ligands by reaction with alkylating or acylating derivatives of the ligands.

As pointed out in the introductory statement, dimeric undecagold clusters such as that described here may be useful in double-labeling scanning transmission electron microscopic experiments, in which one component of a complex structure is labeled with a monomeric cluster and another component with a dimeric cluster. In addition, as labeling reagents for use in low-dose conventional transmission electron microscopy, they should be superior to monomeric undecagold clusters.

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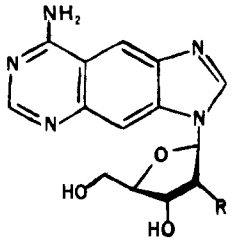
Synthesis and Biochemical Evaluation of 2′-Deoxy-*lin*-benzoadenosine Phosphates†

Ralph A. Lessor, Katharine J. Gibson, and Nelson J. Leonard*

ABSTRACT: 2′-Deoxy-*lin*-benzoadenosine has been synthesized via reductive deoxygenation of 2-(β-D-ribofuranosyl)-8-(methylthio)imidazo[4,5-*g*]quinazoline. The 5′-mono-, 5′-di-, and 5′-triphosphates have been prepared by chemical and/or enzymatic methods. The 5′-diphosphate was found to be a substrate for phosphorylation by pyruvate kinase and was compared with various natural and extended substrates in kinetic assays. When 2′-deoxy-*lin*-benzoadenosine 5′-triphosphate was tested in a nick-translation experiment with *Escherichia coli* DNA polymerase I, a very low level of ^{32}P incorporation from [α-^{32}P]TTP into poly[d(AT)] was observed. Nearest-neighbor analysis indicated that the analogue was not significantly incorporated into internal positions in the polymer. In DNA-sequencing reactions, the analogue caused chain termination at adenine residues, although termination was less uniform and less efficient than that with 2′,3′-dideoxy-ATP. These experiments show that *lin*-benzoadenine can form a widened Watson–Crick base pair with thymine. They strongly suggest, though they do not prove, that the enzyme is able to attach the analogue to DNA.

The utility of dimensionally altered nucleoside and nucleotide analogues as specific biological probes of nucleotide–enzyme interactions has been demonstrated (Leonard, 1982). Specifically, the synthesis and biochemical evaluation of *lin*-benzoadenosine (**1**) and of several ribonucleotides and cofactors



1 R=OH lin-benzoadenosine

2 R=H 2′-deoxy-lin-benzoadenosine

containing this "stretched-out", fluorescent analogue of adenosine have provided useful information concerning the steric requirements for recognition by selected enzymes, the nature of the binding, and the relative position of hydrogen-bonding sites involved in recognition. As an extension of our previous work in this area, we have now prepared 2′-deoxy-*lin*-benzoadenosine (**2**) and its phosphate derivatives for evaluation as enzyme substrates or inhibitors. As potential substrates or inhibitors of enzymes that replicate or repair DNA, these compounds may provide insights into the steric requirements for deoxyribonucleotide recognition during DNA synthesis and into the mechanism of recognition of damaged DNA and its repair.

The synthesis of 2′-deoxyribonucleoside analogues traditionally has been a cumbersome task. Although phase-transfer catalysis of base deoxyribosidation with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-D-*erythro*-pentofuranose and short reaction time favors β anomer formation (Winkeler & Seela, 1983), direct deoxyribosidation with a halo sugar derivative of 2-deoxyribose generally gives mixtures of α and β anomers, as well as positional isomers. This is attributed to the lack of anchimeric assistance that explains the stereospecificity of similar β-ribosidation reactions employing an acyl protecting group on the 2-hydroxyl of the corresponding ribose derivative (Watanabe et al., 1974). In order to avoid this problem by utilizing the readily available β-ribonucleosides, we have previously explored methods for their direct 2′-deoxygenation (Lessor & Leonard, 1981). Free-radical reduction of a thiocarbonyl derivative was selected as the best available method (Barton & McCombie, 1975; Barton & Subramanian, 1977), and a selective deacylation procedure was chosen to differentiate the three hydroxyl groups present in the original

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ribonucleoside (Ishido et al., 1980). Another successful approach to 2′-deoxygenation has been reported by Robins et al. (1983).

Materials and Methods

Melting points were determined on a Büchi capillary melting point apparatus and are uncorrected. Most ^{1}H NMR spectra were recorded on a Varian EM-390 spectrometer with Me_4Si as an internal standard. Column chromatography was performed on Brinkmann 0.05–0.2-mm silica gel unless otherwise noted. Mass spectra were obtained on a Varian MAT 731 spectrometer, coupled with a 620i computer and a STATOS recorder.

3-(3,5-Di-O-acetyl-β-D-ribofuranosyl)-8-(methylthio)-imidazo[4,5-g]quinazoline (**4**) *and 3-(2,5-Di-O-acetyl-β-D-ribofuranosyl)-8-(methylthio)imidazo[4,5-g]quinazoline* (**5**). 8-(Methylthio)-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-imidazo[4,5-*g*]quinazoline (**3**) (Leonard et al., 1976) (105 mg, 221 μmol) was dissolved in 10 mL of anhydrous pyridine. The solution was treated with 42 mg (450 μmol) of hydroxylammonium acetate with stirring under nitrogen for 72 h. The reaction was quenched with acetone (5 mL), and solvent was removed in vacuo. The residue was chromatographed on silica gel, with a 0–4% gradient of methanol in chloroform as the eluting solvent, to afford recovered starting material (35 mg), followed by the products, obtained as an equilibrium mixture of isomers (56 mg, 87% on the basis of unrecovered starting material).

The ^{1}H NMR spectrum indicated the presence of two diacetyl isomers in approximately a 4:1 ratio by integration of the anomeric proton signals, with the 3′,5′-diacetyl isomer **4** predominating: ^{1}H NMR for 3′,5′-diacetyl isomer **4** ($CDCl_3$) δ 8.50 (s, 1 H), 8.22 (s, 1 H), 7.73 (2 overlapping s, 2 H), 5.90 (d, 1 H, $J_{1',2'}$ = 6 Hz, 1′-H), 5.35 (br m, 1 H, 3′-H), 4.82 (dd, 1 H, 2′-H), 4.4 (br m, 3 H, 4′-, 5a′-, and 5b′-H), 2.58 (s, 3 H, SCH_3), 2.2 (2 br overlapping s, acetyl protons); ^{1}H NMR for 2′,5′-diacetyl isomer **5** ($CDCl_3$) δ 8.61 (s, 1 H), 8.32 (s, 1 H), 8.12 (s, 1 H), 7.83 (s, 1 H), 6.12 (d, 1 H, $J_{1',2'}$ = 8 Hz, 1′-H), 5.3 (br m, 2 H, 2′- and 3′-H), 4.4 (br m, 3 H, 4′-, 5a′-, and 5b′-), 2.61 (s, 3 H, SCH_3), 2.2 (2 br overlapping s, acetyl protons); FAB mass spectrum (negative ions), *m/e* 432 (M^-), 417 ($M^- - CH_3$), 389 ($M^- - 43$, $M^- - CH_3CO$), 215 (M^- – sugar); FAB mass spectrum (positive ions), *m/e* 433 ($M^+ + H$), 217 (M^+ – heterocycle).

3-(3,5-Di-O-acetyl-2-O-thiobenzoyl-β-D-ribofuranosyl)-8-(methylthio)imidazo[4,5-g]quinazoline (**6**). A solution of 500 mg (3.5 mmol) of *N,N*-dimethylbenzamide in 10 mL of dry dichloromethane was treated with 2 mL of condensed phosgene at room temperature for 20 h. Solvent and excess phosgene were removed in vacuo, and the residue containing the resulting chloroiminium chloride **9** was dissolved in 10 mL of dry dichloromethane. To this was added a solution of 87 mg (0.201 mmol) of the equilibrium mixture of diacetyl nucleosides described above in 5 mL of dry dichloromethane. The mixture was stirred for 48 h, 1 mL of pyridine was added, and gaseous hydrogen sulfide was bubbled through the mixture for 10 min. The reaction was stirred for 20 min more and then extracted twice with water, dried over sodium sulfate, and evaporated in vacuo.

The yellow-orange residue was dissolved in a minimal amount of chloroform and chromatographed on silica gel. Elution with chloroform afforded two bands. The first was identified as *N,N*-dimethylthiobenzamide by NMR. The second yellow band (10 mg of yellow-orange glass) was tentatively identified as the 3′-thiobenzoyl derivative. Further elution of the column with 2% methanol in $CHCl_3$ afforded a third band (88 mg of yellow-orange glass, 79%), identified as the desired 2′-thiobenzoyl derivative **6** on the basis of further transformation: ^{1}H NMR spectrum of major isomer δ 8.84 (s, 1 H), 8.47 (br s, 1 H), 8.34 (s, 1 H), 8.10 (s, 1 H), 8.01 (m, 2 H, benzoyl), 7.3 (br m, 3 H, benzoyl), 6.45 (m, 2 H, overlapping 1′- and 2′-H), 5.74 (dd, 1 H, 3′-H), 4.3–4.6 (br m, 3 H, 4′-, and 5a′-, and 5b′-H), 2.17 (s, 3 H, SCH_3), 2.29 (s, 3 H, acetyl), 2.08 (s, 3 H, acetyl).

2′-Deoxy-lin-benzoadenosine (**2**). 3-(3,5-Di-*O*-acetyl-2-*O*-thiobenzoyl-β-D-ribofuranosyl)-8-(methylthio)imidazo[4,5-*g*]quinazoline (**6**) (380 mg, 0.688 mmol), the major isomer above, was dissolved in 40 mL of refluxing toluene. Azoisobutyronitrile (AIBN) (5 mg) was added, followed by dropwise addition of a solution of 0.2 g of tri-*n*-butyltin hydride in 40 mL of toluene over a period of 2 h. The mixture was heated at reflux 2 additional h, then another 0.2-g portion of tri-*n*-butyltin hydride was added, and the mixture was refluxed for 4 h. The mixture was cooled to room temperature and evaporated in vacuo.

The ^{1}H NMR spectrum of the crude residue showed two anomeric proton signals, one a doublet of doublets (major product) and the other a doublet. The crude residue was dissolved in ethanol (25 mL), saturated with ammonia at 0 °C, sealed in a pressure vessel, and heated to 130 °C overnight. Upon being cooled, TLC indicated incomplete reaction, so the mixture was resaturated with ammonia at 0 °C and heated to 145 °C for 8 h. The mixture was cooled, and the solvent was removed in vacuo. The residue was dissolved in 400 mL of water, heated with decolorizing carbon, filtered through Celite, and evaporated onto 20 g of cellulose, which was dry loaded onto the top of an 8 × 75 cm column of cellulose powder. The column was eluted with 7:2:1 2-propanol–water–concentrated ammonium hydroxide. Appropriate fractions were pooled and evaporated to give 2′-deoxy-*lin*-benzoadenosine (**2**) (64 mg, 32%), mp 259–270 °C dec. Additional material could be recovered by rechromatographing fractions containing a mixture of 2′-deoxy-*lin*-benzoadenosine and unreduced *lin*-benzoadenosine, to bring the total yield to 89 mg (43%). *lin*-Benzoadenosine (14 mg) was also isolated from the reaction mixture.

^{1}H NMR spectrum of **2** was as follows: (D_2O) δ 8.54 (s, 1 H), 7.84 (s, 1 H), 7.73 (s, 1 H), 7.69 (s, 1 H), 5.56 (dd, 1 H, 1′-H), 4.50 (d, 1 H, *J* = 4 Hz, 4′-H), 4.13 (dd, 1 H, 3′-H), 3.6 (m, 2 H, 5a′- and 5b′-H), 2.7 (m, 1 H, 2′-H), 1.77 (m, 1 H, 2′-H). Decoupling experiments were performed to ascertain correct assignments for the sugar protons. High-resolution FAB MS was as follows: 302.1227 (observed), 302.1201 [calcd for $C_{14}H_{16}N_5O_3$ ($M^+ + H$)]. An ultraviolet spectrum was obtained in 50 mM potassium phosphate, pH 7.

2′-Deoxy-lin-benzoadenosine 5′-Monophosphate (*d-lin-Benzo-AMP*). 2′-Deoxy-*lin*-benzoadenosine (20 mg, 66.4 μmol) was suspended in 2 mL of *m*-cresol and cooled to 0 °C in an ice–water bath. After 15 min, 0.075 mL of pyrophosphoryl chloride was added. The mixture was stirred for 15 min, at which time a clear, yellowish solution had formed. Stirring was continued at 0 °C for 2 h, then the reaction was quenched by addition of ice–water (10 mL), stirred for 5 min, further diluted with water to 75 mL, and extracted with three 25-mL portions of diethyl ether. The aqueous layer was treated with 25 mL of 0.7 M aqueous triethylammonium bicarbonate (TEAB) buffer, pH 7, and then evaporated to dryness.

The residue was dissolved in 300 mL of water and applied to a column of DEAE-Sephadex A-25. Elution with a 0–0.7

M gradient of TEAB was monitored at 346 nm. The appropriate fractions were evaporated to dryness and coevaporated with ethanol (3 × 10 mL). The residue (38 mg) was assayed for phosphate after ashing (Clark + Switzer, 1977) and for its N-heterocycle content by UV spectrophotometry (Leonard et al., 1978). Separate 5′-nucleotidase (from *Crotalus atrox* venom, Sigma) incubations (25 μL) contained 1 mM nucleotide (d-*lin*-benzo-AMP, 3′-AMP, or 5′-AMP), 0.1 unit/mL enzyme (assayed with 5′-AMP), 0.1 M glycine–KOH, pH 9, 40 mM $MgCl_2$, and 4 mM ethylenediaminetetraacetic acid (EDTA). Reaction at 20 °C was monitored on DEAE-cellulose TLC sheets (Baker) with 1 M LiCl as solvent. R_f values were as follows: *d-lin*-benzo-AMP, 0.5; deoxy-*lin*-benzoadenosine, 0.12; 5′-AMP, 0.9; 3′-AMP, 0.75; adenosine, 0.5.

2′-Deoxy-lin-benzoadenosine 5′-Diphosphate (d-lin-Benzo-ADP). 2′-Deoxy-*lin*-benzoadenosine 5′-monophosphate (20 mg, 37 μmol) (as the salt isolated from ion-exchange chromatography above) was dissolved in 1 mL of 2-methyl-2-propanol and 1 mL of water. Morpholine (4 drops) was added, and the mixture was heated at reflux. A solution of *N,N′*-dicyclohexylcarbodiimide (200 mg) in 2-methyl-2-propanol (4 mL) was added in small portions over 3 h. The reaction was monitored by TLC on cellulose plates, with 2:1 2-propanol–0.25 M ammonium bicarbonate as a developing solvent. After an additional 4 h at reflux, complete conversion of the monophosphate to the faster running phosphomorpholidate was observed. The mixture was cooled, and solvent was removed in vacuo. The residue was dissolved in water (30 mL) and washed with three 15-mL portions of diethyl ether. The aqueous layer was evaporated in vacuo.

The residue was dissolved in dry DMF (5 mL) and added to a solution of tributylammonium phosphate (prepared from 35 mg of pyridinium phosphate and 1 equiv of tributylamine in pyridine, then dried by repeated evaporation of pyridine, and dissolved in 15 mL pyridine). The mixture was stirred for 5 days, then evaporated in vacuo, dissolved in water (500 mL), and applied to a DEAE-Sephadex column. Elution with a 0–0.7 M gradient of TEAB and pooling of the appropriate fractions gave, after evaporation and coevaporation with ethanol, a cream solid. The UV spectrum indicated the presence of approximately 30 μmol of *lin*-benzoadenine nucleotide (81% yield); the mass (21 mg) was close to that expected for the tris triethylammonium salt of the diphosphate (23 mg). The material was analyzed by TLC on polyethylenimine (PEI)–cellulose plates (Brinkmann) in 0.3 M potassium phosphate, pH 7, R_f 0.48 with respect to the phosphate front. The phosphate to base ratio was determined as for the monophosphate.

2′-Deoxy-lin-benzoadenosine 5′-Triphosphate (d-lin-Benzo-ATP). Pyruvate kinase was used to phosphorylate d-*lin*-benzo-ADP. The reaction mixture (4 mL) contained 0.3 mM nucleotide, 2.5 mM phospho(enol)pyruvate, and 1 mg/mL pyruvate kinase in a buffer containing 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 10 mM KCl, 5 mM $MgSO_4$, and 2 mM EDTA. Reaction at 20 °C was followed by TLC (PEI–cellulose) as above. After 40 min, the R_f observed for the fluorescent spot had changed from 0.48 to 0.2. The mixture was diluted and chromatographed on a 10-mL DEAE-Sephadex A-25 column with a 200-mL linear gradient from 0.2 to 0.7 M TEAB. The UV spectrum indicated that the product was recovered in only 50% yield, probably because the column selected was too large. The sample also contained about 50% by weight water and/or salt, but control experiments showed that this did not affect the polymerase studies. Purity was checked by TLC and by phosphate to base ratio as for the diphosphate.

Pyruvate Kinase Assays. Assays (1 mL) were run with rabbit muscle pyruvate kinase in the buffer used for the preparation of d-*lin*-benzo-ATP. They also contained 0.2 mM NADH, 0.5 mM phospho(enol)pyruvate, and 12.5 μg/mL lactate dehydrogenase. Substrate concentrations were in the following ranges: ADP, 0.35–1.8 mM; dADP, 0.32–1.7 mM; *lin*-benzo-ADP, 0.65–2.0 mM; 2′-deoxy-*lin*-benzo-ADP, 0.18–0.55 mM. Reaction was initiated by addition of pyruvate kinase and monitored at 370 nm. Data were analyzed by a least-squares linear regression fit of the Lineweaver–Burk plot.

Escherichia coli DNA Polymerase I Studies. Nick-translation experiments were conducted in 15–25-μL reaction volumes with components at the following final concentrations: Tris-HCl, pH 7.4, 60 mM; $MgCl_2$, 6 mM; β-mercaptoethanol, 10 mM; bovine serum albumin, 60 μg/mL; EDTA, 0.2 mM; poly[d(AT)] (P-L Biochemicals), 37 μM base pair; [α-^{32}P]-TTP, 25 μM. dATP and d-*lin*-benzo-ATP concentrations were varied as required. Reaction at 17 °C was initiated by addition of DNA polymerase (a gift of Dr. J. A. Jaehning); because of the DNase sensitivity of the template (Schachman et al., 1960), exogenous DNase was unnecessary. Aliquots of 2 μL were transferred at timed intervals into 8 μL of 0.1 M EDTA, pH 7. The samples were worked up on DE-81 filters (Maniatis et al., 1982). The fluorescent nucleotide composition of nick-translation reactions was examined by PEI–cellulose TLC as before. The radiochemical composition was monitored on PEI–cellulose with 0.5 M LiCl as solvent and thymidine nucleotides as standards; the plate was cut in strips and counted.

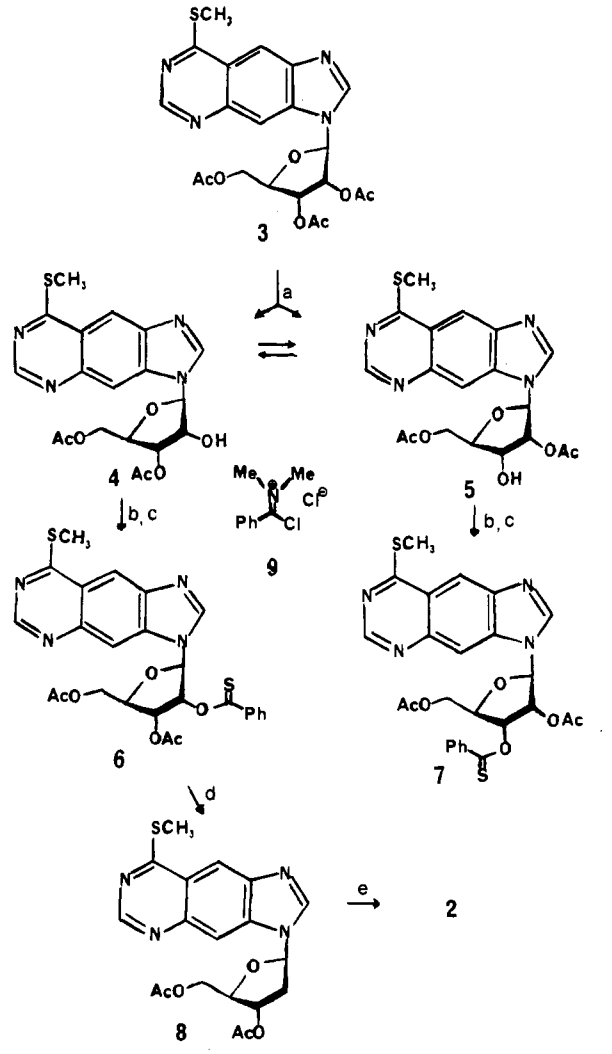
For nearest-neighbor analysis, DNA from nick translations was either precipitated with ethanol or separated from the bulk of unincorporated TTP by spun-column chromatography (Maniatis et al., 1982) in 10 mM triethylammonium bicarbonate, pH 8, and evaporated to dryness. For digestion to 3′-monophosphates, the residue was dissolved in 10 μL of 50 mM glycine–NaOH, pH 9.2, and $CaCl_2$ was added to 10 mM. Micrococcal nuclease was added to 50 μg/mL, and the mixture was incubated at 37 °C for 2 h. The mixture was then made 25 mM in NaH_2PO_4 and 130 mM in ammonium acetate, pH 5.9, spleen phosphodiesterase (0.07 unit) was added, and the mixture was again incubated at 37 °C for 2 h. The samples were then fractionated by TLC [PEI–cellulose, 1 M $(NH_4)_2SO_4$]. R_f values were as follows: 5′-d-*lin*-benzo-AMP, 0.33; 2′-deoxy-3′-AMP, 0.56; 2′-deoxy-5′-AMP, 0.57; TTP, 0.72; TDP and TMP, >0.8; 3′-AMP, 0.5; 5′-AMP, 0.6; 3′-GMP, 0.6; 5′-GMP, 0.6. The plates were cut in strips and counted.

Dideoxy DNA sequencing reactions on M13 DNA with a synthetic oligonucleotide primer were done by the method of Barnes et al. (1983), with an extra reaction in which dideoxy ATP was replaced by an equal concentration of d-*lin*-benzo-ATP. Cold dATP was omitted from both the dideoxy-ATP and the d-*lin*-benzo-ATP reactions; the concentration of the [α-^{32}P]dATP in the assay was ~0.5 μM.

Results and Discussion

The attempt to prepare deoxy-*lin*-benzoadenosine directly from *lin*-benzoadenosine was abandoned when efforts to prepare pure 3′,5′-di-*O*-benzoyl-*lin*-benzoadenosine gave unstable mixtures. We attributed this to the increased nucleophilicity of the exocyclic amino group of *lin*-benzoadenosine compared to adenosine and thus felt that an *S*-methyl-substituted precursor might be more suitable. We first ascertained that the *S*-methyl group of 6-(methylthio)-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purine would survive the

Scheme I[a]



[a] a, H_2NOH-HOAc; b, 9; c, H_2S/pyridine; d, Bu_3SnH, heat; e, NH_3/EtOH.

series of reagents necessary to effect conversion of the ribosyl to the 2′-deoxyribosyl moiety, namely, hydroxylammonium acetate, the chloroiminium chloride **9**, H_2S/pyridine, tributyltin hydride, and heat, then to be replaced in the final treatment with ammonia in ethanol (Lessor, 1983). After this successful conversion, we carried out a corresponding sequence of reactions on the *lin*-benzoadenosine precursor 8-(methylthio)-3-(tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*g*]quinazoline (**3**) (Scheme I). Treatment of **3** (Leonard et al., 1976) with hydroxylammonium acetate (Ishido et al., 1980) afforded a mixture of the 3′,5′- and 2′,5′-di-*O*-acetyl nucleosides **4** and **5** in ca. 4:1 ratio. The mixture was converted to a separable mixture of diacetylthiobenzoyl ribonucleosides by the procedure of Barton & McCombie (1975). Free-radical reductive deoxygenation of the 2′-thiobenzoyl isomer with tri-*n*-butyltin hydride and with AIBN as an initiator afforded the diacetyl deoxyribonucleoside **8** in good yield. The site of deoxygenation was clearly indicated by the presence in the ^1H NMR spectrum of a doublet of doublets for the anomeric proton, indicative of splitting by two different 2′-protons. Treatment of **8** with ethanolic ammonia in a pressure vessel at 150 °C afforded 2′-deoxy-*lin*-benzoadenosine (**2**). The target compound was separated from small quantities of unreduced *lin*-benzoadenosine by chromatography. The identity of the nucleoside was verified by high-resolution mass spectrometry and ^1H NMR. As expected, its UV absorption

Table I: Kinetic Data for Substrates of Pyruvate Kinase

substrate	literature values K_m (mM)	literature values V_{max}^{rel}	obsd values K_m (mM)	obsd values V_{max}[a]	obsd values V_{max}^{rel}
ADP	0.3[b]	1	0.37	67	1
dADP	3.0[c]	~0.5	2.5	12.4	0.19
lin-benzo-ADP	0.7[b]	0.2	0.65	12.5	0.19
d-*lin*-benzo-ADP			0.8	0.7	0.010

[a] In μmol min^{-1} (mg of enzyme)$^{-1}$. [b] Leonard et al. (1978). [c] Plowman & Krall (1967).

maxima were the same as those of *lin*-benzoadenosine (Leonard et al., 1976), and the ratios of peak intensities were within 1.5% of those reported for the parent compound.

The monophosphate was prepared from **2** by the method of Imai et al. (1969). Phosphorylation at the 5′-position was verified by using 5′-nucleotidase. The monophosphate was completely degraded to **2** at a rate ~10% of that for dephosphorylation of 5′-AMP; no adenosine formation was observed in a control containing 3′-AMP, indicating that the enzyme preparation was reasonably free of 3′-phosphatase activity. Assuming the same extinction coefficients as for the corresponding ribonucleotides (Leonard et al., 1978), a P_i to base ratio of 1.56 was obtained, consistent with some P_i contamination. The diphosphate was obtained by chemical phosphorylation of the monophosphate via the morpholidate (Moffatt & Khorana, 1961). The isolated product was homogeneous by TLC, and assays revealed 2.1 P_i per base, verifying that it was indeed the diphosphate. The recovered mass indicated the absence of major amounts of impurities. Pyruvate kinase was used to phosphorylate the diphosphate, affording the triphosphate. The product was homogeneous by TLC; a P_i to base ratio of 3.1 was obtained.

Enzyme Studies. Pyruvate kinase was assayed, by the coupled assay, with ADP, 2′-deoxy ADP, *lin*-benzo-ADP, and 2′-deoxy-*lin*-benzo-ADP. The data are summarized in Table I. These results are easily accommodated by the Sloan & Mildvan (1976) model of the pyruvate kinase active site in which the nucleotide adopts an anti conformation with the diphosphate moiety bound in a relatively selective site and with the heterocycle bound in a less specific binding site whose affinity depends mainly on hydrophobic interactions. The finding that the *lin*-benzo compounds were substrates suggests that the positioning of their diphosphate chains in the active site was fairly similar to that of the natural substrates. However, the lower activity of d-*lin*-benzo-ADP compared to *lin*-benzo-ADP suggests that these two compounds bound in somewhat different conformations even though their overall affinities were much the same. The observation that both analogues bound better than dADP and nearly as well as the preferred substrate ADP may have reflected better accommodation of the extended aromatic *lin*-benzoadenine moiety in the hydrophobic region of the active site.

E. coli DNA polymerase I was tested for its ability to catalyze incorporation of d-*lin*-benzo-ATP into polymer by using the self-complementary alternating copolymer poly[d(AT)] as template/primer. The data are summarized in Table II. The negative control lacking both dATP and analogue (reaction 1) gave ~0.1% incorporation; this value was constant between 5 and 60 min. In the positive control (reaction 2), incorporation increased steadily for 40–60 min and then began to decline. Supplementation with d-*lin*-benzo-ATP (reaction 3) had no effect, showing that neither the analogue nor any possible impurity was noticeably inhibitory. When the analogue replaced dATP (reaction 4), the qualitative progress of

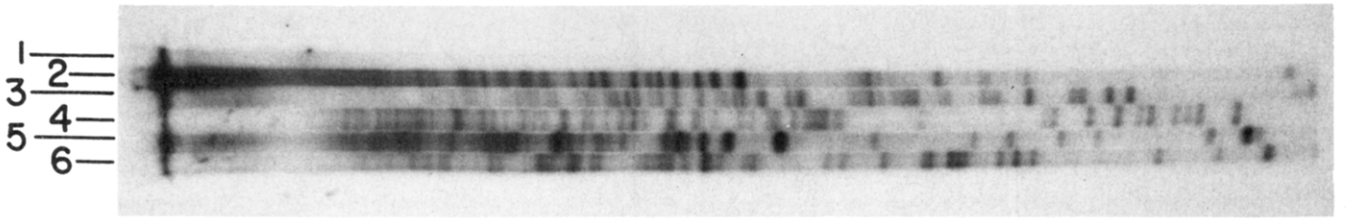


FIGURE 1: Dideoxy DNA sequencing reactions with d-*lin*-benzo-ATP. Terminators in each lane were (1) d-*lin*-benzo-ATP (this reaction also contained 0.1 mM dATP), (2) d-*lin*-benzo-ATP, (3) dideoxy-ATP, (4) dideoxy-TTP, (5) dideoxy-CTP, and (6) dideoxy-GTP. The top of the gel is at the left.

Table II: Nick-Translation Experiments

reaction	no. of determinations	[dATP] (μm)[a]	[d-*lin*-benzo-ATP] (μM)	fraction of ^{32}P incorporated[b]
1	2			0.001
2	6	25		0.46
3	2	25	25	0.52
4	4		25	0.005
5	2		50	0.006
6	2		75	0.005
7	1	0.12	25	0.009
8	1	0.25	50	0.016
9	1	0.38	75	0.023

[a] All assays contained 25 μM [α-^{32}P]TTP and 37 μM (base pair basis) poly[d(AT)]. [b] Values given are those at the time of maximal incorporation. For replicate determinations, averages are given; variability was ±10–15%.

the reaction was the same as in the positive control, but both the initial rate of ^{32}P incorporation and its maximal extent were ~1% of those obtained with dATP. Thin-layer chromatography of the reaction mixture after 1 h showed only d-*lin*-benzo-ATP (estimated >80% pure), and 94% of the counts ran with TTP. The absence of significant monophosphate accumulation suggests that neither compound underwent rapid incorporation followed by excision by the 3′→5′ editing exonuclease activity of the enzyme. When the concentration of d-*lin*-benzo-ATP was increased (reactions 5 and 6), there was no effect. By contrast, when the analogue was deliberately contaminated with 0.5% dATP (reactions 7–9), the peak incorporation corresponded to complete incorporation of the added dATP. Furthermore, in reactions 7–9, maximal incorporation occurred within 5 min, and 40% of those counts were no longer retained on filters after 60 min, presumably as a result of 3′→5′ exonuclease activity. Taken together, these results strongly suggest that the low level of activity seen with d-*lin*-benzo-ATP was not due to adventitious dATP contamination of the analogue.

If d-*lin*-benzo-ATP were incorporated into internal positions in the polymer, some ^{32}P from [α-^{32}P]TTP would have become covalently attached to the 3′-OH of the analogue. If the polymer were then degraded to 3′-monophosphates, 2′-deoxy-3′-[3′-^{32}P]-*lin*-benzo-AMP would be formed. When this was done and the resulting mixtures were analyzed by TLC, essentially no radioactivity was found in the region in which 2′-deoxy-3′-*lin*-benzo-AMP was expected;[1] we estimate that a peak corresponding to 0.03% of the ^{32}P in the assay would have been apparent. We conclude that incorporation of d-*lin*-benzo-ATP into internal positions in the polymer did not occur to a measurable extent.

Although 2′-deoxy-3′-[3′-^{32}P]-*lin*-benzo-AMP was not found, radioactivity comigrating with 2′-deoxy-3′-AMP was found on the chromatograms. The fraction of the total ^{32}P so recovered was approximately the same as the fraction of the label retained on filters after nick translations with d-*lin*-benzo-ATP in place of dATP. A correspondingly smaller amount of 2′-deoxy-3′-[3′-^{32}P]AMP was recovered from reactions containing TTP only. Formation of 2′-deoxy-3′-[3′-^{32}P]AMP could result from [α-^{32}P]TTP addition to nicks having a dA residue on the 3′-OH side; dATP is not required. In the absence of polymerization, however, the 3′→5′ exonuclease activity would tend to excise ^{32}P so incorporated (Brutlag & Kornberg, 1972); the 0.1% incorporation in nick translations containing only TTP may have represented a steady-state balance between the two processes. If so, this balance was perturbed in the presence of d-*lin*-benzo-ATP, and ^{32}P slowly accumulated in the polymer. Since the analogue did not replace dATP as a substrate for polymerization, it would seem that the 3′→5′ exonuclease activity was inhibited.

That the effect of d-*lin*-benzo-ATP was to increase the amount of ^{32}P recoverable as 2′-deoxy-3′-AMP suggested to us that the compound might be acting as a terminator, allowing neither elongation nor 3′→5′ exonucleolytic degradation of polynucleotide chains. To test this idea, d-*lin*-benzo-ATP was included in a set of dideoxy DNA sequencing reactions (Sanger et al., 1977) in place of 2′,3′-dideoxy-ATP. The result is shown in Figure 1. That short pieces of DNA were obtained with the analogue (lane 2) shows the terminator hypothesis to be correct. As expected, termination did not occur in the presence of excess dATP (lane 1). It is probable that covalent attachment of deoxy-*lin*-benzoadenosine to the polymer is involved, though this is not easy to prove without having the analogue in radiolabeled form. A possible alternative is that the analogue was slow to bind, allowing significant primer extension during the first incubation in the sequencing procedure, in which there was excess analogue and a small amount of dATP. Slow release would also be required, so that elongation during the second incubation in the presence of excess dATP did not occur. Transition-state analogues, and compounds that form reversible covalent adducts with enzymes may show such behavior, but we would not expect such behavior from d-*lin*-benzo-ATP.

In these DNA sequencing reactions, each band in the deoxy-*lin*-benzoadenosine lane (2) had a corresponding band in the dideoxyadenosine lane (3), showing that the base-pairing specificity of the analogue was the same as that of adenine. Some expected bands in the deoxy-*lin*-benzoadenosine lane were faint or missing, which suggests that the probability of deoxy-*lin*-benzoadenosine misincorporation was less uniform over all the adenine-requiring sites than dideoxyadenosine misincorporation. The shorter average chain length with dideoxyadenosine than with deoxy-*lin*-benzoadenosine further suggests that dideoxyadenosine was the more efficiently in-

[1] An authentic sample of 2′-deoxy-3′-*lin*-benzo-AMP was not available. However, we used a TLC system in which 2′-deoxy-3′-AMP ran well ahead of 2′-deoxy-5′-*lin*-benzo-AMP and in which 5′-monophosphates generally ran slightly ahead of or even with their 3′ counterparts, so that we would have expected 2′-deoxy-3′-*lin*-benzo-AMP to have been resolved from 2′-deoxy-3′-AMP. All thymidine nucleotides also ran faster than 2′-deoxy-3′-AMP.

corporated of the two analogues, which seems reasonable given its greater structural similarity to dATP.

Watson–Crick pairing of the incoming nucleoside triphosphate to the corresponding base in the template strand is known to be essential to selection by DNA polymerase I among natural deoxyribonucleotides and such analogues as 5-substituted cytosines and uracils (Kornberg, 1980). We have now shown that a tricyclic adenine analogue in which normal base-pairing functionality is preserved can base pair to thymine at the active site of the enzyme, since d-*lin*-benzo-ATP caused chain termination only at adenine residues. Unless unusually stable noncovalent complexes are involved, a plausible rationalization of the data is to suggest that correct base pairing occurs with d-*lin*-benzo-ATP in an anti conformation. One would then expect the triphosphodeoxyribosyl moiety of the molecule to be displaced from its normal position in the active site by the extra 2.4-Å width of the heterocyclic system. Such displacement could make enzymic attachment of the α-phosphate to the primer much more difficult. The 3′→5′ exonuclease activity normally requires an unpaired or mispaired terminus (Kornberg, 1980); it may have failed to excise the unnatural residue because the base-pairing was quite stable, or for steric reasons. Misplacement of the 3′-OH of the deoxy-*lin*-benzoadenosine-terminated primer in the active site could also account for the lack of efficient addition to the analogue.

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