ORGANIC LETTERS

2009
Vol. 11, No. 9
1883−1886

α,β-Difluoromethylene Deoxynucleoside 5′-Triphosphates: A Convenient Synthesis of Useful Probes for DNA Polymerase β Structure and Function

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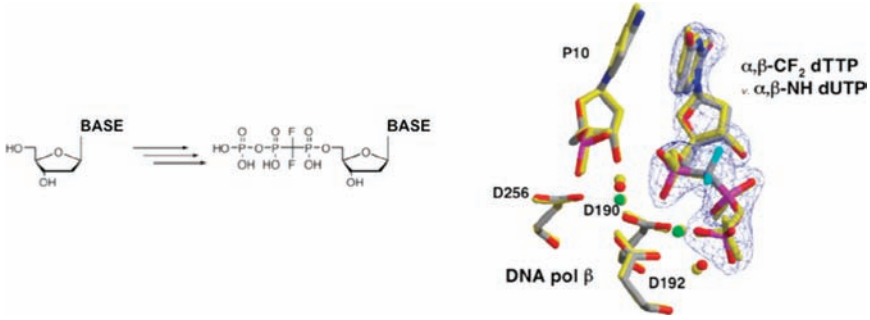
Received February 5, 2009

ABSTRACT



α,β-Difluoromethylene deoxynucleoside 5′-triphosphates (dNTPs, N = A or C) are advantageously obtained via phosphorylation of corresponding dNDP analogues using catalytic ATP, PEP, nucleoside diphosphate kinase, and pyruvate kinase. DNA pol β K_d values for the α,β-CF_2 and unmodified dNTPs, α,β-NH dUTP, and the α,β-CH_2 analogues of dATP and dGTP are discussed in relation to the conformations of α,β-CF_2 dTTP versus α,β-NH dUTP bound into the enzyme active site.

In an ongoing multidisciplinary study of structure and function of DNA polymerase β, a eukaryotic enzyme primarily involved in filling short DNA gaps,[1] we required a series of α,β-methylene-substituted analogues of deoxynucleoside 5′-triphosphates (dNTPs). When the $P_α$-O-$P_β$ bridging oxygen in a natural mononucleotide substrate is replaced by an imido (NH)[2−4] or methylene (CXY)[4,5] group, the P-N or P-C bond should resist cleavage in the nucleotidyl transfer reaction catalyzed by the enzyme. As a result, these analogues will remain intact in stable ternary DNA complexes with the polymerase and therefore should be useful to probe pre-chemistry enzyme-complex function and structure, as recently shown with in an X-ray crystallographic study of α,β-NH dUTP with DNA pol β.[6] Information about such complexes provides a reference point for theoretical analysis of the chemical mechanism[7] for the complete transfer of a monophosphate nucleoside donor to the sugar acceptor in the active site. As probes for the mechanism of polymerase catalysis and its relationship to polymerase fidelity, α,β-methylene dNTP analogues permit exploration of stereoelectronic effects on active site interac-

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10.1021/ol701755k CCC: $40.75
Published on Web 04/07/2009

tions, by making appropriate substitutions X,Y on the adjacent P_α CXY bridging carbon. The largest obtainable electron-withdrawing effect with minimal steric perturbation can be achieved using X,Y = F, resulting in analogues in which the bisphosphonate group is expected to be less basic than the pyrophosphate moiety in the natural dNTPs.[8,9]

In this Letter, we describe the first synthesis of α,β-CF_2 dCTP **6**, using a modified chemical-enzymological approach that also can be applied to synthesis of α,β-CF_2 dATP **3**, affording these compounds in sufficient purity to virtually eliminate detectable contaminating substrate activity in polymerase inhibition kinetics assays. DNA pol β K_d values were determined for these analogues and compared to K_d values for α,β-CF_2 dTTP **9**, the natural substrates dATP, dCTP and dTTP, α,β-NH dUTP **10**, and the dATP and dGTP α,β-CH_2 analogues (**11**, **12**). We also describe the ternary complex of **9** bound with template-primer DNA into the active site of DNA polymerase β, as determined by X-ray crystallography, and discuss its structural implications.

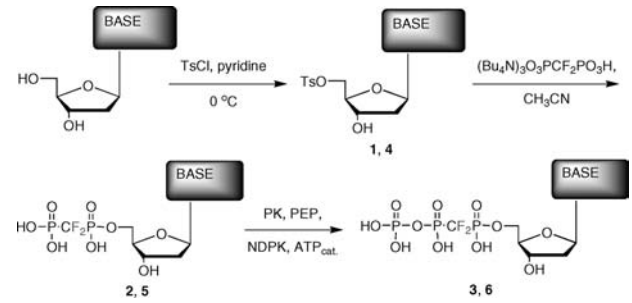
An obvious route to α,β-methylene-dNTP analogues entails coupling a particular methylenebis(phosphonate) derivative to the target nucleoside, followed by phosphorylation[10] to add the terminal γ-phosphate. Several α,β-CXY NDPs (X,Y = H or F) were previously synthesized via tosylation of the 5′-OH in the protected ribonucleoside with tosyl chloride and (dimethylamino)pyridine, followed by deprotection and displacement of the tosyl moiety with the appropriate tris(tetrabutylammonium)[11] bisphosphonate. A similar approach was used to prepare an α,β-CF_2 dGDP (N^2-(*p*-*N*-*n*-butylphenyl) derivative),[12] dATP and dTTP,[13] and related nucleotide analogues.[14] In a synthesis of AZT 5′-carbonylbis(phosphonate), trimethyl (diazomethylene)bis(phosphonate) was coupled to AZT under Mitsunobu conditions, but subsequent deprotection (demethylation) at phosphorus was required.[15] Direct 5′-coupling of 2−6 equiv of methylenebis(phosphonic dichloride) with unprotected nucleosides also has been demonstrated.[16]

Chemical phosphorylation of α,β-CH_2 dADP and dTDP has been carried out via the *p*-nitrobenzyl-phosphoromorpholidates.[13] Unfortunately, subsequent deprotection by hydrogenolysis of the *p*-nitrobenzyl group restricts the pyrimidine substrate, because the cytosine ring of dC is prone to reduction under these conditions.[17] Side products from the phosphorylation step typically contaminate the α,β-methylene dNTP analogue product. Carbonyldiimidazole (CDI) activation[18] of the dGDP derivative referred to above has been utilized as an alternative route.[12] We found phosphorylation of our dNDP substrates using CDI to be problematic because of the reactivity of the imidazolate, which tended to give unwanted nucleoside phosphorylation at the 3′-OH and other side products.

As an alternative to syntheses of α,β-imido dNTPs using CDI to activate the NDP analogue for coupling with tributylammonium phosphate,[3,19] Kenyon proposed enzymatic phosphorylation by either creatine kinase (CK)[2,19] or pyruvate kinase (PK) and phosphoenolpyruvate (PEP).[19] However, inadequate CK and PK activity in attempted PEP phosphorylation of our dNDP intermediates led us to consider adaptation of a method for phosphorylation of azole carboxamide riboxynucleoside 5′-diphosphates (NDPs) by ∼ stoichiometric ATP and nucleoside diphosphate kinase (NDPK)[20a] previously applied to synthesis of **11**,[20b] in which PEP and PK are included to recycle the ADP produced in the phosphoryl transfer and drive the reaction to completion.

Synthesis of α,β-dNTPs. We first converted dA or N^4-benzoyl-dC **7** to the corresponding 5′-tosylates **1** or **4**, respectively (see Scheme 1), by reaction with tosyl chloride in pyridine (75−80%).[13]

Scheme 1. Synthesis of α,β-Methylene dNTP Analogues[a]



[a] **1**, **2**, and **3**: base = adenine. **4**: base = N^4-Bz-cytosine. **5** and **6**: base = cytosine. Note: before tosylation, dC was converted to N^4-Bz-deoxycytidine **7** with benzoic anhydride, DIEA in pyridine, microwave irradiation (2 min, 300 W). Prior to the enzymatic phosphorylation, the N^4-protected dCDP analogue **8** obtained directly from **4** was debenzoylated to **5** using methanolic ammonia.

The NH_2 group of dC was protected via facile microwave-induced reaction with benzoic anhydride and diisopropylethylamine (DIEA) in pyridine,[21] to give **7** (76%). When purifying **4** from the reaction mixture, we initially used conventional extraction with aq $NaHCO_3$ (pH ∼8.3) but obtained low isolated yields (∼20%). The pK_a of the N^4-proton in **4** was estimated (ACDLabs pKaDB 8.01 software program) to be ∼8. Accordingly, we changed to a slightly acidic aqueous workup (citric acid buffer, pH ∼4.5), which increased the isolated yield to ∼70%.

The purified tosylates were converted to the dNDP α,β-difluoromethylene analogues **2** or **5** via condensation with the tris(tetrabutylammonium) salt[13] of difluoromethylenebis(phosphonic acid) (DFBP). The N^4-benzoyl dCDP analogue **8** obtained as the direct tosylation product from **4** was then deprotected in methanolic ammonia to give **5** quantitatively, which was purified by preparative ion-exchange HPLC. DFBP was obtained by

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bromotrimethylsilane dealkylation[22] of tetra-isopropyl (difluoro)-methylenebis(phosphonate),[8] synthesized by fluorination of the carbanion of tetra-isopropyl methylenebis(phosphonate).[7,23]

Phosphorylation to the dNTP analogues **3** or **6** was cleanly achieved using NDPK and a *catalytic* amount of ATP, regenerated with 2.5 equiv of PEP with PK in 50 mM HEPES buffer (75−90% by HPLC and ^{31}P NMR). Importantly, the latter modification renders unnecessary the use of an affinity column[20] to purify the product from excess ATP.

Purification of α,β-dNTPs. To obtain product free of detectable nucleotide contaminants, we found that separation on DEAE Sephadex or Dowex[3,5,9,12−14,24−30] or single-pass preparative HPLC using a C-18 or ion-exchange[3,5,9,12−14,24−31] column was not sufficient. However, dual-HPLC (ion exchange, then C-18) provided highly purified products based on analytical HPLC and ^{1}H, ^{31}P, and ^{19}F NMR analysis (Supporting Information).

This overall synthesis/purification route has the advantage of being applicable to both purine and pyrimidine examples, including the previously unavailable α,β-CF_2 dCTP analogue **6**. The reactions are relatively clean (particularly compared to standard CDI phosphorylation), do not require protection/deprotection chemistry in the phosphate moieties, eliminate the problem of excess ATP in the enzymatic phosphorylation, and after the dual-HPLC purification provide exceptionally pure analogues suitable for polymerase inhibition studies (see below). The dual-HPLC purification protocol was also effective in reducing impurities in α,β-CF_2 dTTP **9** prepared by the *p*-nitrobenzyl-phosphomorpholidate method (see below).

DNA Synthesis Assays. DNA synthesis was assayed on four single-nucleotide gapped DNA substrates where the templating base in the gap was complementary to the incoming dNTP. The DNA sequence was as described previously[32] with the core sequence identical to that used for crystallization.

Initial DNA polymerase inhibition studies on α,β-CF_2 dCTP, **6** at 1 mM showed no detectable background DNA synthesis with 0.5 nM enzyme, and only a trace with 50 nM enzyme, representing <0.01% of the inhibitor (Figure 1A). Comparable absence of background synthesis was evident in the assay using α,β-CF_2 dATP, **3** (Figure 1B). For comparison, with a preparation of inhibitor **9** obtained via the *p*-nitrobenzyl phosphoromorpholidate method, at 50 nM pol β most of the primer strand in the assay has been extended, with some incorporation still detectable using the lower concentration of enzyme (Figure 1B). After dual-HPLC purification, this artifact was no longer observable with 0.5 nM enzyme, although incorporation at 50 nM enzyme was more evident than for **6** and **3** (Supporting Information).

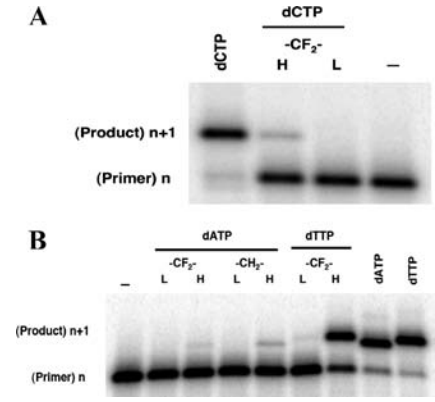

Figure 1. Gapped DNA synthesis assay with DNA pol β for dNTPs (dCTP, dATP, dTTP) and α,β-CXY analogues. Primer (*n*) extension was assayed in the presence of low (L, 0.5 nM) or high (H, 50 nM) pol β and 1 mM analogue for 5 or 10 min, respectively. (A) dCTP versus **6**. (B) **3** versus **11**, **9**, dATP, and dTTP. The mobility of the extension product (*n* + 1) with dCTP, dATP, or dTTP serves as a reference, where most of the primer (200 nM) is extended after a 21 min incubation with 50 nM enzyme and 100 μM dNTP.

Inhibition of natural nucleotide insertion by the different α,β-modified dNTP analogues was investigated at various concentrations of inhibitor (*I*) and subsaturating dNTP substrate (*S*). Steady-state kinetic parameters (k_m, K_m) were determined by fitting the rate data to the Michaelis equation, and the inhibitor constant K_i (=K_d) was determined by fitting the inhibition data to the equation for competitive inhibition (Supporting Information). Both fits used nonlinear regression methods. Equilibrium dissociation constants (K_d) for the natural dNTPs were taken from previously published data [dATP;[33] dCTP;[34] dTTP[35]]. The K_i's for **10**, **11**, and **12** were reported previously.[6,36]

The CF_2-analogues readily inhibit pol β single-nucleotide insertion (Figure 2). However, their binding affinities are at least

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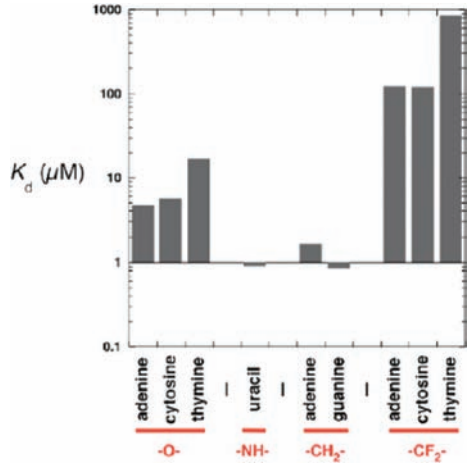

Figure 2. DNA pol β dissociation constants (K_d) for three natural dNTPs (dATP, dCTP, dTTP), for α,β-NH dUTP (**10**), the α,β-CH_2 analogues of dATP and dGTP (**11**, **12**), and the α,β-CF_2 analogues of dATP, dCTP, and dTTP (**3**, **6**, **9**). K_d values determined by single-turnover analysis.

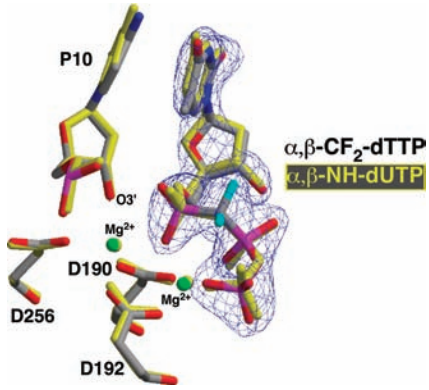

Figure 3. Superposition of the active site of the ternary substrate complex of pol β with incoming **9**, α,β-CF_2-dTTP (gray carbons), and the corresponding ternary complex with **10**, α,β-NH-dUTP (yellow atoms; pdb ID 2FMS). Active site carboxylates (D190, D192, D256) that coordinate two magnesium ions are shown, as well as the 3′-OH of the primer terminus (P10). The fluorine atoms of **9** are cyan. Electron density for the 2Fo-Fc simulated annealing map (blue) is contoured to 5σ illustrating the high quality of the electron density.

an order of magnitude lower than those of the natural nucleotides. Interestingly, the -NH- and -CH_2- analogue exhibited the tightest binding (~10-fold tighter than natural dNTPs), despite polarity differences between the α,β-NH and α,β-CH_2 groups. This pattern contrasts with the results obtained by O'Hagan for NADH-dependent glycerol 3-phosphate dehydrogenase with -CH_2- and -CF_2- phosphonate analogues of *sn*-glycerol-3-phosphate,[37] which respectively had the same and 3.5-fold greater K_m values than the natural substate. In a study by Berkowitz on substrate mimics for glucose 6-phosphate dehydrogenase, the K_m of the α-CF_2 analogue was an order of magnitude greater than the K_m of glucose 6-phosphate, but that of the α-CH_2 derivative was also higher (4-fold).[38]

Crystallization and Analysis of α,β-Modified dNTP Analogues Complexed with DNA Primer and DNA pol β. The crystal structure of the ternary complex of pol β with the incoming analogue **9** opposite dA represents the precatalytic state of the nucleotidyl transfer reaction for correct incorporation, containing all atoms required for catalysis including the two catalytic metal ions. As expected, the substitution of the CF_2 for the bridging oxygen prevented dissociation of the pyrophosphate leaving group, trapping the complex. All correlated atoms in the active site of this structure superimpose well with previously determined ternary complex structures of pol β where the reaction was trapped by deletion of the nucleophilic 3′-OH on the primer terminus[39] or by using a nitrogen in place of the α,β bridging oxygen[1,4,6] (Figure 3).

In summary, we have synthesized a series of α,β-CF_2 dNTP analogues. Two of these, **3** and **6**, were synthesized by an advantageous chemical-enzymatic method that was used to obtain the previously unavailable dCTP analogue, **6**.[40] The isolation method provides the inhibitors essentially free of contaminating "dNTP" activity. K_d values for these and comparable analogues, combined with an X-ray crystallographic study of **9** bound into a pol β ternary complex, indicate that CXY modification in the P_α-O-P_β bridging position of dNTPs where X,Y = F or H can modulate binding properties over a wide range of K_d while substantially retaining a dNTP-like conformation.

Acknowledgment. We thank Dr. Ron New (Mass Spectrometry Facility, University of California, Riverside) for assistance in obtaining high resolution mass spectra of nucleotide analogues, Ms. Keriann Oertell (University of Southern California) for verifying the DNA synthesis background activity in **9** after dual-HPLC purification, and Prof. George L. Kenyon (University of Michigan) for helpful discussions. This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences and by NIH program project grant 5-U19-CA105010.

Supporting Information Available: Detailed synthetic procedures, compound characterization data including NMR and HRMS spectra, details of enzyme studies, crystallographic methods and statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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