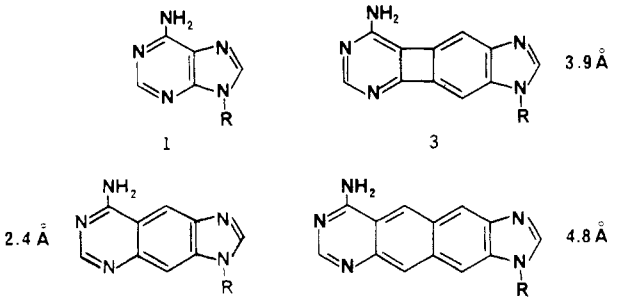
Dimensional probes of enzyme–coenzyme binding sites are related to the natural coenzymes by defined dimensional changes in the molecules. The importance of ATP (adenosine 5′-triphosphate), among the natural coenzymes, as a source of energy for living cells is illustrated by the calculation[1] that a person uses and resynthesizes his own body weight of ATP daily. One-sixth of all known enzymes require ATP or a related adenine-containing cofactor (1), such as AMP, ADP, cyclic AMP, NAD^+, NADPH, FAD, or coenzyme A; yet for none of these is the binding site of the adenine moiety described more fully than as a "pocket", "slot", or "surface".

The best information provided by X-ray analysis for an enzyme–ATP complex is a composite structure for hexokinase,[2,3] in which "the adenine is situated in a shallow depression on the surface of the large lobe of the enzyme at the entrance to its deep cleft."[2] Binding of the substrate glucose to the bottom of the cleft causes a 12° rotation of one lobe of hexokinase relative to the other, closing the cleft,[4,5] Since the distance between the γ-phosphate of the ATP and the 6-hydroxy of glucose is nearly 6 Å (at 3-Å resolution), transfer of the γ-phosphate to glucose[6] requires that the Mg^{2+}-ATP and the glucose be brought closer together. To determine the extent of further conformational change and to establish the active-site geometry, a crystal structure of the ternary glucose–enzyme–Mg^{2+}-ATP complex would be required.[7]

Because of the absence of highly resolved X-ray structure determinations of various crystalline enzyme–ATP complexes, we decided to assess the size of the space available for adenine with dimensional probes. While natural nucleoside triphosphates other than ATP have been found, not surprisingly, to be mainly inactive with ATP-requiring enzymes and while many analogues of adenine nucleotides have been made and tested for enzyme activation and inhibition, we have been concentrating on stretched-out analogues that are unique in retaining the terminal pyrimidine and imidazole rings. Thereby, the normal hydrogen-bonding sites, e.g., N1, N^6, and N7, of the adenine moiety are retained, while a spacer is formally inserted between the terminal rings. The spacer has dimensions known from X-ray structure determinations of corresponding hydrocarbon systems. Thus, the formal insertion of a benzene ring into the center of the adenine ring system (2) stretches

DIMENSIONAL PROBES OF ADENINE BINDING SITES



the original (1) linearly by 2.4 Å, insertion of a benzocyclobutadiene unit (3) stretches it by 3.9 Å, and insertion of a naphthalene unit (4) stretches it by 4.8 Å. Although the heteroaromatic ring systems in 2–4 differ electronically from adenine, which will be reflected in their basicity, nucleophilicity, and π-binding characteristics, at least such differences are related to one major structural feature. The degree of interaction of compounds in this series with appropriate enzymes reveals the effect of incrementally greater spacing between N7 and 6-NH_2, and N9 and N3 of the original adenine ring. We have synthesized *lin*-benzoadenine nucleotides (2), which act as cofactors in enzyme reactions and have spectroscopic properties, including useful fluorescence, that help define their environments.

Our experiments with dimensional probes have the following five levels: (1) synthesis and characterization of the heterocyclic nuclei, previously unknown in the cases of 2–4; (2) N-ribosidation, generally directed toward the five-membered ring by deactivation of the pyrimidine ring; (3) mono-, di-, and triphosphorylation, purification, and structure roof of the resulting ribonulceotides; (4) determination of the enzyme binding and kinetics relative to the natural substrate or cofactor; and (5) NMR spectroscopy for conformational analysis and fluorescence spectroscopy for metal ion and enzyme binding.

Synthesis. The synthesis of *lin*-benzoadenine (2, R = H) started with nitration of 7-chloro-4-quinazolone (5)[8] and separation of mononitro isomers by fractional crystallization. The predominant 7-chloro-6-nitro-4-quinazolone (6) was elaborated to 8-(methylthio)-imidazo[4,5-*g*]quinazoline (7, R = H) in five steps.[9]

Nelson J. Leonard was born in Newark, NJ, in 1916. He received his undergraduate education at Lehigh University. After 2 years at Oxford University as a Rhodes Scholar and graduate work at Columbia University, where he received the Ph.D. degree, he went to the University of Illinois for postdoctoral work with Roger Adams. Except for U.S. Government service and for visiting professorships, he has been ever since on the faculty of the University of Illinois, where he is now Professor of Chemistry and Biochemistry and a Member of the Center for Advanced Study. This article is based in part on his address on the occasion of receiving the Roger Adams Award in Organic Chemistry at the 27th National Organic Chemistry Symposium, American Chemical Society, 1981.

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(2) M. Shoham and T. A. Steitz, *J. Mol. Biol.*, **140**, 1 (1980).
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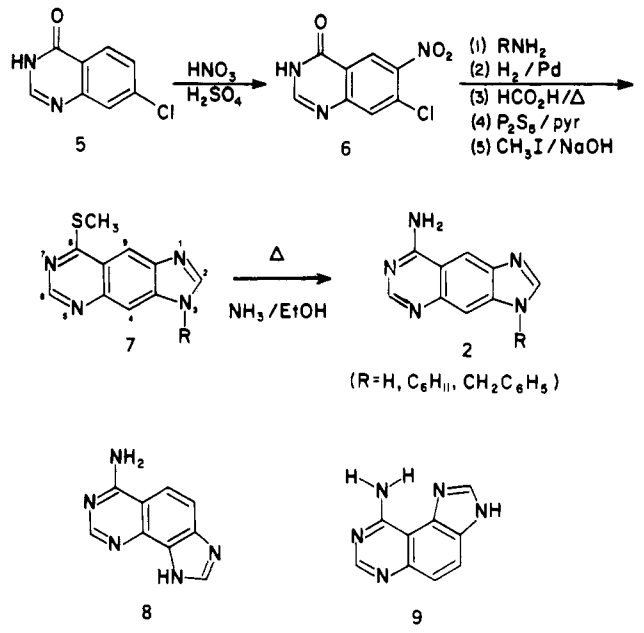


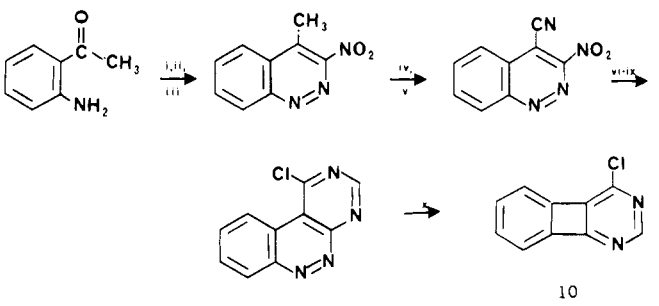
The methylthio substituent was used for displacement by ammonia (→**2**, R=H) and primary amines[10] and for deactivation of the pyrimidine ring, thereby guiding alkylation[9] and N-ribosidation[11] to the imidazole ring. *dist*-Benzoadenine (**8**) was made in a manner analogous to *lin*-benzoadenine (**2**, R=H) from the minor isomer obtained in the nitration of 7-chloro-4-quinazolone (**5**), and *prox*-benzoadenine (**9**) was made from the intermediate formed in the nitration of 6-acetamido-4-quinazolone.[12,13]

A route to the *lin*-benzocyclobutadienoadenine (*lin*-bcb-adenine) compounds (**3**)[13] involves the pyrolysis of an internal pyrazine ring as precursor to the four-membered ring. For example, the synthesis of 4-chloro-1,3-diazabiphenylene (**10**) is outlined in Scheme I.[14] Further steps necessary for elaboration of the terminal imidazole ring include nitration, analogous to the β-nitration observed for biphenylene,[15] selective reduction, followed by nitration, reduction, and ring closure. The chloro group in **10** will serve the same dual role as the methylthio group in **7**. The extrusion of nitrogen should also be possible when an imidazole ring is already in place in the molecule, as projected through **11** in Scheme II.[14]

An effective synthesis for the construction of the *lin*-naphthopurine ring system, as in **4**, had to be regioselective, providing appropriate tetra-β-substituted naphthalene intermediates. Using 3,4-bis(trimethylsilyl)bicyclo[4.2.0]octa-1,3,5-triene (**12**),[16] we depended upon the trimethylsilyl groups to undergo selective substitution by bromo and nitro (electrophilic desilylation) of two of the eventual four β-positions of the naphthalene ring (Scheme III) faster than competitive α substitution (electrophilic displacement of hydrogen).[17] Ammonia displacement and acetylation gave the N-protected intermediate **13**, permitting selective radical bromination on the four-membered ring, which was followed by treatment with silver acetate to provide the mixed acetoxy derivatives. When these (**14**), which are thermally equivalent to acetoxy-*o*-xylylene intermediates (**15**), were heated in dimethyl acetylenedicarboxylate, they underwent cycloaddition and loss of acetic acid to yield one tetra-β-substituted naphthalene, **16**. It was found important to construct the imidazole ring, as in **17**, prior to formation of the pyrimidine ring.[17] The intermediate **18** (with the 3-Ac isomer) was pivotal in conversions of *lin*-naphthohypoxanthine (**19**) and *lin*-naphthoxanthine (**20**) and should also lead to *lin*-naphthoadenine (**4**, R = H) and *lin*-naphthoguanine.

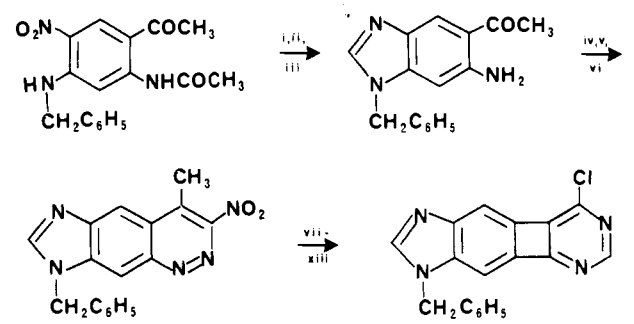
N-Ribosidation. In the *lin*-benzo series, the N3 position is the desired location for the ribofuranosyl group (**2**, R = ribosyl) for concordance with adenosine. Since the N ribosidation of intermediate **7** (R = H) occurred at both N1 and N3, chromatographic separation and identification of isomers was necessary. Differentiation at the 8-(methylthio)-3(1)-(β-D-tri-*O*-

Scheme I[a]



[a] i, $NaNO_2/HCl$, ii, $CH_3NO_2/NaOH$; iii, $DABCO/H_2O$; iv, *i*-AmONO/HCl; v, Ac_2O; vi, H_2SO_4; vii, $SnCl_2/HCl$; viii, HCOOEt/NaOEt; ix, $POCl_3$, $PhNEt_2$; x, 600-650 °C, silica tube, 0.01 mmHg.

Scheme II[a]



[a] i, H_2/Ni; ii, $CH(OMe)_3$, HCOOH; iii, HCl, EtOH; iv, $NaNO_2/HCl$; v, CH_3NO_2/KOH; vi, $DABCO/H_2O$; vii, *i*-AmONO/HCl; viii, Ac_2O; ix, $SnCl_2/HCl$; x, KOH, EtOH, H_2O; xi, HCOOEt/NaOEt; xii, $POCl_3$, $PhNEt_2$; xiii, 650 °C, silica tube, 0.01 mmHg. Debenzylation may have to precede stages xii and xiii.

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(13) The prefix *lin* refers to the linear relationship of the three rings in compound **2**; *prox* for proximal and *dist* for distal refer to the spatial relationship of the amino group in compounds **9** and **8**, respectively, with respect to the imidazole ring. The fusion prefixes benzo, etc., when used with *lin*, *dist*, and *prox* do not mean fusion, but insertion. The term "benzo" presents no ambiguity, for only when the ring is central does it contain no nitrogens and is accordingly "benzo". The same is true of the term "naphtho", as used for **4**, **19**, and **20**. For "benzocyclobutadieno", we are testing the abbreviation "bcb".
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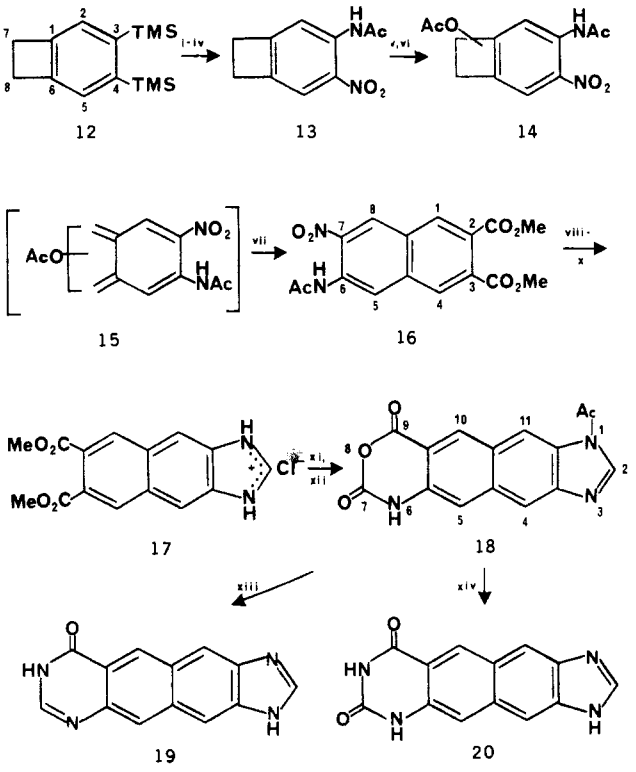
Scheme III[a]



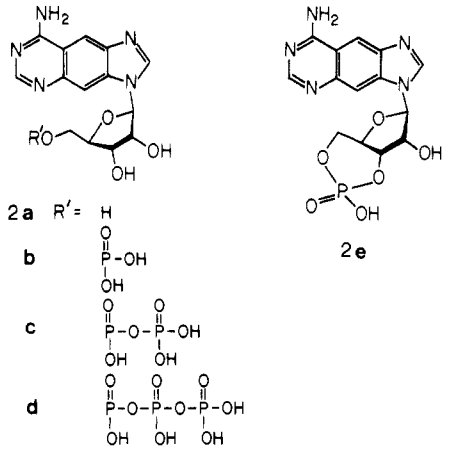
[a] i, Br_2, Py, CCl_4; ii, HNO_3, Ac_2O, HOAc; iii, NH_3, *n*-BuOH, bomb; iv, AcCl; v, NBS, CCl_4, $h\nu$, $(PhCO_2)_2$; vi, AgOAc, HOAc; vii, DMAD, ST; viii, HCl, MeOH; ix, H_2, Pd/C, MeOH; x, $CH(OMe)_3$, HCO_2H; HCl/MeOH; xi, NaOH, MeOH; H_3O^+; Ac_2O, xii, Me_3SiN_3, Py; xiii, formamide; xiv, urea.

acetylribofuranosyl)imidazo[4,5-*g*]quinazoline (**7**, R = 2′,3′,5′-tri-*O*-acetylribofuranosyl) stage was possible by means of ultraviolet spectroscopic comparison with the 1- and 3-benzyl substituted derivatives, the latter being available by unequivocal synthesis from compound **6** and benzylamine. The other isomer was shown to be 1-substituted by unequivocal ^{15}N labeling of the 1-position and ^{13}C NMR spectroscopy, in which the ^{15}N–^{13}C coupling constant for the α carbon of the N substituent showed a clear doublet.[18] The method of structure assignment by ^{15}N–^{13}C coupling is general and offers a positive means of identifying the site of N-alkylation or N-glycosidation in similar heterocycles.

Treatment of compound **7** (R = 2′,3′,5′-tri-*O*-acetylribofuranosyl) with ethanolic ammonia at 150 °C effected concomitant deblocking of the sugar group and displacement of methylthio by amino to afford *lin*-benzoadenosine (**2**, R = ribosyl). The β configuration at the anomeric carbon was established by the ^1H NMR spectra of this product and its isopropylidene derivative.[11]

For the synthesis of the related *lin*-benzoguanosine, *lin*-benzoinosine, and *lin*-benzoxanthosine, N-ribosidation of the mercuric salt of 6-(ethylthio)-imidazo[4,5-*g*]quinazolin-8-one (see **7** for numbering) gave a common intermediate in which the ethylthio group was displaced by ammonia to give *lin*-benzoguanosine or was removed reductively to give *lin*-benzoinosine.[19] An authentic sample of *lin*-benzoinosine was produced by the facile deamination of *lin*-benzoadenosine (**2**, R = ribosyl) by adenosine deaminase from calf intestinal mucosa.[11] *lin*-Benzoinosine was oxidized by xanthine oxidase to *lin*-benzoxanthosine, completing the benzo series related to the natural ribonucleosides adenosine, guanosine, inosine, and xanthosine. A new general method of converting ribonucleosides to deoxyribonucleosides (**1**, **2**, R = 2-deoxyribosyl) had to be devised for efficient entry into the deoxy series.[20]

Phosphorylation. It was desirable that phosphorylation of **2a** be efficient and specific and, if possible, that it proceed without the necessity of protection–deprotection steps. Unprotected *lin*-benzoadenosine (**2a**) was converted to *lin*-benzo-AMP (**2b**) by reaction



with pyrophosphoryl chloride in *m*-cresol.[21] The integrity of the 5′-phosphate was established by its ^{31}P NMR spectrum and by its complete reversion to *lin*-benzoadenosine on incubation with 5′-nucleotidase, a highly specific enzyme.[22-24] The 5′-monophosphates of the closely related *lin*-benzoguanosine, *lin*-benzoinosine, and *lin*-benzoxanthosine were obtained by the same method.[25] By using pyrophosphoryl chloride with *lin*-benzoadenosine in the absence of solvent at low temperature, we obtained *lin*-benzoadenosine 3′-(2′),5′-bisphosphate, which was of interest as a donor in the T4-induced RNA ligase reaction that linked the 3′,5′-bisphosphate to the 3′ end of oligonucleotides.[26]

lin-Benzo-ADP (**2c**) and *lin*-benzo-ATP (**2d**) were prepared from *lin*-benzo-AMP[22,23] by the phosphoromorpholidate method.[27] Once we discovered that *lin*-benzo-ADP (**2c**) was active as a substrate with rabbit muscle pyruvate kinase and phosphoenolpyruvate, this convenient enzymatic route from the di- to the triphosphate enabled us to maintain our supply of *lin*-benzo-ATP by recycling after testing the cofactor in

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Table I
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	265	8.1	2.2	2.0
lin-benzoadenosine (**2a**)		7.5	5.3	1.7
lin-benzoadenine (**2**, R = H)	346	6.6	6.2	1.7
dist-benzoadenine (**8**)			no reaction	
prox-benzoadenine (**9**)			no reaction	

[a] Wavelength monitored. [b] Change in molar extinction coefficient. [c] Expressed as moles per minute per 1 unit of enzyme.

various ATP-requiring enzyme systems. The 5′-di- and triphosphates of *lin*-benzoguanosine, *lin*-benzoinosine, and *lin*-benzoxanthosine were made by similar procedures.[25]

lin-Benzo-cAMP (**2e**) was synthesized from *lin*-benzoadenosine via the 5′-[(trichloromethyl)-phosphonate] derivative,[28] with 3′-O anionic displacement of the trichloromethyl group. The structure and purity of this cAMP analogue were established by HPLC, ^{31}P NMR spectroscopy, electrophoresis, and its conversion to **2b** on incubation with beef heart nucleotide 3′,5′-phosphodiesterase, an enzyme that plays an important role in regulating intracellular cAMP.[29]

Enzyme Binding and Kinetics. When the syntheses of the laterally extended analogues of the natural purines, nucleosides, and nucleotides had been performed, the next question was whether they would be active as substrates or cofactors in various enzyme systems. Adenosine deaminase from calf intestinal mucosa,[30] which possesses broad substrate specificity, converts *lin*-benzoadenosine (**2a**) to the corresponding *lin*-benzoinosine (**24**). The apparent Michaelis constants and relative velocities (Table I) indicate that *lin*-benzoadenosine reacts at about 85% of the V_{max} of the natural substrate adenosine, with a K_m value of the same order as that for adenosine.[11] The facile deamination of *lin*-benzoadenosine by adenosine deaminase indicates that the enzyme can accept a substrate larger than the normal substrate adenosine. The anti conformation of the ribose attachment is favored as in adenosine. That *lin*-benzoadenine (**2**, R = H) is hydrolyzed to *lin*-benzohypoxanthine (**22**) at the same rate as *lin*-benzoadenosine is hydrolyzed to *lin*-benzoinosine is surprising, since adenine itself is not converted to hypoxanthine (**21**) by this enzyme. Moreover, the angular benzoadenines **8** and **9** do not react under comparable conditions.

Xanthine oxidase from buttermilk, a complex enzyme that catalyzes the air-oxidation of hypoxanthine (**21**) to xanthine to uric acid and that has broad substrate specificty,[31,32] also catalyzes the oxidation of *lin*-benzohypoxanthine (**22**) to *lin*-benzoxanthine to *lin*-benzouric acid (Figure 1). While there are no previous

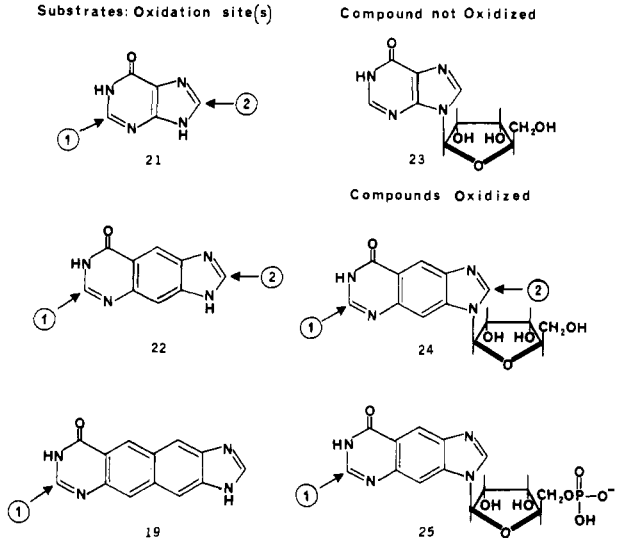

Figure 1. Reaction with xanthine oxidase.

reports of ribonucleoside oxidation using xanthine oxidase, i.e., inosine (**23**) is not a substrate, and 7- and 9-substituted hypoxanthine derivatives are inactive twoard xanthine oxidase,[33] *lin*-benzoinosine (**24**) undergoes oxidation to *lin*-benzoxanthine and to the *lin*-benzo analogue of 9-ribosyluric acid.[11] Thus, when unfavorable steric interaction of the hydrophilic ribosyl group in inosine is displaced 2.4 Å by the benzene-ring spacer in *lin*-benzoinosine, the possibilities of binding in the hydrophobic pocket of the enzyme and of oxidation are restored. Even the ribonucleotide analogue *lin*-benzo-IMP (**25**) is oxidizable to *lin*-benzo-XMP with xanthine oxidase and oxygen.[25] In this case, oxidation occurs in the pyrimidine ring, whereas further oxidation at the imidazole carbon is inhibited by the presence of the added phosphate group. We extended our examination of the dimensional tolerance of the binding pocket in xanthine oxidase with *lin*-naphthohypoxanthine (**19**) and *lin*-naphthoxanthine (**20**). *lin*-Naphthohypoxanthine (**19**) is oxidized to *lin*-naphthoxanthine (**20**) but no furhter. the V_{max} values increase in the order hypoxanthine (**21**) < *lin*-benzohypoxanthine (**22**) < *lin*-naphthohypoxanthine (**19**).[17] The K_m values for *lin*-benzohypoxanthine and *lin*-naphthohypoxanthine are similar. *lin*-Naphthohypoxanthine functions as a competitive inhibitor of the oxidation of hypoxanthine by xanthine oxidase, and *lin*-naphthoxanthine functions as a noncompetitive inhibitor. The limiting lateral extension of the substrate for xanthine oxidase to be able to act on the imidazole ring appears to be between 2.4 and 4.8 Å. Further refinement of the limit should be possible as *lin*-bcb-hypoxanthine becomes available (series represented by **3**).

The *lin*-benzo-cAMP (**2e**) analogue of cAMP maximally activates protein kinase from brain and from skeletal muscle.[34] Addition of low levels of *lin*-benzo-cAMP does not inhibit activation of protein kinase by cAMP, while *lin*-benzoadenosine (**2a**) inhibits kinase activity slightly less than does adenosine. Thus, *lin*-benzoadenosine and its 3′,5′-monophosphate interact

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with cAMP-dependent protein kinase, an enzyme that plays a pivotal role in many hormonal responses,[35] showing that the active sites can accommodate the lateral extension of the purine ring by 2.4 Å. The interaction of the catalytic subunit and holoenzyme of cAMP-dependent protein kinase with *lin*-benzoadenine nucleotides has been examined by Hartl and Roskoski.[36]

The allosteric activation of aspartate transcarbamylase (ATCase) occurs with *lin*-benzo-ATP (**2d**) to the same extent as it does with ATP, and the association constant is similar to those previously obtained for ATP at a variety of temperatures, buffers, and pH's.[37] The enzyme ATCase from *Escherichia coli* catalyzes the conversion of carbamyl phosphate and aspartate to carbamyl aspartate and phosphate. This reaction is the first step in the pyrimidine biosynthetic pathway, and, in prokaryotes, it is subject to allosteric inhibition and activation. Cytidine nucleotides, the end products of the pathway, inhibit ATCase, while adenine nucleotides activate the enzyme.[38,39] From the fluorescence of *lin*-benzo-ATP in the presence of ATCase (see below), we deduced the absence of π interactions between the activator and aromatic residues in the protein and the involvement of N7 of *lin*-benzo-ATP (analogous to N1 of ATP) in the enzymatic binding. Both the activation and the binding are consistent with the London–Schmidt hypothesis for enzyme activation or inhibition.[40]

Values for binding (K_m) and maximum velocity (V_{max}) for *lin*-benzo-ADP (**2c**) with pyruvate kinase allowed us to use a coupled assay for *lin*-benzo-ADP formation involving pyruvate kinase and lactate dehydrogenase.[23] Such formation occurs when *lin*-benzo-ATP serves as a substrate for phosphofructokinase (PFK). Other phosphoryl transfer enzymes with which *lin*-benzo-ATP substitutes for ATP with varying degrees of efficiency include yeast hexokinase, phosphoglycerate kinase, acetate kinase, and adenylate kinase.[22,23] In all cases, utilization of *lin*-benzo-ATP (**2d**) represents the largest known dimensional deviation from the natural cofactor ATP. The structural requirements for inhibitory activity of yeast PFK by higher concentrations of nucleoside triphosphate are more stringent than for catalytic activity, yet *lin*-benzo-ATP inhibits the enzyme to approximately the same degree as ATP. The ability of *lin*-benzo-ATP to phosphorylate 3-phosphoglyceric acid catalyzed by yeast 3-phosphoglycerate kinase permits equilibrium of the γ (terminal) phosphate in the nucleoside triphosphate with $^{32}PO_4^{3-}$, and [γ-^{32}P]-*lin*-benzo-ATP can be prepared thereby. The conformational change for bringing the phosphoglycerate site and the ATP site together[41] will apparently accommodate the wider nucleus represented by *lin*-benzo-ATP.

Adenylate kinase (rabbit muscle) catalyzes the transfer of phosphate between Mg^{2+}-ATP plus AMP and Mg^{2+}-ADP plus and ADP. Our results (Table II)[23] support the two-site model in which one site is specific for AMP or ADP and the other, less demanding, is specific for ATP or ADP.[42-44] In the crystal, native adenylate kinase (pig muscle) has an open cleft, and AMP causes the cleft to close.[45] In aqueous solution, the relation of the highly specific AMP binding site to one of the ADP sites is shown unequivocally by using a mixture of *lin*-benzo-ADP (**2c**) and ADP with the adenylate kinase. *lin*-Benzo-ADP alone remains unchanged with the enzyme, while the mixture comes to equilibrium with five species present, AMP, ADP, ATP, *lin*-benzo-ADP, and *lin*-benzo-ATP, but no *lin*-benzo-AMP. Thus, one of the ADP binding sites is highly specific and corresponds to the AMP binding site, in accord with the principle of microscopic reverisibility. Since the sugar/base conformation of *lin*-benzo-AMP (**2b**) is anti and since the compound possesses the requisite NH_2 group,[44] the extension of the heteroaromatic ring system[23,46] may account for its failure as a substrate for adenylate kinase.[23] By contrast, the enzyme's hydrophobic pocket for ATP[47] accommodates the laterally extended *lin*-benzo-ATP.

Table II
Adenylate Kinase[a]

$$Mg^{2+}\text{-ATP} + AMP \underset{\text{myokinase}}{\rightleftharpoons} Mg^{2+}\text{-ADP} + ADP$$

\+ *lin*-benzo-ATP + AMP
\- *lin*-benzo-ATP + *lin*-benzo-AMP
\- ATP + *lin*-benzo-AMP
\- *lin*-benzo-ADP
\+ *lin*-benzo-ADP + ADP

[a] A plus sign means reaction; a minus sign means no reaction took place.

Because the route to *lin*-benzo-ATP (**2d**) and other products in this series (**2**) guarantees freedom from contamination by natural adenine ribonucleotides, there can be no uncertainty when the analogues show enzymatic activity. Although *lin*-benzo-ADP (**2c**) is not converted to the 5′-triphosphate (**2d**) with intact rat liver mitochondria,[48] fragments of mitochondrial particles will phosphorylate *lin*-benzo-ADP and hydrolyze *lin*-benzo-ATP. Purified mitochondrial ATPase is inhibited more potently by *lin*-benzo-ADP than by ADP. The inactivity of the ADP analogue with intact mitochondria is due to its failure to be transported across the inner membrane at an appreciable rate, i.e., to serve as a substrate for the adenine nucleotide carrier.

A *lin*-benzoadenine ribonucleotide moiety may be incorporated in an oligonucleotide by single addition with T4 RNA ligase:[26,49]

$$(Ap)_3C + pNp \xrightarrow[ATP]{\text{RNA ligase}} (Ap)_3CpNp$$

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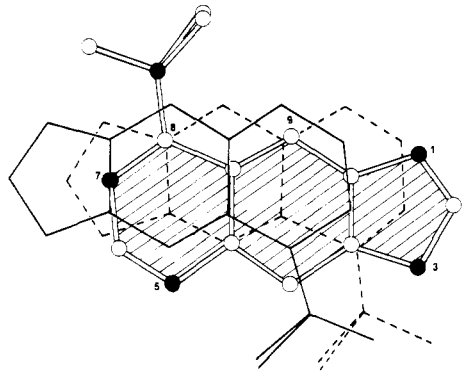


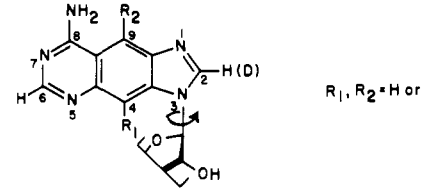
Figure 2. Intermolecular stacking pattern in crystals of the *N,N*-dimethyl derivative of *lin*-benzoadenine, 8-(dimethylamino)imidazo[4,5-*g*]quinazoline (●, N; ○, C). One of the methyl groups attached to N^8 avoids interaction with the peri-H at C-9 to some extent by displacement above or below the heteroaromatic plane in a disordered arrangement around the side-chain nitrogen (shown).

In the presence of excess enzyme, *lin*-benzoadenosine 3′,5′-bisphosphate is incorporated at the 3′ end of the tetranucleotide receptor in 92% yield. It is possible to make poly(*lin*-benzoadenylic acid) from *lin*-benzo-ADP (**2c**) using polynucleotide phosphorylase from *Micrococcus luteus*, which is primer independent,[22,23] in the presence of Mn^{2+}, but the rate of polymerization is slower than that for the formation of poly(adenylic acid) from ADP under the same conditions. For polymerization of *lin*-benzo-IDP to poly(*lin*-benzoinosinic acid) with polynucleotide phosphorylase, it was necessary to use a GpU primer in the presence of Mg^{2+}.[25] *lin*-Benzo-ATP does not replace ATP for T7 phage DNA-directed RNA synthesis in vitro and does not inhibit ATP incorporation into RNA,[23] nor does *lin*-benzo-ATP get incorporated by primase, a rifampicin-resistant RNA polymerase, into a polynucleotide chain, the synthesis of which is template directed.[50] These experiments indicate that normal Watson–Crick base pairing is exacting in template-directed polymerizations.

Spectroscopic Properties. ^{31}P NMR spectra of the *lin*-benzoadenine ribonucleotides confirmed their structures[23] and established or reaffirmed certain generalities. Thus, the 5′-P signal experiences little change from the average value of 3.88 ppm downfield from 85% H_3PO_4, for many compounds observed[26] and is distinct from the chemical shift of 1.7 ppm upfield for both *lin*-benzo-cAMP and cAMP.[23] Among the ribonucleotide 3′,5′- and 2′,5′-bisphosphates observed, the 2′-P signal is consistently upfield from the 5′-P resonance, and the 3′-P signal is shifted downfield by ~0.20 ppm.[26]

In the series *lin*-benzo-AMP, -ADP, and -ATP (**2b–d**) in aqueous solution, the UV-spectroscopically determined pK_a values give a unique response to the presence and conformation of the phosphate side chain.[22,51] When no intramolecular interaction can occur between phosphate and base, as in *lin*-benzoadenosine (**2a**) and *lin*-benzo-cAMP (**2e**), the N^+–H pK_a value is 5.6, unchanged in 5 mM Mg^{2+}, whereas the values of **2b–d** (7.6, 7.3, 7.1, respectively) indicate that the phosphates in these molecules are involved in base protonation–deprotonation. The decrease in pK_a values of *lin*-benzo-ADP (**2c**) and *lin*-benzo-ATP (**2d**) to 6.9 and 6.6, respectively, in the presence of 5 mM Mg^{2+} is parallel to, but greater than, that observed for ADP and ATP[52] and indicates the formation of Mg^{2+} complexes with the pyrophosphate unit[53] of **2c** and **2d**. The pK_a values are also lowered in the presence of Mn^{2+} and Co^{2+}. Association of *lin*-benzo-ATP (**2d**) with quaternary ammonium micelles reduced the N^+–H pK_a value to that of *lin*-benzoadenosine (**2a**) by breaking the interaction between the phosphate and the base. The rise in fluorescence polarization of *lin*-benzo-ATP in the presence of $CetNMe_3Cl$ micelles confirms the electrostatic binding.

During the synthesis of the heteroaromatic ring of *lin*-benzoadenine nucleotides, each of the C protons could be replaced specifically by deuterium of a different extent (**26**), and the relative intensities allowed



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unequivocal assignment of the individual proton resonances throughout the series **2a–e** and in P^1, P^2-di-*lin*-benzoadenosine 5′-pyrophosphate.[54] This technique greatly reduces the number of experiments and variables involved and prevents misassignments of the signals upon changing the multiplicity of phosphate groups and solution concentration. As infinite dilution is approached, the 6-H becomes a "monitor" of charge (protonation takes place on the pyrimidine ring), the 4-H is sensitive to phosphate ionization and is therefore indicative particularly of the syn conformation that predominates under acidic conditions (pD ~4.0), and the 2-H is responsive to the anti conformation (pD ~ 8.5) of **2b–d**.

When the base is unprotonated, *lin*-benzoadenine nucleotides are stacked in aqueous solution, with association constants of at least one order of magnitude greater than those of the corresponding adenine nucleotides.[54] Head to tail orientations of stacked *lin*-benzoadenine nucleotides in aqueous solution were indicated by the deuterium substitution effect on relaxation times (DESERT).[55] The intramolecular stacking pattern of the *N,N*-dimethyl derivative of *lin*-benzoadenine [8-(dimethylamino)imidazo[4,5-*g*]quinazoline] in the crystal is a head to tail alternate stack (Figure 2).[56]

The fluorescence of these active, stretched-out derivatives of the nucleic acid bases, nucleosides, and nucleotides increases their utility. For example, the fluorescence quantum yield of *lin*-benzo-AMP (**2b**) is 0.44 and its fluorescence lifetime is 3.7 ns at pH 8.5 in 50 mM Tris-HCl buffer,[11,22,51] and the nucletotides show

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Table III
Binding of Divalent Cations to *lin*-Benzoadenine Nucleotides

nucleotide	association constant (M^{-1} at 23 °C)		
	Co^{2+}	Mn^{2+}	Mg^{2+}
lin-benzo-AMP (**2b**)	10^3	10^3	
lin-benzo-ADP (**2c**)	7.1×10^4	9.0×10^4	6.4×10^3
lin-benzo-ATP (**2d**)	1.1×10^6	1.2×10^6	8.1×10^4

sensitivity of the fluorophore to environmental conditions, such as divalent metal ions and stacking. The association constants of paramagnetic ions, such as Co^{2+} and Mn^{2+}, to the *lin*-benzoadenine nucleotides were determined by the decrease in fluorescence intensity of the *lin*-benzoadenine moiety with increasing concentration of the metal ion, corrected for any collisional quenching. The association constants of diamagnetic metal ions, such as Mg^{2+}, which is not a fluorescence quencher, were obtained by competition studies. The association constants determined for the *lin*-benzoadenine nucleotides **2b–d** (Table III) were greater than those for the corresponding adenine nucleotides and were, in decreasing order of magnitude, *lin*-benzo-ATP > *lin*-benzo-ADP > *lin*-benzo-AMP.[24,51] Fourier transform ^1H NMR of *lin*-benzo-ATP in the presence of Co^{2+} showed the greatest broadening of the 2-H signal (**26**), corresponding to the 8-H in ATP. *lin*-Benzo-cAMP (**2e**), serving as a control, showed no significant broadening of the ^1H signals even at high Co^{2+}/nucleotide concentrations. Our results indicate that the positioning of the ring and the conformation of the phosphate chain of *lin*-benzo-ATP chelated with paramagnetic ions involve N1, just as ATP can involve the equivalent N7 of the natural ring system.

The nucleotides of *lin*-benzoguanine, *lin*-benzoxanthine, and *lin*-benzohypoxanthine have fluorescence quantum yields in aqueous solution of 0.39, 0.55, and 0.04 and fluorescence lifetimes of 6, 9, and ~1.5 ns, respectively.[25] The *lin*-naphtho compounds are brilliantly fluorescent, as indicated by their high fluorescence quantum yields and lifetimes in 95% ethanol purged of oxygen: *lin*-naphthohypoxanthine (**19**): λ_{em} 460 nm, τ 25 ns, Φ 0.70; *lin*-naphthoxanthine (**20**): λ_{em} 460 nm, τ 33 ns, Φ 0.88.[17] Since both *lin*-benzopurines and *lin*-naphthopurines exhibit long-wavelength absorption bands well beyond the region of absorption of proteins and nucleic acids, these fluorophores can be selectively excited at long wavelenghts. Fluorescence quenching occurs when one unit of *lin*-benzo-AMP (**2b**) is stacked over another, as in an aqueous solution of the anhydride, P^1,P^2-di-*lin*-benzoadenosine 5′-pyrophosphate. The hydrolytic cleavage of this compound with snake venom phosphodiesterase results in an increase in fluorescence intensity of approximately two orders of magnitude.[22] Stacking occurs in the polymer, poly(*lin*-benzoadenylic acid), made from *lin*-benzo-ADP using polynucleotide phosphorylase, as shown by its relative nonfluorescence and by the regeneration of fluorescence by mixed enzymatic cleavage to *lin*-benzoadenosine.[22,23] It is therefore predictable that positioning of a *lin*-benzoadenosine system in close complexing proximity to other aromatic moieties—for example, adenosine, nicotinamide, isoalloxazine, tryptophan, tyrosine, or phenylalanine— will result in fluorescence quenching. Fluorescence quenching and increases in fluorescence polarization can provide additional information concerning the interaction of the *lin*-benzoadenine nucleotides with selected enzymes.

Fluorescence Properties and Enzyme Interactions. When *lin*-benzo-ATP (**2d**) was prepared from *lin*-benzo-ADP (**2c**) with pyruvate kinase in the presence of phosphoenolpyruvate, the fluorescence polarization of bound *lin*-benzo-ATP increased only slightly,[51] supporting the earlier conclusion that pyruvate kinase possesses a flexible binding pocket for the adenine moiety (of ATP).[57–59] Greater fluorescence polarization was observed with *lin*-benzo-ADP (**2c**) and hexokinase, which was raised further by D-lyxose, the phosphorylatable competitive inhibitor of glucose phosphorylation.[60] This effect indicates enhanced binding of **2c**, as observed by other means in the natural series.

Fluorescent *lin*-benzo-ATP activates aspartase transcarbamylase (ATCase) to the same extent as ATP. The association constant of 5×10^{-3} M^{-1} at pH 8.7 and 4 °C determined by fluorescence polarization is similar to values previously obtained for ATP.[37] The average angle of rotation of *lin*-benzo-ATP bound to ATCase is ~35°. Most of this large rotation is due to rotation of the *lin*-benzo-ATP molecule bound to the allosteric site. The allosteric site binds both nucleotide activators and inhibitors loosely and is also flexible enough to accommodate the lateral extension of the adenine ring by 2.4 Å. The fluorescence emission of *lin*-benzo-ATP is not quenched when bound to ATCase, which indicates the absence of π interactions between the activator and tyrosyl residues in the protein. By contrast, the fluorescence emission of *lin*-benzo-ADP (**2c**) is strongly quenched by purifed mitochrondrial ATPase, which provides evidence of strong complexation of **2c** with this enzyme.[48]

In the design of inhibitors of adenylate kinase, the 1000-fold greater inhibition of A(5′)p_5(5′)A compared with A(5′)p_4(5′)A[5,61–64] suggests that the additional phosphate of the former, which lengthens the phosphate chain by about 2.7 Å, is crucial for strong inhibition. We asked the question whether there would be a similar inhibition-enhancing effect of stretching one of the terminal adenines by 2.4 Å.[24,65] Accordingly, we synthesized P^1-(*lin*-benzo-5′-adensoyl)-P^4-(5′-adenosyl)-tetraphosphate (**27a**) and P^1-(*lin*-benzo-5′-adenosyl)-P^5-(5′-adenosyl)pentaphosphate (**27b**) from *lin*-benzoadenosine 5′-monophosphoromorpholidate with adenosine 5′-triphosphate and with adenosine 5′-tetraphosphate. These mixed dinucleoside oligophosphates are competitive with respect to ATP and AMP and are potent inhibitors of porcine muscle adenylate kinase, with association constants of 2×10^6 M^{-1} for the tetraphosphate and 2×10^5 M^{-1} for the pentaphosphate, respectively, as determined by kinetics and fluorescence

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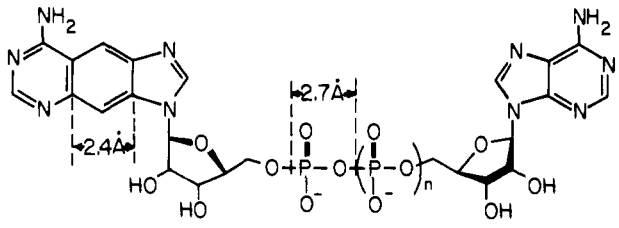

27a, n = 3
b, n = 4

experiments. These compounds (**27a,b**) are intramolecularly stacked when free in aqueous soluion, as judged by their low fluoresence quantum yield and short lifetime compared with *lin*-benzo-AMP. When adenylate kinase is present, the fluorescence quantum yields and lifetimes of **27a** and **27b** are increased. The reversal of quenching signifies that the intramolecular stacking has been broken and that these inhibitors are bound to the enzyme in an "open" or "extended" form of the oligophosphate chain. The difference in the extent of the reversal of fluorescence quenching for **27a** and **27b** by adenylate kinase is indicative of different modes of association at the enzyme active site for these two inhibitors.[65] *lin*-Benzo-A(5′)p_4(5′)A (**27a**) may derive its stronger association to adenylate kinase, compared with that of **27b**, from mimicking more closely the interactions of ATP with the enzyme, hence leading to stronger stacking interaction with Tyr-95, resulting in partial quenching of the *lin*-benzoadenine fluorescence not observed for **27b** with the enzyme. According to the X-ray structure of crystalline adenylate kinase, AMP and ATP bind at opposite ends of the cleft, with their phosphates extending toward each other and in the center of the cleft.[5] Recently, the complex between the inhibitor Ap_5A and human adenylate kinase has been crystallized,[45] and determination of this structure should be helpful in further definition of the geometry achieved during catalysis.

lin-Benzo-ATP (**2d**) has been shown to be an acceptable substrate for light production in the firefly luciferase system.[66] This ATP analogue displays strong enzyme binding and a reduced rate of enzyme catalysis compared with ATP. Variations in the color of the bioluminescence emission suggest that a lateral extension in the purine base induces an incremental change in the conformation of luciferase in the vicinity of the excited light emitter.

Enzyme Flexibility. The interaction of glucose and of ATP with yeast hexokinase mentioned at the outset[2,4,5,7] results in an "induced fit", as postulated originally by Koshland,[67] who also asked whether "changes of a few ångstroms in distance may be sufficient to prevent enzyme action."[68] We seek an answer in dimensionally quantitative terms with specific sets of enzymes and cofactors. We are encouraged in this enterprise by the demonstrated[69] conformational fluctuation and flexibility of proteins,[70] as opposed to mechanical rigidity, and by a dynamic description of enzyme action.[71]

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Intramolecular [2 + 2] Photoaddition/Cyclobutane-Fragmentation Sequence in Organic Synthesis†

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During the last decade, synthesis has advanced at an ever increasing pace toward the objective of constructing complex organic molecules in a minimum number of steps in high overall yield. There is no doubt that this progress is due largely to the challenge presented by the variety of fascinating structures found in nature. Moreover, this progress may be partially ascribed to the development and refinement of key reactions, which provide most of the required structural complexity efficiently with predictable and high regio- and stereochemical control.

At the outset of our work we felt that intramolecular [2 + 2] photoadditions of enones to olefins, combined with subsequent cyclobutane cleavage reactions, might

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† Dedicated to Professor Vladimir Prelog on the occasion of his 75th birthday.

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