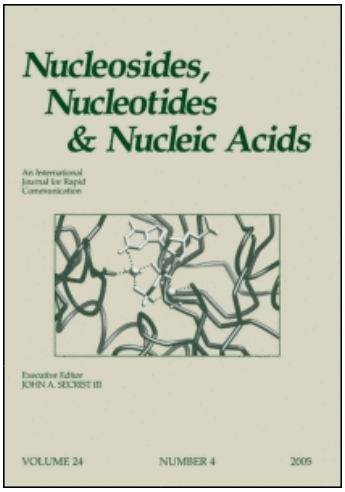

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SYNTHESIS OF γ-PHOSPHATE-LINKED NUCLEOSIDE AFFINITY CHROMATOGRAPHY RESINS FOR PROTEIN PURIFICATION, INCLUDING RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE

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ABSTRACT: Seven nucleotides linked through the γ-phosphate to diamine hydrocarbons were synthesized and coupled to Sepharose for use in protein purification affinity chromatography. The synthesis involved converting the nucleotides to nucleoside-5'-trimetaphosphates using dicyclohexyl carbodiimide, followed by nucleophilic ring opening of the trimetaphosphate with an α,ω-diamino hydrocarbon to generate a γ-phosphoamide linkage in each nucleotide.

Introduction

Ribonucleoside triphosphate reductase (RTPR, EC 1.17.4.2) from *Lactobacillus leichmannii* is a 5'-deoxyadenosylcobalamin-dependent (AdoCbl, Coenzyme B_{12}) enzyme which converts ribonucleoside triphosphates (NTP) to deoxyribonucleosides triphosphates (dNTP),[1,2] a process essential for DNA synthesis. Our interests in adenosylcobalamin-dependent enzymes[3-6] has caused us to re-examine the eleven reported isolation procedures for RTPR before beginning mechanistic and structural studies on this enzyme.[7]

In the past, two major investigators have worked with RTPR. First, in their pioneering work, Blakley and co-workers discovered the enzyme in 1965 and studied it until 1983.[8,9] During this time, a dGTP-based affinity column resin was developed and recommended for the purification of RTPR, along with a lowered pH and the presence of a reducing agent. Stubbe and co-workers, who have worked with RTPR from 1981 to the present,[10,11] did not incorporate the dGTP-based affinity chromatography into their RTPR purification methods after 1984; instead they adopted a modification of the procedure developed by Chen *et al.* (1974).[12-14]

Recently, the gene for the *L. leichmannii* RTPR was cloned and overexpressed into *E. coli*.[15] After purification using ion-exchange and hydroxylapatite columns, the enzyme

was judged to be >95% pure, as visualized by SDS-PAGE stained with Coomassie dye. However, single turnover studies of wild type RTPR and cysteine mutants result in a value less than the predicted stoichiometric turnover number.[16] For prereduced wild type RTPR, 2.0 equivalents of product should theoretically be produced in the absence of external reductants, but only 1.4 equivalents of dCTP was produced per RTPR. Similarly, cysteine to serine RTPR mutants C731S, C736S, and C731/736S has single turnover values of 0.8, 0.6, 0.7 respectively, less than the expected value of 1.0 equivalents. The reason for this discrepancy is not understood, but is suggestive that 20-40% of the RTPR is inactivated, with the isolated protein being, therefore, inhomogeneous.

A search of the RTPR literature raised the possibility that the inactivated RTPR could possibly be removed by affinity chromatography. Blakley's γ-phosphate-linked dGTP-Sepharose resin separated weakly binding, low-activity forms of the enzyme from a tightly binding high-activity form.[1,17-19] RTPR presumably interacts with the dGTP-Sepharose through the specificity allosteric site, or h-site. Interestingly, the low activity forms were shown to be only partially convertible to the high-activity enzyme by treatment with a reducing agent and rechromatography,[18,19] suggesting that some, but not all, of the low activity form results from reversible oxidation of sulfhydryl groups.

Hence, an important question became whether or not affinity chromatography columns, for example, analogous to the dGTP-Sepharose affinity column developed by Blakley,[17] would further improve the purification of cloned and overexpressed RTPR[15] by eliminating some of the protein's apparent microheterogeneity.

Blakley's γ-phosphoester-linked, Sepharose-NH-$(CH_2)_6$-O-dGTP

In his landmark 1978 *Methods in Enzymology* paper,[19] Blakley reported his purification scheme employing a γ-phosphoester-linked, Sepharose-NH-$(CH_2)_6$-**O**-dGTP-based affinity chromatography column (with its –O-P– phosphate link to the dGTP) and recommended this dGTP-based affinity column resin for the purification of RTPR. One might have expected this to become the accepted RTPR purification scheme, but only two other researchers have ever used this column in the intervening 19 years, and then only for the ribonucleotide reductases from *Corynebacterium nephridii*[20] and *E. coli*.[21]

Our critical analysis of the RTPR literature reveals that a dGTP affinity column is very likely *required* to provide the highest purity RTPR. However, and although the synthesis of the particular dGTP column described by Blakley, Sepharose–NH–$(CH_2)_6$-**O**-dGTP, is done on a gram scale and uses inexpensive reagents, several problems are apparent with the synthesis; these problems may explain why Blakley's dGTP-based column has not been adopted by subsequent RTPR researchers despite its publication 19 years ago. In

particular, and with the advantage of 19 years of additional literature, the following problems with the synthesis can be identified: (i) the multistep, convergent synthesis involves 11 chemical reaction steps, 8 column purifications, 4 recrystallizations, and numerous concentration, drying, and other manipulations; (ii) the synthesis uses the toxic reagent S-ethyl trifluorothiol acetate; (iii) the synthesis is in low (unreported overall) yield; and (iv) the synthetic intermediates and product were not characterized by the modern techniques of organic chemistry.[17,19] Additionally, the elution behavior of the dGTP-based affinity column was reported by Blakley to be batch dependent,[1,17-19] and the preparative columns were rather large and long (2.5 x 95 cm) so that the column did not behave as a typical affinity chromatography column. Therefore, an alternative synthetic route to an analogous γ-phosphate modified nucleotide for generation of the dGTP-based affinity column was sought and is developed herein.

Aminophenol Ester-linked dATP

An alternative affinity resin for the purification of ribonucleotide reductases involves esterification of p-nitrophenol to the γ-phosphate of dATP, followed by the reduction of the nitro group to an amine. After Knorre et. al. (1976) demonstrated that NTP γ-phosphoesters can be obtained by using Et_3N as a base catalyst,[22] this method has been used as an alternative synthesis to that proposed by Berglund and Eckstein[23] for the generation of ATP- and dATP-aminophenol-Sepharose. This aminophenol-linked dATP has been used to purify the Fe-dependent ribonucleotide reductase from a variety of sources,[24-26] the anaerobic S-adenosylmethionine-dependent ribonucleotide reductase from *E. coli*,[27] and the adenosylcobalamin-dependent ribonucleotide reductase from *Pyrococcus furiosus*.[28]

In general, ribonucleotide reductases have three nucleotide-binding sites, two for allosteric effectors and one for the substrate. The interaction between the above enzymes and the dATP-aminophenol affinity resin occurs at the ATP/dATP binding site which controls the overall activity of the enzyme, also known as the allosteric activity site or the l-site.[29,30] This site has been located at the amino-terminal region of the Fe-dependent R1 subunit,[31,32] a region with sequence homology to all of the dATP-aminophenol-Sepharose purified ribonucleotide reductases.[28,32]

Our interest lies in the adenosylcobalamin-dependent ribonucleoside triphosphate reductase (RTPR) from *L. leichmannii*; however, RTPR does *not* bind to this aminophenol ester-linked dATP resin.[33] Perhaps this is due to RTPR lacking the activity site which would allow inhibition by dATP, a postulate consistent with the absence of a strong dATP inhibition.[14,34,35] Additionally, RTPR does not contain any sequence homology to the amino-terminal region of other ribonucleotides which retain the dATP regulation of

activity.[28,32] In short, the dATP-aminophenol-Sepharose can not be used for the purification of RTPR, nor perhaps even for other ribonucleotide reductases.[36]

Knorre's Synthesis of γ-Phosphoamide-linked Nucleotides

Our synthetic approach to the generation of a γ-phosphoamide-linked dGTP ligand for the purification of RTPR is based on Knorre et al.'s 1976 general synthetic method for the generation of γ-phosphate modified nucleotides.[22] This procedure, further developed experimentally in later work,[37] involves the conversion of a NTP to a nucleoside-5'-trimetaphosphate intermediate by dehydration with dicyclohexylcarbodiimide (DCC), Scheme 1.[38] (Note that the later publication[37] provides additional, needed experimental detail over the initial, brief communication,[22] and hence it should be consulted for details about Knorre et al.'s procedures.)

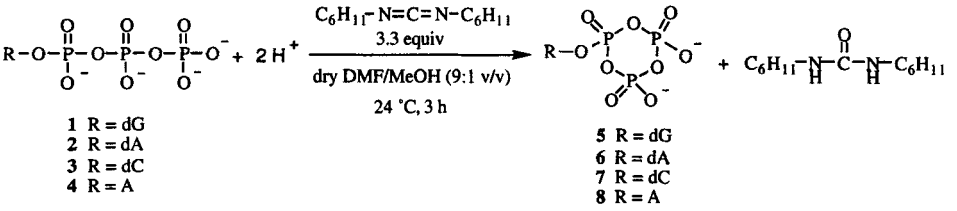
Nucleoside-5'-trimetaphosphates have been used for the generation of a variety of irreversible inhibitors and affinity labeling reagents of use in investigations of specific interactions of NTPs and dNTPs with proteins.[37,39] The reaction of nucleoside-5'-trimetaphosphate with amines results in ring opening and the generation of NTP γ-phosphoamidates, Scheme 2.[22,40,41] This synthetic route provides substantial improvement over Blakley's method, being simpler and more versatile in terms of nucleotides or linkers which can be employed.

Ligand Coupling to Chromatography Support

An additional issue with Blakley's affinity column synthesis is the use of cyanogen bromide, NCBr, activation via the classical 1970 Cuatrecasas method.[42] Due to the strongly basic reaction medium used, greater than 80% of the reactive cyanate ester generated is subsequently hydrolyzed, producing a matrix highly contaminated by carbamates, Scheme 3.[43] Kohn and Welcheck developed an alternative activation procedure for Sepharose that generates the reactive cyanate ester under conditions where it is more stable, Scheme 4.[44] This "cyano-transfer" method eliminates the base hydrolysis reaction by utilizing Et_3N at neutral conditions to increase the electrophilicity of the cyanate by forming the complex $[NC\text{-}NEt_3]^+Br^-$. Herein we utilize this chemistry which offers improvements in 1) lower amounts of toxic NCBr required, 2) higher levels of activation, and 3) elimination of carbamate contaminates within the resin.

The Present Studies, the Synthesis and Characterization of Seven γ-Phosphoamide-Linked Nucleotide Chromatography Resins

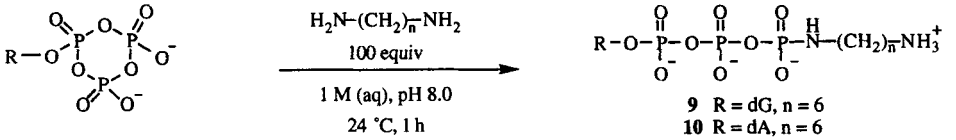
Herein we report the synthesis and characterization of a 2'-deoxyguanosine-5'-(γ-amidohexamethylene-6-amine)-triphosphate-Sepharose for use in the affinity



1 R = dG
2 R = dA
3 R = dC
4 R = A

5 R = dG
6 R = dA
7 R = dC
8 R = A

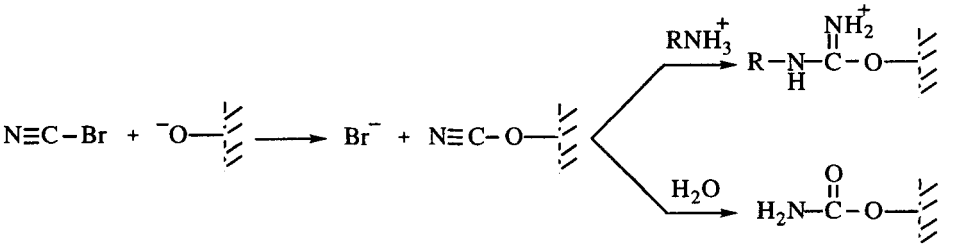
Scheme 1: Conversion of triphosphate nucleosides to nucloeside-5'-trimetaphosphates by dehydration with dicyclohexylcarbodiimide.



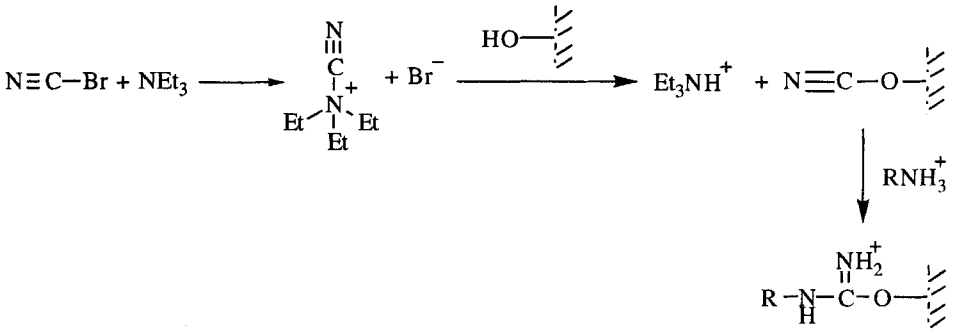
5 R = dG
6 R = dA
7 R = dC
8 R = A

9 R = dG, n = 6
10 R = dA, n = 6
11 R = dC, n = 6
12 R = A, n = 6
13 R = dG, n = 2
14 R = dG, n = 4
15 R = dG, n = 12

Scheme 2: Ring opening of the nucleoside-5'-trimetaphosphates with excess α,ω-diamino hydrocarbon to generate the γ-phosphoamide products.



Scheme 3: NCBr activation of Sepharose under strongly basic conditions to produce the desired isourea linkage (top), or with H_2O the undesired carbamates (bottom).



Scheme 4: Cyano-transfer activation of Sepharose at neutral pH.

chromatography purification of RTPR. While this resin is analogous to that made previously by Hoffmann and Blakley,[17] we report a simpler more versatile ligand synthesis and better Sepharose activation chemistry; in addition, we have characterized our products by modern methods (^{31}P NMR, mass spectroscopy, and for the most important compound, **9**, elemental and thermogravimetric analysis).

Furthermore, we synthesized a series of ligands with six carbon diamino spacer containing different nucleotides (dGTP, dCTP, dATP and ATP) and three additional dGTP analogs with varying diamino spacer lengths. These latter materials demonstrate the ease and versatility of the synthetic procedure, and were prepared in order to possibly optimize the affinity purification of RTPR and to gather information about the interaction between RTPR and deoxynucleotides at the allosteric site.

Elsewhere we will report the investigation of RTPR's interaction with these various γ-phosphoamide-linked affinity columns and the resultant purity of the enzyme, the key result being our finding that RTPR purified with the dGTP-based resin is of a higher purity.[7]

Results and Discussion

Synthesis

The synthesis of the γ-phosphoamide nucleotides was accomplished as outlined back in Schemes 1 & 2. Prior to the first step, the sodium salt of the each nucleotide (**1-4**) must be converted to the Et_3NH^+ salt to enhance their solubility in the aprotic organic solvent, DMF. The nucleotide Et_3NH^+ salt is very hygroscopic; thus, in order to minimize the additional amount of water present, the DCC reaction was performed in a nitrogen drybox. The nucleotides were dissolved in dry DMF/methanol (9:1 v/v), the methanol being added to further increase the nucleotide's solubility.

In the first step of the synthesis, Scheme 1, DCC cyclization of the nucleoside triphosphates (**1-4**) yields the corresponding trimetaphosphate intermediates (**5-8**).[38] Note that the nucleoside triphosphates (**1-4**) likely contain at least two H_2O molecules as stable hydrates. An excess of DCC is required due to the H_2O sensitivity of this first step, which can cause hydrolysis of DCC and the trimetaphosphate intermediate.[37] Although pyridine hydrochloride is sometimes added to the reaction mixture[45,46] to maintain a weakly acidic medium (which in turn increases the reaction rate[37]), neither we, nor others,[47,48] utilize this step because the reaction is complete within three hours without it.

In the second step of the synthesis, Scheme 2, the trimetaphosphate ring is then opened by its addition to aqueous solutions of diamino hydrocarbons resulting in the corresponding γ-amidates (**9-15**).[22] (This process is facilitated by the presence of polar solvent which is necessary to facilitate the solvation of the γ-phosphate leaving group.[37]) Note that a large excess of the diamino hydrocarbon is utilized to prevent the formation of bis-nucleotide adducts through attachment at both amino sites of the α,ω-diamine.[46]

In our studies, diamino hydrocarbons with an even number of carbons were used. Aqueous solutions of 1 M concentration were achievable for 2-8 carbon-length chains. However, diamines containing 10 or more methylene carbons were, as expected, not very soluble in water. Hence, for the synthesis of **15**, having a 12 carbon $NH_2(CH_2)_{12}NH_2$ spacer, a mixed solvent of isopropanol/water (1:1) was used, which allowed a concentration of 0.5 M in diamine. After evaporating the reaction solution to dryness, the desired product, **15**, was recovered by twice extracting the precipitate with water.

Purification

Purification of the desired γ-phosphoamide products, **9-15**, was accomplished by anion exchange chromatography on diethylaminoethyl (DEAE) Sepharose. One must first filter the product mixtures, **9-14**, and then dilute them 45-fold with H_2O to reduce the ionic strength of the excess diamine sufficiently to allow the nucleotide products to bind to the DEAE resin. In the case of **15**, after filtration, the water extract noted earlier can be directly loaded onto the DEAE resin. Purified products (**9-15**) were obtained using a 0 - 1 M linear gradient of $Et_3NH^+HCO_3^-$ buffer, pH 7.5, FIG. 1. The compounds separate as expected on the basis of increasing negative charge. At pH 7.5, **9-15** have an overall -2 charge and are therefore eluted as the first major peak, while the unreacted starting material, **1-4**, having an overall -4 charge are eluted later along with a variety of small impurity peaks.[49] Isolated yields of **9-15** were typically 40-70%.

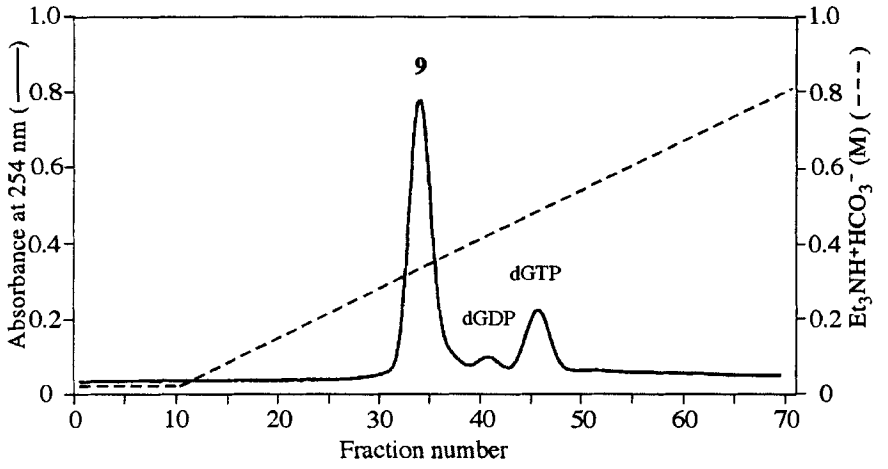


FIG. 1: Purification of 2'-deoxyguanosine-5'-(γ-amidohexamethylene-6-amine)-triphosphate (9) on DEAE Sepharose with a 0-1 M gradient of $Et_3NH^+HCO_3^-$ buffer, pH 7.5.

Characterization

The identity and purity of the isolated products, **9-15**, were confirmed by ^{31}P NMR and electrospray mass spectroscopy. In the ^{31}P NMR of **9-15**, the change of the γ-phosphate's chemical shift to a higher frequency is characteristic of the formation of the phosphoamide bond at the γ position, FIG. 2.[22,40,41] While many of the ^{31}P spectrums indicate 95-99% purity, occasionally, detectable ^{31}P signal impurities as seen in FIG. 2 are observed, presumably resulting from degradation or incomplete separation of impurities on the DEAE chromatography column. Electrospray mass spectroscopy demonstrates that the predicted mass for each target compound, **9-15**, is in fact obtained.

In the case of **9**, the most important affinity column ligand for purifying RTPR,[7] the product was further characterized by a full elemental analysis and thermogravimetric analysis. The excess $Et_3NH^+HCO_3^-$ was removed (as volatile Et_3, H_2O, and CO_2) from **9** by repeated evaporation in ethanol, and then the Et_3NH^+ salt of **9** was dried overnight under vacuum over P_2O_5. Elemental analysis on C, H, and N ($\pm \leq 0.19$ for each element), confirmed the purity of **9**, and indicated the presence of two Et_3NH^+ molecules and two H_2O molecules. Thermogravimetric analysis confirms the presence of the two H_2O molecules (calculated for 2 H_2O, 4.27% weight loss; found 4.16%) in the sample prepared for the elemental analysis.

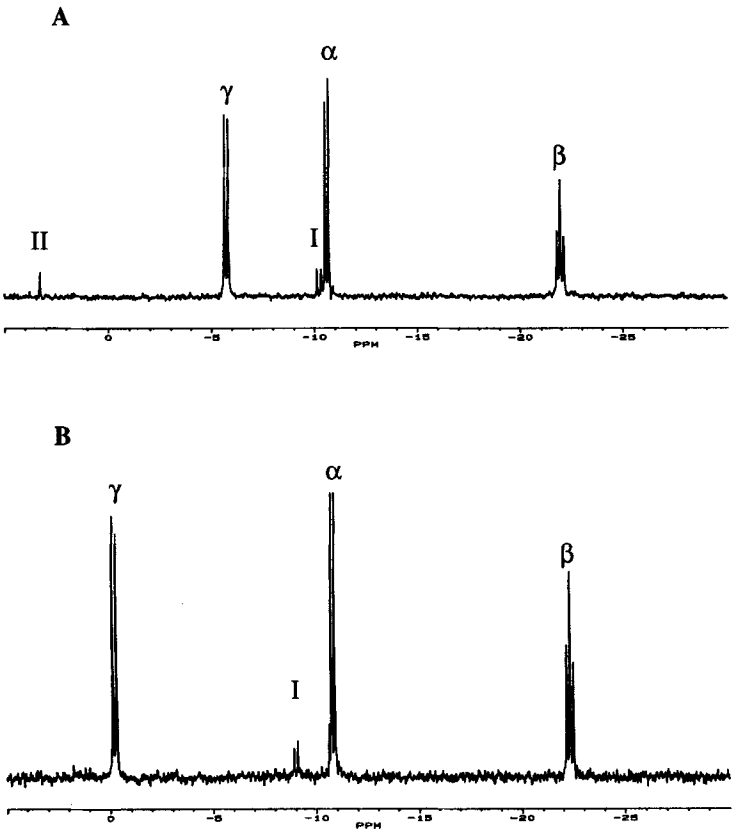


FIG. 2: $\{^1H\}$-^{31}P NMR (121.5 MHz) of dGTP Compounds in D_2O Solvent. (A) The Et_3NH^+ salt of dGTP. (B) The Et_3NH^+ salt of **9**. The additional peaks are occasionally seen, I) is dGTP impurity, II) is a small phosphate impurity. See the experimental section for further details.

Coupling to Sepharose

The Sepharose was activated for coupling of the γ-phosphoamides, **9-15**, by the superior "cyano-transfer" activation method using triethylamine (and the *in situ* formed $[NC\text{-}NEt_3]^+Br^-$), all under neutral conditions. Note that this method quantitatively generates only the desired cyanate ester, -OCN, and eliminates the competing hydrolysis reaction of this ester.[43,44] An added feature of this method is the 10-fold reduction in the amount of the highly toxic NCBr over that required by other methods.[42,50]

Directly after activation of the Sepharose, the amount of cyanate ester present was determined to be 62 μmol per mL of wet Sepharose using the 1,3-dimethylbarbituric acid in

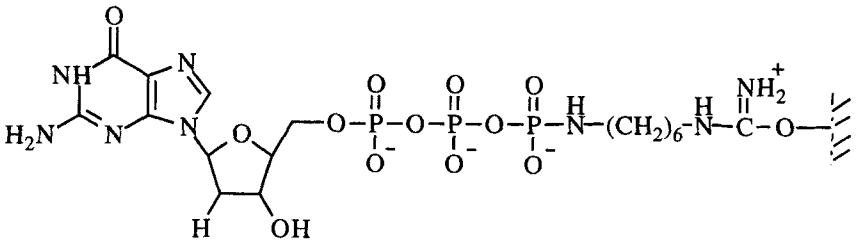


FIG. 3: 2'-Deoxyguanosine-5'-(γ-amidohexamethylene-6-amine)-triphosphate-Sepharose

pyridine assay.[43] Each ligand, **9-15**,was dissolved in 0.1 M $NaHCO_3$ buffer (pH 8.3) and reacted as a 50% slurry with activated Sepharose for 24 hours at 4 °C. The Sepharose was washed extensively to remove unbound ligand and then any remaining cyanate ester is quenched by 1 M ethanolamine (pH 8).

The amount of each ligand coupled to the Sepharose was estimated by cleavage of the ligand from a small amount of the Sepharose using the established method of 10% formic acid.[51] This cleavage reaction works via protonation of the phosphoamide nitrogen ($pK_a \approx 3$), which in turn makes the phosphoamide bond sensitive to acid-catalyzed hydrolysis and thereby releases the nucleoside triphosphate.[45] Ligand densities ranged from 0.4 - 2.9 μmol per mL of wet Sepharose.

The resultant columns with the ligands, **9-15**, attached to Sepharose are stored at 4 °C in 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA. Hydrolysis of the isourea linkage occurring above pH 5,[52] plus the relatively unstable nature of nucleoside triphosphates,[49] causes some slow leaching of the ligand from the support.

Lastly, and although NCBr-activated Sepharose has been the matrix of choice for the preparation of affinity chromatography supports for all of the previous affinity column methods for the purification of ribonucleotide reductases,[17,20,24,25,27,29,53] we did briefly evaluate two alternative activated matrix supports, Reacti-Gel® (Pierce) and 3M Emphase® (Pierce). However, the best results were obtained with the NCBr activated Sepharose, and thus only those results are reported herein.

Summary and Conclusions

By (a) adapting earlier chemistry of Knorre et al.,[22] and then (b) employing the modern NCBr replacement reagent $NC\text{-}NEt_3^+$,[44] we have developed a 2 step, generally 38-75% yield synthesis of *any* desired dNTP nucleoside triphosphate with *any* desired length α,ω-

diamine linker, H_2N-$(CH_2)_n$-NH_2, for example, the optimum n = **6** column material, Sepharose-O($C{=}NH_2^+$)-$(CH_2)_6$-**NH**-dGTP. The basic chemistry is completely different than that used previously[17] in that (i) it involves a trimetaphosphate (i.e., cyclic anhydride) intermediate, and (ii) yields a -NH-phosphate (not -**O**-) linked dGTP. In addition, the chemistry is much more versatile, allowing us to rapidly synthesize 7 different columns (four dGTP ligands of variable H_2N-$(CH_2)_n$-NH_2 linkers lengths (n = 2,4,6 and 12 carbons); and a six carbon linker for dATP, dCTP, and ATP).

Affinity chromatography is a powerful purification technique that can separate proteins with high specificity based on their biological functionality.[54-56] Nucleotides are substrates or effectors for hundreds of proteins, and thus have great potential as ligands for affinity chromatography.[37,57] In principle, our protocol is useful for the rapid development of an optimized affinity support for the purification of other proteins that interact with nucleotides or analogous compounds.

In work nearing completion, we have used these affinity resins for the purification of ribonucleotide reductase from *L. leichmannii*, and for the characterization of RTPR's interaction with deoxynucleotides at the allosteric site.[7]

Experimental

Reagents. Commercial reagents were used without further purification unless otherwise stated. Diethylaminoethyl (DEAE) Sepharose (fast-flow), Sepharose 4B, cyanogen bromide, and all nucleotides (sodium salts) were obtained from Sigma. 1,4-Diaminobutane, 1,6-diaminohexane, 1,8-diaminooctane, 1,10-diaminodecane, 1,12-diaminododecane, and dicyclohexylcarbodiimide (DCC) were purchased from Aldrich. Ethylenediamine was purchased from Mallinkrodt and triethylamine was purchased from Fisher. Dowex AG 50W-X8 was obtained from Bio-Rad Laboratories. Dimethylformamide (DMF), dimethylsulfoxide (DMSO), and methanol (all HPLC grade) were dried for at least 48 h prior to use over molecular sieves 4Å (Linde) or 3Å (Mallinkrodt) which had been activated earlier drying overnight (12 hr) in a vacuum oven (150 °C, 10^{-1} mm Hg). In the early part of these studies, solvents were checked for dryness by UV-visible spectrometry using Reichard's dye.[58] The DCC was freshly sublimed (34 °C, 10^{-3} mm Hg) just before use. Distilled water was purified by a Barnstead NANOpure system.

Instrumental Analysis. UV-visible analyses were conducted with a Hewlett Packard 8452A diode array spectrometer. At all stages, nucleotides were detected and measured quantitatively using their ultraviolet absorbance and the following extinction coefficients

(dGTP: ε_{253} = 13,700 $M^{-1}cm^{-1}$; dCTP: ε_{272} = 9,100 $M^{-1}cm^{-1}$; ATP, dATP: ε_{259} = 15,400 $M^{-1}cm^{-1}$)[49] by taking aliquots diluted into aqueous solutions. Electrospray mass spectroscopy was performed on a Fisions VG Quattro-SQ instrument using negative ion detection, and with a matrix consisting of a 50/50 solution of acetonitrile / 0.1% aqueous NH_4OH. Elemental analysis was performed by Atlantic Microlabs, Norcross, GA. Thermogravimetric analysis was performed by Rose Consulting, Half Moon Bay, CA.

The ^{31}P NMR spectra were obtained from 15-30 mg portions of dried product dissolved in ca. 1.5 mL D_2O (Cambridge Isotopes), obtained on a Bruker AC-300E (121.5 MHz). All ^{31}P NMR chemical shifts (δ) were referenced externally to 85% aqueous H_3PO_4, were collected with at least 512 transients per spectrum, and are reported in ppm. Each isolated nucleotide had characteristic triplet (α), doublet (β), and doublet (γ) resonance patterns, with ^2J- coupling constants of 19-21 Hz.

General Procedures. Nucleotide triphosphate salts are unstable at room temperature and, therefore require storage at -20 °C.[49] Aqueous solutions of these compounds are particularly susceptible to hydrolysis. Care was taken to keep aqueous solutions at room temperature for as short a time as possible. Rotoevaporation at 20×10^{-3} mm Hg was done using a cold water bath (10-15 °C), and vacuum drying was done using a Schlenk line (10^{-3} mm Hg) and a Schlenk tube at room temperature.[59]

Conversion of nucleotides to Et_3NH^+ salts. Stock triethylammonium bicarbonate ($Et_3NH^+HCO_3^-$) buffer was prepared by bubbling CO_2 into a 1.0 M aqueous Et_3NH^+ solution until a pH of 7.5 was reached.[60] The sodium salt of each nucleotide investigated (0.15 mmol) was dissolved in 10 mL 0.1 M $Et_3NH^+HCO_3^-$ buffer (pH 7.5) and loaded on a 2.5 cm × 11 cm column packed with Dowex AG 50W-X8 cation exchange resin which had been pre-equilibrated with 0.1 M $Et_3NH^+HCO_3^-$ buffer. The nucleotides were washed through the column with 0.1 M $Et_3NH^+HCO_3^-$ buffer (60 mL). Six 10 mL fractions were collected. The presence of nucleotides in each fraction was determined by spotting on Silica gel 60F-254 TLC plates (EM Industries) and examining for fluorescence quench by a hand-held UV-lamp. Nucleotide containing fractions were combined and transferred to a 100 mL round-bottomed flask and the volume was reduced by rotary evaporation. Then the solution was transferred to a 25 mL sidearm Schlenk flask, attached to a line equipped with a Welch Duo seal vacuum pump (10^{-3} mm Hg),[59] and dried to a residue at room temperature. The solid residue was stored at -20 °C under dry argon until use. The Et_3NH^+ cation exchange column was regenerated between uses by first passing 50 mL 1 M $Et_3NH^+HCO_3^-$ buffer (pH 7.5) through the column followed by 750 mL of 0.1 M $Et_3NH^+HCO_3^-$ buffer (pH 7.5).

*Generation of the trimetaphosphate nucleosides intermediates, **5-8**.* In a Vacuum Atmospheres inert atmosphere (nitrogen) drybox (i.e., to mitigate against adventitious water), 1.5 mL dry DMF/methanol (9:1 v/v) was added to 0.15 mmol of nucleoside triphosphate (**1-4**) (Et_3NH^+ salt) in a 25 mL sidearm Schlenk flask with a magnetic stir bar and stirred until dissolved. Freshly sublimed 1,3-dicyclohexylcarbodiimide (102 mg, 0.5 mmol, 3.3 equiv) was added to the solution and stirred for at least 3 hr. Under the conditions used, a pale-yellow color occasionally developed and a white precipitate formed (1,3-dicyclohexylurea) by the end of the reaction time. The stopcock was closed and the flask was removed from the drybox. The flask was then connected via its side-arm to a Schlenk vacuum line and the solution was evaporated to dryness.

Occasionally, no precipitate would be observed after the 3 hour reaction period. In these cases, 2-3 eq. additional DCC was added to the reaction mixture. The flask was then stirred for an additional 2-3 hours during which a precipitate formed. Such reactions generally resulted in lowered yields and may indicate a problem with trace H_2O being present.

*Generation of phosphoamide products, **9-14**.* Under Ar, 1.5 mL dry DMF/methanol (9:1 v/v) was added to the dry nucleoside trimetaphosphate (**5-8**) reaction mixture, and the mixture was stirred until dissolved. Using a 5 mL syringe, the DMF solution was then added dropwise with stirring to 15 mL of a 1 M aqueous solution in air of the chosen diamine hydrocarbon buffered to pH 8-9 with CO_2 (1,6-hexanediamine, for **9-12**; ethylenediamine, for **13**; 1,4-diaminobutane, for **14**). The reaction flask was rinsed with an additional 1.5 mL dry DMF/methanol (9:1 v/v) which was added to the diamine hydrocarbon solution. A white precipitate (1,3-dicyclohexylurea)[37] was observed upon addition of the DMF solution to the aqueous diamine solution. The reaction mixture was stirred at room temperature for 1 hour, then filtered through a medium glass frit to remove the precipitate. The DMF/H_2O solution was diluted 45-fold with deionized H_2O (800 mL) to reduce the ionic strength, then loaded on the DEAE Sepharose anion exchange column, or stored frozen at -20 °C for purification at a later time.

DEAE Sepharose Chromatography. The phosphoamide products, **9-15**, were isolated using column chromatography at 4 °C as follows. The aqueous solution was loaded (~3 mL/min) onto a diethylaminoethyl (DEAE) Sepharose fast-flow column (2.5 cm × 20 cm), and in the Et_3NH^+ form. Detection at 254 nm was accomplished using a flow-though UV-monitor (Bio-Rad Model EM-1). After the loading was complete, 100-200 mL of H_2O was used to wash through any nonbinding material. The products adhering to the column

were eluted with a buffer gradient (0 to 1 M $Et_3NH^+CO_3^-$ buffer, pH 7.5, 600 mL total), see FIG. 1. The first major band (sometimes preceded by a minor contaminant band) contains the desired phosphoamide product, which was pooled and evaporated to dryness using rotoevaporation at 10-15 °C.

2'-deoxyguanosine-5'-(γ-amidohexamethylene-6-amine)-triphosphate (9),
$[Et_3NH^+]_{3-x}[H]_x[H_2N(CH_2)_6NH\text{-}P(O)_2OP(O)_2OP(O)_2O\text{-}dG]$ •yH_2O •$zEt_3NH^+HCO_3^-$
Compound (**9**) was isolated as a white Et_3NH^+ salt, 75% yield; {^{1}H} - ^{31}P NMR: δ [D_2O] = -0.218 (γ) d, ($^2J_{PP}$ = 21.2 Hz); δ = -22.36 (β) t, ($^2J_{PP}$ = 20.3 Hz); δ = -10.82 (α) d, ($^2J_{PP}$ = 19.4 Hz); mass spectrum: *m/z* $[H_2•M]^-$ (ES) calcd for $C_{16}H_{29}N_7O_{12}P_3$ 604.4 (found: 604.0). Bulk $Et_3NH^+HCO_3^-$ was removed by multiple evaporations in ethanol and the material was vacuum dried overnight at ambient temperature over P_2O_5.[61] Anal.: for (**9** • 2 Et_3NH^+ • 2 H_2O) $C_{28}H_{64}N_9O_{14}P_3$ calcd C, 39.86%; H, 7.65%; N, 14.94%; found: C, 39.98%; H, 7.46%; N, 14.94%. Thermogravimetric analysis: calcd weight loss for 2 H_2O is 4.27%, observed is 4.16% upon heating from 25-250 °C at a 5 °C per min in a dry nitrogen environment; decomposition starts at 155 °C.

2'-deoxyadenosine-5'-(γ-amidohexamethylene-6-amine)-triphosphate (10),
$[Et_3NH^+]_{3-x}[H]_x[H_2N(CH_2)_6NH\text{-}P(O)_2OP(O)_2OP(O)_2O\text{-}dA]$ •yH_2O •$zEt_3NH^+HCO_3^-$
Compound (**10**) was isolated as a white Et_3NH^+ salt, 38% yield; {^{1}H} - ^{31}P NMR: δ [D_2O] = -0.120 (γ) d, ($^2J_{PP}$ = 20.3 Hz); δ = -22.26 (β) t, ($^2J_{PP}$ = 20.2 Hz); δ = -10.76 (α) d, ($^2J_{PP}$ = 19.3 Hz); mass spectrum: *m/z* $[H_2•M]^-$ (ES) calcd for $C_{16}H_{29}N_7O_{11}P_3$ 588.4 (found: 588.0).

2'-deoxycytidine-5'-(γ-amidohexamethylene-6-amine)-triphosphate (11),
$[Et_3NH^+]_{3-x}[H]_x[H_2N(CH_2)_6NH\text{-}P(O)_2OP(O)_2OP(O)_2O\text{-}dC]$ •yH_2O •$zEt_3NH^+HCO_3^-$
Compound (**11**) was isolated as a white Et_3NH^+ salt, 39% yield; {^{1}H} - ^{31}P NMR: δ [D_2O] = -0.082 (γ) d, ($^2J_{PP}$ = 20.6 Hz); δ = -22.22 (β) t, ($^2J_{PP}$ = 21.1 Hz); δ = -10.74 (α) d, ($^2J_{PP}$ = 19.5 Hz); mass spectrum: *m/z* $[H_2•M]^-$ (ES) calcd for $C_{15}H_{29}N_5O_{12}P_3$ 564.4 (found: 564.0).

Adenosine-5'-(γ-amidohexamethylene-6-amine)-triphosphate (12).
$[Et_3NH^+]_{3-x}[H]_x[H_2N(CH_2)_6NH\text{-}P(O)_2OP(O)_2OP(O)_2O\text{-}A]$ •yH_2O •$zEt_3NH^+HCO_3^-$
Compound (**12**) was isolated as a white Et_3NH^+ salt, 54% yield; {^{1}H} - ^{31}P NMR: δ [D_2O] = -0.040 (γ) d, ($^2J_{PP}$ = 20.8 Hz); δ = -22.18 (β) t, ($^2J_{PP}$ = 20.0 Hz); δ = -10.74 (α) d, ($^2J_{PP}$ = 19.4 Hz); mass spectrum: *m/z* $[H_2•M]^-$ (ES) calcd for $C_{16}H_{29}N_7O_{12}P_3$ 604.4 (found: 604.1).

2'-deoxyguanosine-5'-(γ-amidoethylene-2-amine)-triphosphate (13).
$[Et_3NH^+]_{3-x}[H]_x[H_2N(CH_2)_2NH\text{-}P(O)_2OP(O)_2OP(O)_2O\text{-}dG]$ •yH_2O •$zEt_3NH^+HCO_3^-$
Compound (**13**) was isolated as a white Et_3NH^+ salt, 69% yield; {^{1}H} - ^{31}P NMR: δ [D_2O] = -1.39 (γ) d, ($^2J_{PP}$ = 20.1 Hz); δ = -21.94 (β) t, ($^2J_{PP}$ = 20.1 Hz); δ = -10.60 (α) d, ($^2J_{PP}$ = 20.0 Hz); mass spectrum: *m/z* $[H_2•M]^-$ (ES) calcd for $C_{12}H_{21}N_7O_{12}P_3$ 548.3 (found: 548.3).

2'-deoxyguanosine-5'-(γ-amidotetramethylene-4-amine)-triphosphate (14).
$[Et_3NH^+]_{3-x}[H]_x[H_2N(CH_2)_4NH\text{-}P(O)_2OP(O)_2OP(O)_2O\text{-}dG]$ •yH_2O •$zEt_3NH^+HCO_3^-$
Compound (**14**) was isolated as a white Et_3NH^+ salt, 65% yield; {^{1}H} - ^{31}P NMR: δ [D_2O] = -0.531 (γ) d, ($^2J_{PP}$ = 21.1 Hz); δ = -22.34 (β) t, ($^2J_{PP}$ = 20.3 Hz); δ = -10.79 (α) d, ($^2J_{PP}$ = 19.9 Hz); mass spectrum: *m/z* $[H_2•M]^-$ (ES) calcd for $C_{14}H_{25}N_7O_{12}P_3$ 576.3 (found: 576.0).

Generation of phosphoamide product 15. This product required a slightly modified procedure due to the insolubility of the 1,12-diaminododecane in water. The modified procedure uses isopropanol/water instead.

To the dry nucleoside trimetaphosphate (**5**) reaction mixture, 1.5 mL dry DMF was added and the mixture was stirred until dissolved. Using a 5 mL syringe, the DMF solution was then added dropwise with stirring to 30 mL of 0.5 M 1,12-diaminododecane in isopropanol:water (1:1 v/v). The reaction mixture was allowed to stir at room temperature for 1 hour, then filtered through a medium glass frit to remove the precipitate. The solution was evaporated to dryness utilizing the vacuum of a Schlenk line. The solid was extracted twice with 50 mL water to recover **15** from the 1,12-diaminododecane. The aqueous solution was filtered and then frozen until isolation using DEAE Sepharose anion exchange chromatography.

2'-deoxyguanosine-5'-(γ-amidododecamethylene-12-amine)-triphosphate (15).
$[Et_3NH^+]_{3-x}[H]_x[H_2N(CH_2)_{12}NH\text{-}P(O)_2OP(O)_2OP(O)_2O\text{-}dG]$ •yH_2O •$zEt_3NH^+HCO_3^-$
Compound (**15**) was isolated as a white Et_3NH^+ salt, 69% yield; {^{1}H} - ^{31}P NMR: δ [D_2O] = -0.35 (γ) d, ($^2J_{PP}$ = 21.1 Hz); δ = -22.41 (β) t, ($^2J_{PP}$ = 20.1 Hz); δ = -10.97 (α) d, ($^2J_{PP}$ = 19.2 Hz); mass spectrum: *m/z* $[H_2•M]^-$ (ES) calcd for $C_{22}H_{41}N_7O_{12}P_3$ 688.5 (found: 688.5).

Coupling the Aminophosphoamide to Sepharose. Triethylammonium bicarbonate was removed from the purified aminophosphoamide products, (**9-15**), by three cycles of

dissolving them in ethanol (10 mL) and rotary evaporation to dryness to prepare the ligand for coupling.

Sepharose 4B was activated according to the optimized "cyano-transfer" activation method developed by Kohn and Wilchek (1982),[44] by adding NCBr at 37.5 mg per mL of wet Sepharose 4B. (WARNING: cyanogen bromide is a highly toxic, volatile solid.) After the activation reaction was complete, the reaction mixture was poured into ice cold washing medium (acetone: 0.1 N HCl = 1:1), and the amount of active cyanate ester (R-OCN) formed on the Sepharose was determined immediately, and found to be 62 μmol per mL Sepharose by the colorimetric assay method of Kohn and Wilchek using 1,3-dimethylbarbituric acid in pyridine.[43] This activated Sepharose 4B was then washed just prior to use with cold 60% aqueous acetone, 30% aqueous acetone, and then water.

Each nucleotide (**9-15**) was then dissolved in sufficient 0.1 M $NaHCO_3$ (pH 8.3) to yield an approximately 15 - 20 mM solution. An equal volume of wet packed activated Sepharose was added to the ligand solution to produce a 50% slurry. After rotation (8 rpm) for 24 hr at 4 °C, the resin was washed extensively with 0.1 M $NaHCO_3$ (pH 8.3) buffer to recover uncoupled ligand. Any remaining cyanate esters were quenched using a 1 M ethanolamine (pH 8) solution for 1 hour at 24 °C. The resins were washed with 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, and stored at 4 °C.

*Quantification of amount of **9-15** coupled to Sepharose.* The amount of **9-15** coupled to the Sepharose was determined by quantification of the amount of nucleoside triphosphate released from the Sepharose by formic acid hydrolysis of the phosphoamide bond.[51] Each nucleotide-Sepharose (50 μL of 50% aqueous suspension by volume) was added to 950 μL 10% formic acid and incubated at 37 °C; after 2 hrs no further release of nucleotide was observed and thus the hydrolysis was judged complete. The solution was centrifuged briefly to pellet the Sepharose before a 200 μL aliquot was removed and neutralized with 800 μL 1 M potassium phosphate (pH 7.3). The UV-visible absorbance was measured on the sample, and the amount of nucleoside triphosphate released was calculated from the corresponding nucleotide triphosphate extinction coefficient. Sepharose affinity column ligand densities by the above procedure were: **9**, 2.9 μmol/mL; **10**, 2.6 μmol/mL; **11**, 1.6 μmol/mL; **12**, 2.1 μmol/mL; **13**, 1.4 μmol/mL; **14**, 2.0 μmol/mL; **15**, 0.4 μmol/mL.

Supplemental Materials Available

The following supplemental materials are available by request from the authors: additional experimental details for controls, the evaluation of the alternative activated matrix supports, and the ^{31}P NMR spectra of **10-15**.

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