Nucleoside carbonyl(di- and triphosphates)†

Ivan B. Yanachkov, James M. Stattel and George E. Wright *‡

Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical School, Worcester, MA 01655, USA

Received (in Cambridge, UK) 15th March 2001, Accepted 15th August 2001
First published as an Advance Article on the web 19th October 2001

The first examples of nucleoside di- and triphosphates containing the electrophilic and potentially reactive carbonyl group in place of a phosphoanhydride oxygen are reported, using the DNA polymerase inhibitors N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPdGTP) and N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-diphosphate (BuPdGDP) as platforms. The P^2,P^3-carbonyltriphosphonate, BuPdGMPPCOP, was obtained by reaction between the phosphoro-*N*-methylimidazolidate of the monophosphate BuPdGMP and carbonyldiphosphonic acid (PCOP), and it was isolated by preparative reverse phase chromatography. The carbonyldiphosphonate analogue, BuPdGMPCOP, was obtained by displacement of the 5′-mesyl group of the corresponding 5′-mesylnucleoside with carbonyldiphosphonate. While BuPdGMPCOP was stable in aqueous solutions, BuPdGMPPCOP hydrolyzed to BuPdGMP and PCOP with a half-life of 3 hours. Both BuPdGMPPCOP and BuPdGMPCOP were potent, competitive inhibitors of human DNA polymerase α.

Introduction

Nucleoside di- and triphosphates (NDPs, NTPs) are key cellular metabolites. Analogues of these nucleotides with a modified polyphosphate group have played important roles in elucidating the structure, function, and location of enzymes and proteins involved in NDP/NTP binding.[1] Such modifications include substitutions of the bridged (phosphoroanhydride) or non-bridged (terminal) oxygen atoms of the polyphosphate chain. The substitutions of the non-bridged oxygens are restricted mainly to phosphorothioates,[1a] while the phosphoroanhydride oxygen has been replaced with methylene, halomethylene, and imino groups. The resulting analogues in many cases retain their affinity for the corresponding enzymes or proteins, but often cannot serve as substrates (*e.g.* non-hydrolyzable NTP analogues), thus inhibiting the corresponding processes.

In this paper we present a new type of nucleoside polyphosphate analogue in which a phosphoroanhydride oxygen is replaced by a potentially reactive carbonyl group. This type of compound might not only serve as a non-hydrolyzable analogue, but the highly electrophilic carbonyl group may also interact with nucleophilic centers near polyphosphate binding sites, providing a new mode of inhibition. Indication of this kind of interaction has been observed with carbonyldiphosphonic acid (**6**, PCOP), a carbonyl analogue of pyrophosphate, which has inhibitory activity toward many enzymes having a pyrophosphate binding site. Among these[2] are pyrophosphate-dependent phosphofructokinase, aspartate transcarbamylase, inorganic pyrophosphatase, and a large number of viral and cellular DNA polymerases.[3]

Specifically, we describe the synthesis and properties of N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-(P^1,P^2-carbonyldiphosphonate) (**15**, BuPdGMPCOP) and N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-(P^2,P^3-carbonyltriphosphonate) (**8**, BuPdGMPPCOP), and their inhibitory properties toward human DNA polymerase α. The parent nucleotides, N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-diphosphate (**14**, BuPdGDP), and N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (**7**, BuPdGTP)[4] are potent inhibitors of the B family of DNA polymerases, and have been widely used in study of the identity and roles of DNA polymerases in cellular DNA synthesis.[1b]

Results and discussion

Synthesis of BuPdGMPPCOP (8)

P^2,P^3-Modified nucleoside triphosphates are synthesized by condensation of activated nucleoside monophosphates with the corresponding pyrophosphate analogues (Scheme 1). The most common method is the imidazolidate method of Hoard and Ott,[5] in which the nucleoside 5′-monophosphate is activated *in situ* to the phosphoroimidazolidate by reaction with an excess of carbonyldiimidazole. Reactions of the imidazolidate of BuPdGMP (**2**) with the tributylammonium (TBA) salt of PCOP (**6**) showed consumption of the activated monophosphate, but the only products that were detected (TLC) and isolated from the reaction mixtures were BuPdGMP (**1**) and PCOP (**6**) itself. The same result was found when the synthesis of BuPdMPPCOP (**8**) was attempted from the phosphoromorpholidate of BuPdGMP (**3**), prepared by the method of Moffat and Khorana.[6] Both imidazolidate and morpholidate methods require long reaction times (more than 12 h) during which time decomposition of PCOP (**6**) or the product was possible. Consequently, we applied a fast and mild method for synthesis of nucleoside triphosphates, recently developed in this laboratory.[7] In this method, nucleoside 5′-monophosphates are activated as the phosphoro-*N*-methylimidazolidates by treatment with *N*-methylimidazole in the presence of 2,2′-dipyridyl disulfide and triphenylphosphine.[8] The activated nucleotide is isolated by precipitation with diethyl ether, and coupled with inorganic pyrophosphate or a pyrophosphate analogue. For example, the phosphoro-*N*-methylimidazolidate of BuPdGMP (**4**) reacted with an excess of tributylammonium pyrophosphate (**5**) in less than 5 minutes to give the triphosphate BuPdGTP (**7**) in 69% yield (isolated). However, when the same intermediate

† Electronic supplementary information (ESI) available: figures showing the time course of the reaction of the phosphoroimidazolidate of BuPdGMP (**2**) with PCOP (**6**), and reaction of *N*-methylphosphoroimidazolate of BuPdGMP (**4**) with 1 eq. of **6**. See http://www.rsc.org/suppdata/p1/b1/b102467c/

‡ *Present address*: GLSynthesis, Inc., One Innovation Drive, Worcester, MA 01605, USA

 DOI: 10.1039/b102467c

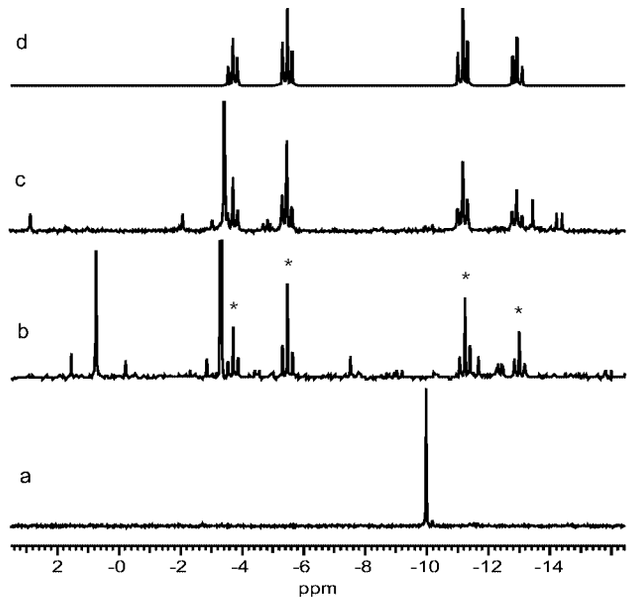
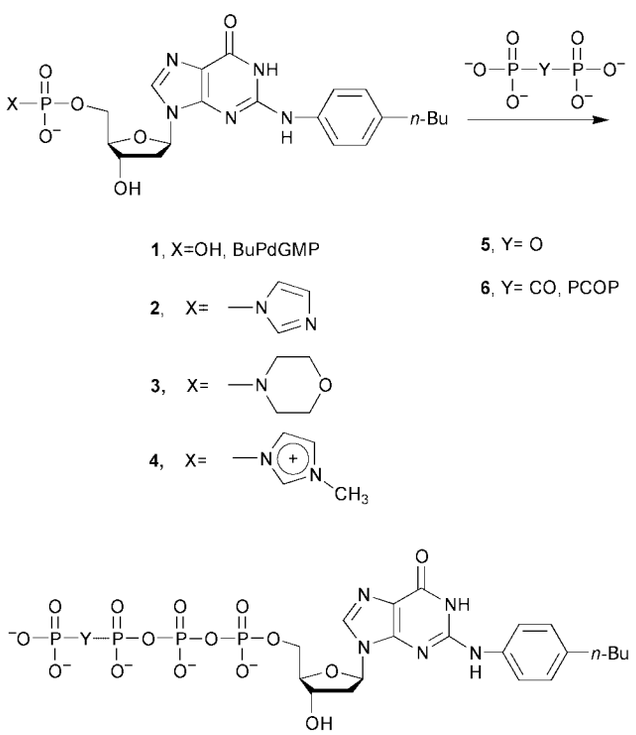


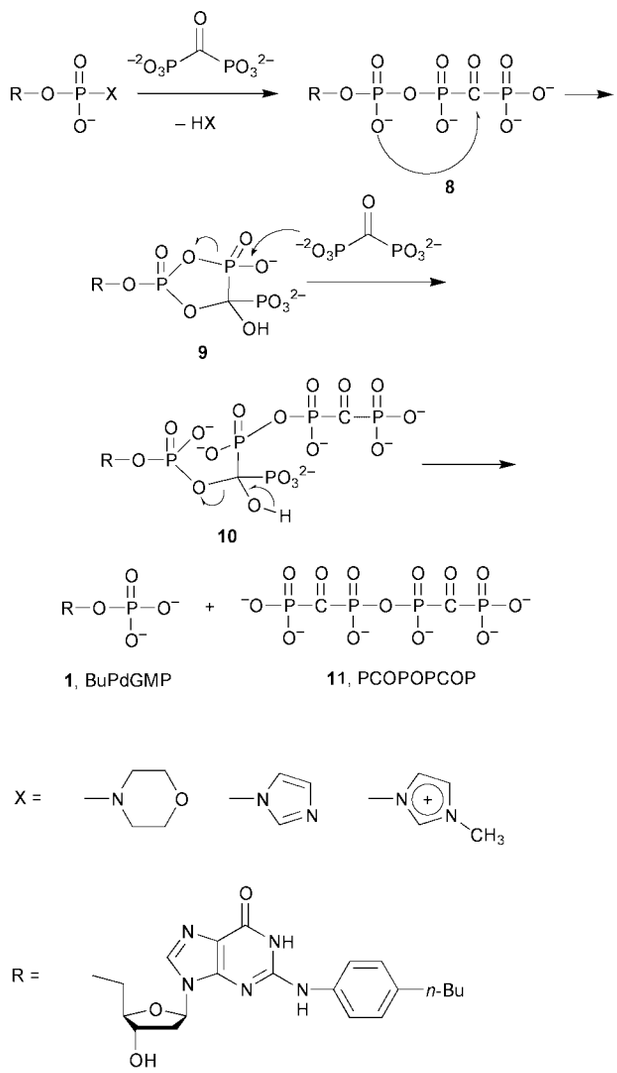
Fig. 1 ^{31}P NMR spectra: a) phosphoro-*N*-methylimidazolidate of BuPdGMP (**4**); b) reaction of **4** with 5 eq. of PCOP (**6**) after 6 min; c) reaction of PCOP (**6**) with 0.5 eq. EDC after 10 min; d) simulated spectrum of PCOPOPCOP (**11**).



Scheme 1

was reacted with PCOP (**6**) under these conditions only the monophosphate BuPdGMP (**1**) and PCOP were isolated after aqueous workup.

To shed light on these unexpected outcomes, the reaction of the purified *N*-methylimidazolidate of BuPdGMP (**4**) with the TBA salt of PCOP (**6**) was followed by ^{31}P NMR spectroscopy (Fig. 1). The signal for the *N*-methylimidazolidate **4** at −10 ppm (spectrum a) disappeared after 6 minutes, and a signal for BuPdGMP (**1**) at 0.8 ppm appeared, together with an interesting set of four "triplets" at −3.6, −5.4, −11.2 and −13.0 ppm (denoted with asterisks in spectrum b).§ The appearance of the latter signals did not change upon turning the proton decoupler off. It was proved that the four "triplets" are part of a single spin system by a ^{31}P–^{31}P COSY experiment and by a homonuclear ^{31}P experiment where, on positioning the decoupler between one pair of "triplets", the other pair collapsed to a singlet (results not shown). The same spin system was generated when the TBA salt of PCOP (**6**) reacted with 0.5 eq. of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (Fig. 1, spectrum c), suggesting that the spectrum resulted in both cases from the anhydride of **6**, PCOPOPCOP (**11**, Scheme 2).¶ The



Scheme 2

calculated ^{31}P spectrum of this compound as a symmetrical ABB′A′ system (Fig. 1, spectrum d), after iterative fitting to spectrum c, revealed $^{2}J_{AB} = {}^{2}J_{A'B'} = 212$ Hz, $^{3}J_{BB'} = 38$ Hz, and $^{4}J_{AB'} = 0$ Hz.

A plausible mechanism for the formation of the anhydride PCOPOPCOP (**11**) in this reaction is illustrated in Scheme 2. First, PCOP (**6**) reacts with the activated nucleoside monophosphate giving the expected nucleoside P^2,P^3-carbonyltriphosphonate **8**. The highly electrophilic P^2,P^3 carbonyl group of **8** is attacked by the α-phosphate to form a cyclic hemiacetal

§ The same result was observed when the imidazolidate of BuPdGMP was reacted with an excess of PCOP. ^{31}P NMR spectra of the time course of the reaction are presented in the ESI.

¶ This compound hydrolyzed to PCOP when triethylammonium bicarbonate (TEAB) buffer was added to the reaction mixture, as observed by ^{31}P NMR. Attempts to isolate it by ion exchange chromatography resulted in the formation of only PCOP.

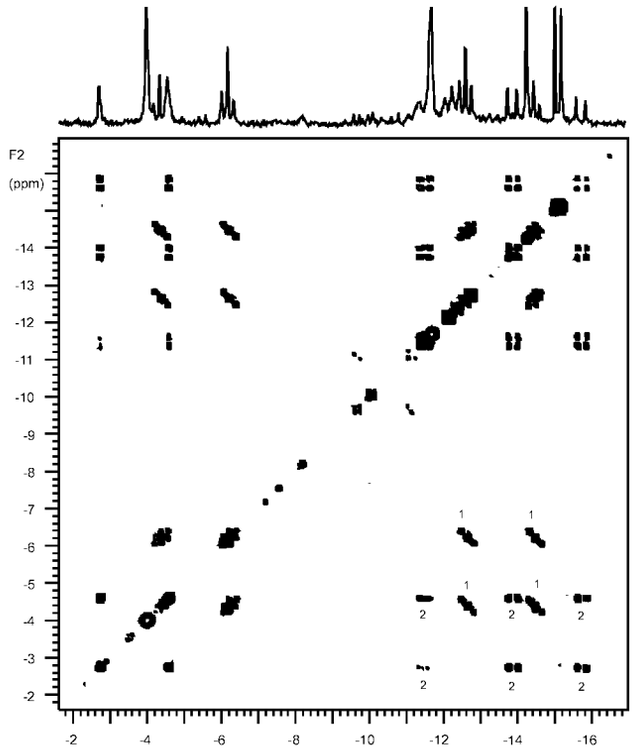


Fig. 2 ^{31}P–^{31}P COSY spectrum of the reaction of the phosphoro-*N*-methylimidazolidate of BuPdGMP (**4**) with 1 eq. of PCOP (**6**). The crosspeaks for the anhydride PCOPOPCOP (**11**) are denoted by 1, while the crosspeaks for BuPdGMPPCOP (**8**) are denoted by 2.

9. In compound **9** the neutral α-phosphate becomes a good leaving group, making the β-phosphate susceptible to nucleophilic attack, analogous to the well known susceptibility of the cyclic trimetaphosphates of nucleosides toward nucleophilic opening of the anhydride ring.[9] Phosphorolysis of **9** by another molecule of PCOP (**6**) breaks the α–β anhydride bond and opens the hemiacetal ring giving intermediate **10**; subsequent elimination releases the nucleoside monophosphate BuPdGMP (**1**) and the anhydride PCOPOPCOP (**11**). Our inability to isolate PCOPOPCOP (**11**) is probably due to its facile cyclization and hydrolysis in aqueous solution by a mechanism analogous to the one shown in Scheme 2.

According to Scheme 2, a possible approach to avoid the decomposition of **8** was to use a limiting amount of PCOP during its synthesis. A drawback of this approach is that BuPdGMPPCOP (**8**) (or the products of its decomposition) can react with the activated BuPdGMP or with compound **9**. Indeed, when the phosphoro-*N*-methylimidazolidate **4** was treated with an equimolar amount of the TBA salt of PCOP (**6**), the ^{31}P spectrum of the reaction mixture became complex (see ESI). A ^{31}P–^{31}P COSY spectrum of this mixture (Fig. 2) revealed, among other signals, the spectrum of PCOPOPCOP (**11**) (cross peaks labeled 1) together with a spin system of three ^{31}P signals in agreement with the spectrum expected for BuPdGMPPCOP (**8**) (cross peaks labeled 2). This compound was successfully isolated from the reaction mixture by preparative reverse phase HPLC in 17% yield and characterized by ^{1}H, ^{31}P NMR and FAB mass spectra. The ^{31}P spectrum of **8** (Fig. 3) consists of a doublet of doublets centered at −10.14 ppm assigned to P^1 ($^2J_{P1P2} = 29.7$ Hz; $^4J_{P1P3} = 7.0$ Hz; the line width of this signal increased considerably when the decoupler was turned off); a doublet of doublets at −9.15 and −10.90 ppm, assigned to P^2 ($^2J_{P1P2} = 29.7$ Hz; $^2J_{P2P3} = 207$ Hz); and a doublet of doublets centered at −1.49 ppm, assigned to P^3 ($^2J_{P2P3} = 207$ Hz; $^4J_{P3P1} = 7.0$ Hz). BuPdGMPPCOP (**8**) is stable as a solid when stored at −76 °C. In aqueous solution at room temperature it hydrolyzed to BuPdGMP (**1**) and PCOP (**6**) (HPLC)

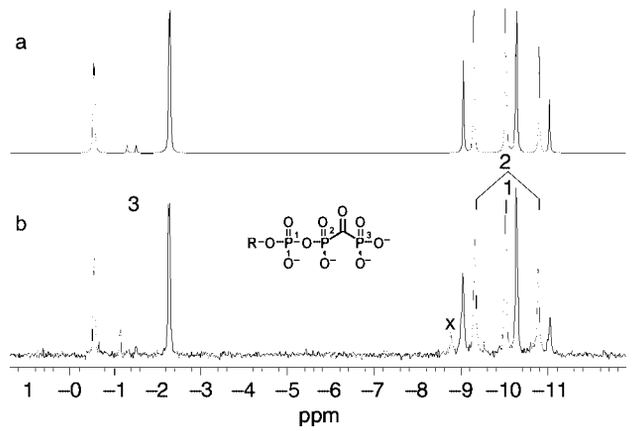


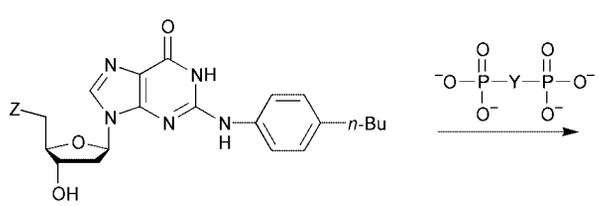
Fig. 3 ^{31}P NMR proton decoupled spectrum of BuPdGMPPCOP (**8**) in 20 mM EDTA in D_2O: a) recorded spectrum (x denotes a signal from contamination); b) calculated spectrum.

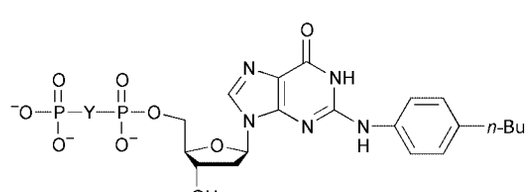
with a half-life of 3 h. The much higher susceptibility of BuPdGMPPCOP to hydrolysis, when compared with other triphosphates or triphosphate analogues, could be explained by reactions similar to those in Scheme 2, involving cyclization followed by hydrolytic ring opening of the cyclic intermediate.

Synthesis of BuPdGMPCOP (15)

The methods employed for the synthesis of nucleoside diphosphates containing a modified phosphoroanhydride oxygen include condensation of a nucleoside (protected on the base and 3′(2′) positions of the sugar) with methylene or halomethylene pyrophosphate analogues in the presence of an excess of carbodiimide,[10] and nucleophilic displacement of the 5′-tosyl group of 5′-tosylnucleosides by modified pyrophosphates.[11]

While the application of the condensation method was not successful (results not shown), we used a modification of Poulter's displacement procedure for the synthesis of the carbonyl analogue of BuPdGDP (**15**, Scheme 3). Reaction of the





Scheme 3

TBA salt of PCOP (**6**) with N^2-(4-butylphenyl)-5′-mesyl-2′-deoxyguanosine (**13**)[12] in MeCN gave BuPdGMPCOP (**15**), isolated as a yellow solid in 93% yield by ion-exchange chromatography and characterized by its ^{1}H, ^{31}P, ^{13}C NMR and FAB mass spectra. The ^{1}H NMR spectrum did not differ from that of the corresponding diphosphate, BuPdGDP (**14**). In the ^{31}P

Table 1 Inhibitor potency against human DNA polymerase α

Inhibitor	Apparent K_i/nM [a]
BuPdGTP, **7**	0.31
BuPdGMP, **1**	24
BuPdGMPPCOP, **8**	0.72 [b]
BuPdGMPCOP, **15**	1.4 [b]

[a] Assays were performed as described in the Experimental section in the absence of the competitive substrate dGTP ("truncated" assay). [b] Assays were performed with HEPES buffer (see text).

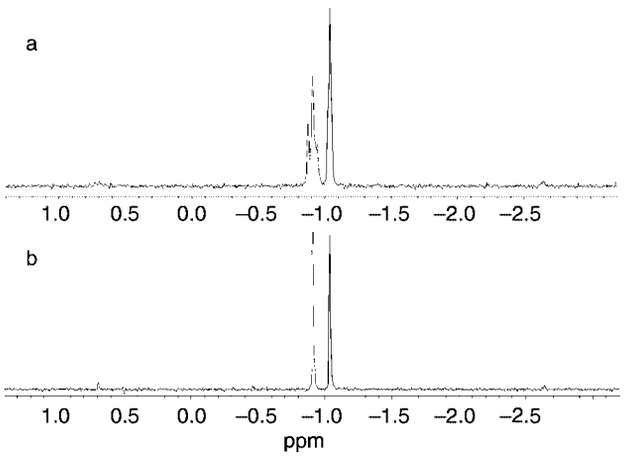


Fig. 4 ^{31}P NMR spectrum of BuPdGMPCOP (**15**) in D_2O: a) proton coupled spectrum; b) proton decoupled spectrum.

NMR spectrum, P^1 and P^2 had close chemical shifts and were strongly coupled, with $^2J_{PP}$ = 193 Hz, forming an interesting AB spin system (Fig. 4). Because of this strong P^1P^2 interaction, not only was the three bond P^1–H5′,5″ coupling observable (4.2 Hz), but also an unusual five bond P^2–H5′,5″ coupling of 1.9 Hz. The same interesting feature was present in the ^{13}C spectrum, where C-4′ and C-5′ showed the influence not only of P^1, but also of P^2. In addition the ^{13}C signal from the carbonyl group of the PCOP moiety was present as a doublet of doublets far downfield at 237.5 ppm with $^2J_{PC}$ = 117.5 and 125.7 Hz. As a solid, BuPdGMPCOP (**15**) was stable for six months when stored at −76 °C, and an aqueous solution of the nucleotide was stable for at least 48 hours at room temperature (HPLC).

DNA polymerase inhibition

We tested BuPdGMPCOP (**15**) and BuPdGMPPCOP (**8**) as inhibitors of the catalytic subunit of human DNA polymerase α,[13] generously donated by Dr Teresa Wang (Stanford University). Solutions of the compounds freshly prepared in HEPES buffer were assayed using activated DNA under previously established "truncated" conditions,[3b] *i.e.* lacking the competitive substrate dGTP, to determine directly the apparent K_i values. BuPdGMPCOP (**15**) and BuPdGMPPCOP (**8**) exhibited potencies similar to that of BuPdGTP (**7**) under these conditions (Table 1), suggesting that both compounds bind with similar affinity to the polymerase active site. Experiments with variable concentrations of dGTP demonstrated competitive kinetics for both inhibitors (results not shown).

Conclusions

In the present work we focused on the synthesis and study of carbonyl analogues of BuPdG di- and triphosphates, in order to exploit the products as DNA polymerase α inhibitors. The methodology developed for these syntheses can be implemented for the synthesis of carbonyl analogues of other nucleoside di- and triphosphates, both natural and modified. The resulting compounds could be potentially of great utility in biochemistry and cell biology.

Experimental

General

All NMR experiments were recorded with a VARIAN Unity 300 spectrometer, except for the ^{31}P homonuclear decoupling experiments which were obtained with a VARIAN Unity 500 spectrometer. Proton and carbon chemical shifts are reported from internal TMS or 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium (DSS), and phosphorus chemical shifts are from external 85% orthophosphoric acid. The NMR parameters of the second order ^{31}P systems were calculated after fitting to the experimental data by use of VARIAN spin simulation software.

DMF was SureSeal® from Aldrich, and DMF-d_7 and DMSO-d_6 (Cambridge Isotopes) were dried additionally by storing over 3 Å molecular sieves. All moisture sensitive reactions were carried out by the syringe technique under dry nitrogen. The NMR tubes and the reaction flasks were dried at 120 °C overnight under vacuum and cooled under nitrogen.

Tetrasodium carbonyldiphosphonate was prepared by the method of Wang *et al.*[14] N^2-(4-Butylphenyl)-2′-deoxyguanosine 5′-phosphate (BuPdGMP, **1**) was prepared as the TBA salt as described.[4]

N^2-(4-Butylphenyl)-2′-deoxyguanosine 5′-(P^2,P^3-carbonyltriphosphonate) (BuPdGMPPCOP, 8)

Tetrasodium carbonyldiphosphonate (2.5 hydrate, 0.25 mmol, 80.5 mg) was dissolved in 1 mL of water and loaded on a 1.5 × 25 cm column of Dowex 8 × 50 in the pyridinium form at 4 °C. The yellow fraction was eluted with ice-cold water and was immediately coevaporated under vacuum with tributylamine (0.119 mL) at a bath temperature of 20 °C. The residue was dissolved in 1 mL of water and lyophilized for 24 h, then dissolved in 1.5 mL of dry DMF and evaporated at rt and high vacuum.

Triethylammonium N^2-(4-butylphenyl)-2′-deoxyguanosine 5′-phosphate (BuPdGMP, **1**) (0.25 mmol, 135 mg) was dissolved in 2.5 mL of dry DMF, and the solution was concentrated under vacuum at rt to about two thirds of its original volume. Triphenylphosphine (196.5 mg, 0.75 mmol) and 2,2′-dipyridyl disulfide (168 mg, 0.75 mmol) were dissolved in 1 mL of dry DMF under nitrogen; *N*-methylimidazole (0.2 mL, 0.25 mmol) was added, and the solution was added to the solution of **1** in a 50 mL screw cap centrifuge tube under nitrogen. After 8 min the phosphoro-*N*-methylimidazolidate of BuPdGMP (**4**) was precipitated by addition of cold dry diethyl ether (30 mL). After centrifugation and decanting of the solvents it was resuspended in another portion of ether, centrifuged, and the ether decanted. The residue was dried under a gentle steam of nitrogen (until disappearance of the smell of ether, ~10 min) and dissolved in 1 mL of dry DMF. To this solution was added a solution of tetrabutylammonium carbonyldiphosphate (**6**) in 1 mL of dry DMF. After 5 min the ^{31}P NMR spectrum of an aliquot of the reaction mixture showed complete consumption of the phosphoro-*N*-methylimidazolidate **4** and formation of BuPdGMPPCOP (**8**), BuPdGMP (**1**), the anhydride PCOPOPCOP (**11**) and PCOP (**6**), together with some unidentified byproducts. The reaction mixture was concentrated to one third of its original volume under high vacuum at rt and diluted with 10 mL ice-cold 0.1 M TEAB (pH 7.3). BuPdGMPPCOP (**8**) was isolated by reverse phase preparative HPLC: column, Dynamax C18 5 μ, 21 × 250 mm; eluent, 20 mL min^{-1} of 75% 0.1 M TEAB (pH 7.35)–25% MeCN. BuPdGMPPCOP (**8**) and BuPdGMP (**1**) had retention times of 10.3 and 15.8 min, respectively, and the ratio of the two peaks was 28 : 72 (detection at 254 nm). The fractions containing the product were collected on ice and combined to give (by UV) a

yield of 43.7 μmol (17%) of **8**. This solution was immediately evaporated to one fifth of its volume under high vacuum at rt, and the residue was frozen and lyophilized. A portion of this product was converted to the sodium salt by passage through a column of Dowex 8 × 50 (0.5 × 2.0 cm) in the sodium form at 4 °C, elution with water, and lyophilization. The ^{1}H NMR spectrum in D_2O was identical to that of BuPdGMP (**1**). ^{31}P NMR (in 20 mM EDTA in D_2O, pH 8): −1.49 (dd, P^3, J_{P2P3} = 207.5 Hz, J_{P1P3} = 7.0 Hz), −9.95 (dd, P^2, $^3J_{P1P2}$ = 29.7 Hz), −10.14 (dd, P^1; ^{1}H coupled, multiplet). FAB MS (TEA salt) 753.3 $[M + TEA + H]^+$.

N^2-(4-Butylphenyl)-2′-deoxyguanosine 5′-carbonyldiphosphonate (BuPdGMPCOP, 15)

Tetrasodium carbonyldiphosphonate (161 mg, 0.5 mmol) was dissolved in ice-cold water (2 mL), and the solution was passed through a column of Dowex 8 × 50, H^+ form, and eluted with ice-cold water until the eluate became neutral. The eluate was immediately adjusted to pH 6.15 with aqueous 40% tetrabutylammonium hydroxide (*ca*. 1 mL) and lyophilized. The yellow residue was dissolved in dry MeCN (1 mL), and N^2-(4-butylphenyl)-5′-mesyl-2′-deoxyguanosine (**13**,[12] 48 mg, 0.1 mmol) was added. The reaction mixture was concentrated on a rotary evaporator (rt, 20 Torr) until it became a thick oil. After storage at rt for 24 h there was almost no starting material left (TLC). The reaction mixture was diluted with 0.1 M TEAB and chromatographed on a DEAE-Sephadex column (2 × 20 cm) with a linear gradient of 0.1–1.0 M TEAB during 16 h at a flow rate of 2.67 mL min^{-1}. The yellow fractions (fractions 136–159, 16 mL each) were combined and coevaporated with *n*-BuOH, redissolved in water, and lyophilized to give 81.8 mg of **15** as a yellow solid [93% as tris(TEA) salt]. For spectral characterization a portion of this product was converted to the sodium salt by passing through a column of Chelex in the Na^+ form. ^{1}H NMR (D_2O): 7.94 (s, 1H, 8-H), 7.37, 7.11 (m, 2H each, Ph), 6.25 (t, 1H, H-1′; $J_{1'2'} = J_{1'2''}$ = 6.9 Hz), 4.62 (m, 1H, 3′-H), 4.06 (m, 1H, 4′-H), 4.03 (m, 1H, 5′-H), 3.96 (m, 1H, 5″-H), 2.63 (m, 1H, 2′-H, $J_{2'3'}$ = 7.1 Hz), 2.46 (t, 2H, Bu-1, *J* = 7.5 Hz), 2.35 (m, 1H, 2″-H, $J_{2'3'}$ = 4.1 Hz, $J_{2'2''}$ = 14.0 Hz), 1.44 (m, 2H, Bu-2), 1.18 (m, 2H, Bu-3), 0.76 (t, 3H, CH_3, *J* = 7.4 Hz). ^{31}P NMR (D_2O): 1.33 (d, P^1, J_{P1P2} = 193 Hz; ^{1}H dec. off, dt, J_{PH} = 4.2 Hz), −0.62 (d, P^2; $^3J_{P1P2}$ = 193 Hz, ^{1}H dec. off, dt, $^5J_{PH}$ = 1.9 Hz). ^{13}C NMR (D_2O): 237.5 (dd, P*C*OP, J_{PC} = 117.5 and 125.7 Hz), 161.4 (C-1), 152.1 (C-4), 151.0 (C-2), 138.1 (Ph-4), 137.2 (Ph-1), 136.4 (C-8), 128.7 (Ph-3,5), 120.2 (Ph-2,6), 117.3 (C-5), 85.8 (dd, C-4′, $^3J_{CP1}$ = 5.7, $^5J_{CP2}$ = 1.9 Hz), 83.1 (C-1′), 70.9 (C-3′), 65.1 (br s, C-5′, $^2J_{CP1}$ = 5 Hz, estimated from the linewidth), 39.0 (C-2′), 34.4 (Bu-1), 33.3 (Bu-2), 22.0 (Bu-3), 13.6 (Bu-4). FAB MS (of TEA salt) $[M + H]^+$ = 572, $[M + TEA + H]^+$ = 673, $[M + 2TEA + H]^+$ = 774.

Enzyme assays

Materials. dNTPs were from New England Biolabs, [^{3}H]dTTP was from New England Nuclear, and calf thymus DNA was from Worthington. Other reagents were from Sigma. Activated DNA was prepared as described.[15] Recombinant human DNA polymerase alpha, expressed as a single polypeptide, was the generous gift of Dr Teresa S.-F. Wang, Stanford University.

Assays. Assays of DNA polymerase activity were done in 25 μL volumes containing 20 mM Tris-Cl (pH 8.0) or 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[ethane-2-sulfonic acid] (HEPES; pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 0.1 mg mL^{-1} bovine serum albumin (BSA), 0.4 mg mL^{-1} activated calf thymus DNA, 25 μM dATP and dCTP and 10 μM [^{3}H]dTTP (1250 cpm pmol^{-1}). Reactions were initiated by the addition of enzyme (final concentration 5–10 nM) and incubated for 10 min at 30 °C. Reaction mixtures were quenched by addition of 1 mL of 10% trichloroacetic acid–100 mM sodium pyrophosphate, and acid-insoluble product was collected on Whatman GF/A filters, washed three times with 0.1 M HCl–100 mM sodium pyrophosphate, and counted in 1 mL Optifluor.

Inhibitor assays. Compounds were tested in triplicate by the addition of ten-fold serial dilutions of inhibitors stored in 50 mM Tris-Cl or 50 mM HEPES (pH 7.5) to assay mixtures. For determinations of apparent K_i values, five concentrations of inhibitor were used in the absence of dGTP ("truncated" assay conditions[3b]). For competition experiments, inhibitor assays were done at five concentrations of dGTP, with dATP, dCTP and [^{3}H]dTTP concentrations fixed at 50 μM; results were plotted (SigmaPlot) as 1/pmol dGMP incorporated *vs*. 1/[dGTP]. Regression lines were obtained using the Macintosh (HyperCard) program Enzyme Kinetics (D. G. Gilbert, Indiana University).

Acknowledgements

This work was supported by grant GM 21747 from the National Institutes of Health.

References

1 For reviews see: (*a*) F. Eckstein and J. B. Thomson, *Methods Enzymol.*, 1995, **262**, 189 and references cited therein; (*b*) N. C. Brown and G. E. Wright, *Methods Enzymol.*, 1995, **262**, 202; (*c*) K. H. Scheit, *Nucleotide Analogues*, Wiley, New York, 1980; (*d*) G. M. Blackburn, M.-J. Guo, A. G. McLennan, in *Ap$_4$A and Other Dinucleoside Polyphosphates*, ed. A. G. McLennan, CRC Press, Boca Raton, 1992, pp. 305–342; (*e*) R. Engel, *Chem. Rev.*, 1977, **77**, 349; (*f*) R. G. Yount, *Adv. Enzymol.*, 1975, **43**, 1.
2 (*a*) Z.-Y. Peng, J. M. Mansour, F. Araujo, J.-Y. Ju, C. E. McKenna and T. E. Mansour, *Biochem. Pharmacol.*, 1995, **49**, 105; (*b*) N. Laing, W. W. C. Chan, D. W. Hutchinson and B. Öberg, *FEBS Lett.*, 1990, **260**, 206; (*c*) I. N. Smirnova, N. A. Kudryavtseva, S. V. Komissarenko, N. B. Tarusova and A. A. Baikov, *Arch. Biochem. Biophys.*, 1988, **267**, 280.
3 (*a*) G. E. Wright, U. Hübscher, N. Khan, F. Focher and A. Verri, *FEBS Lett.*, 1994, **341**, 128; (*b*) N. N. Khan, L. J. Reha-Krantz and G. E. Wright, *Nucleic Acids Res.*, 1994, **22**, 232; (*c*) C. E. McKenna, J. N. Levy, L. A. Khawli, V. Harutunian, T. G. Ye, M. C. Starnes, A. Bapat and Y. C. Cheng, *ACS Symp. Ser.*, 1989, **401**, 1; (*d*) T. A. Rozovskaya, N. B. Tarusova, Sh. K. Minasyan, A. M. Atrazhev, M. K. Kukhanova, A. A. Kraevskii, Z. G. Chidgeavadze and R. S. Beabealashvilli, *FEBS Lett.*, 1989, **247**, 289; (*e*) P. A. Cload and D. W. Hutchinson, *Nucleic Acids Res.*, 1983, **11**, 5621; (*f*) B. Eriksson, G. Stening and B. Öberg, *Antiviral Res.*, 1982, **2**, 81; (*g*) B. Eriksson, B. Öberg and B. Wahren, *Biochim. Biophys. Acta*, 1982, **696**, 115; (*h*) B. Eriksson, A. Larsson, E. Helgstrand, N. G. Johansson and B. Öberg, *Biochim. Biophys. Acta,*, 1980, **607**, 53; (*i*) R. V. Talanian, N. C. Brown, C. E. McKenna, T. G. Ye, J. N. Levy and G. E. Wright, *Biochemistry*, 1989, **28**, 8270.
4 G. E. Wright and L. W. Dudycz, *J. Med. Chem.*, 1984, **27**, 175.
5 D. E. Hoard and D. G. Ott, *J. Am. Chem. Soc.*, 1965, **87**, 1785.
6 J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 1961, **83**, 649.
7 Y. Yanachkov, J. Y. Pan, M. Wessling-Resnick and G. E. Wright, *Mol. Pharmacol.*, 1997, **51**, 47.
8 V. F. Zaritova, T. S. Godovicova, I. V. Kutiavin, L. M. Khalimskaya, in *Biophosphates and Their Analogues—Synthesis, Structure, Metabolism and Activity*, ed. K. S. Bruzik and W. J. Stec, Elsevier, Amsterdam, 1987, pp. 149–164.
9 D. G. Knorre, V. A. Kurbatov and V. V. Samukov, *FEBS Lett.*, 1976, **70**, 105.
10 T. C. Myers, K. Nakamura and A. B. Danielzadeh, *J. Org. Chem.*, 1965, **30**, 1517.
11 (*a*) V. M. Dixit and C. D. Poulter, *Tetrahedron Lett.*, 1984, **25**, 4055; (*b*) V. J. Davisson, D. R. Davis, V. M. Dixit and C. D. Poulter, *J. Org. Chem.*, 1987, **52**, 1794.
12 I. Yanachkov and G. E. Wright, *Nucleosides Nucleotides*, 1994, **13**, 339.
13 W. C. Copeland and T. S.-F. Wang, *J. Biol. Chem.*, 1991, **266**, 22739.
14 T. S. T. Wang, R. A. Fawwaz, L. J. Johnson, G. E. Mojdehi and P. M. Johnson, *J. Nucl. Med.*, 1980, **21**, 767.
15 N. N. Khan, G. E. Wright, L. W. Dudycz and N. C. Brown, *Nucleic Acids Res.*, 1984, **12**, 3695.