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α,β -Difluoromethylene deoxynucleoside 5'-triphosphates: a convenient synthesis of useful probes for DNA polymerase β structure and function

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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 (NDPK) and pyruvate kinase (PK). DNA pol β K_d values for the α,β -CF₂ and unmodified dNTPs, α,β -NH dUTP, and the α,β -CH₂ analogues of dATP and dGTP are discussed in relation to the conformations of α,β -CF₂ dTTP v. 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 gaps¹, we required a series of α,β -methylene-substituted analogues of deoxynucleoside 5'-triphosphates (dNTPs). When the

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Supporting Information Available General methods used; detailed synthetic procedures and characterization data for **1** – **9**; analytical and preparative HPLC methods; HPLC studies of conversions of **1** to **2**, **2** to **3**, **7** to **4**, **4** to **5**, **5** to **6**; ¹H NMR spectra of **1**–**7** and **9**; ³¹P and ¹⁹F NMR spectra of **2**, **3**, **5**, **6**, **9**; ¹³C NMR spectrum of **9**; analytical HPLC traces for **3**, **6** and **9**; HRMS spectra for **3** and **6**, and LRMS spectrum for **9**; DNA synthesis gel analysis of **9** before and after HPLC purification; protocol and inhibition plot for inhibition of pol β dCTP incorporation by **6**; crystallographic methods and statistics for the ternary pol β complex with **9**; and supplementary literature references are available online at <http://pubs.acs.org>.

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 mechanism⁷ 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 interactions, 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 article, we describe the first synthesis of α,β -CF₂ dCTP **6**, using a modified chemical-enzymological approach that also can be applied to synthesis of α,β -CF₂ 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 -CF₂ dTTP **9**, the natural substrates dATP, dCTP and dTTP, dUTP, α,β -NH dUTP **10**, and the dATP and dGTP α,β -CH₂ 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 phosphorylation¹⁰ 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 a α,β -CF₂ dGDP (N²-(*p*-N-n-butylphenyl) derivative)¹², dATP and dTTP,¹³ and related nucleotide analogues.¹⁴ In an 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 two to six equivalents of methylenebis(phosphonic dichloride) with unprotected nucleosides also has been demonstrated.¹⁶

Chemical phosphorylation of α,β -CH₂ 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) activation¹⁸ of the dGDP derivative referred to above has been utilized as an alternative route¹². We found phosphorylation of our dNDP substrates using CDI to be problematic due to 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 **1120b**, 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 N⁴-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

The NH₂-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. NaHCO₃ (pH ~ 8.7), but obtained low isolated yields (~20%). The pK_a of the N⁴-proton in **4** was estimated (ACDLabs pKaDB 8.01 software program) to be ~8. Accordingly, we changed to a slightly acidic aqueous work-up (citric acid, 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) salt¹³ of difluoromethylenebis(phosphonic acid) (DFBP). The N⁴-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 bromotrimethylsilane (BTMS) dealkylation²² of tetraisopropyl (difluoro)methylenebis(phosphonate) (TiPDFBP)⁸, synthesized by fluorination of the carbanion of tetraisopropylmethylenebisphosphonate.^{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 eq. of PEP with PK in 50 mM HEPES buffer (75–90% by HPLC and ³¹P-NMR). Importantly, the latter modification renders unnecessary use of an affinity column²⁰ to purify the product from excess ATP.

Purification of α,β -dNTPs

In order to obtain product completely free of biochemically 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 ¹H, ³¹P and ¹⁹F NMR analysis.

This overall synthesis/purification route has the advantage of being applicable to both purine and pyrimidine examples, including the previously unavailable α,β -CF₂ 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 -CF₂ 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 previously³² with the core sequence identical to that used for crystallization. Enzyme activities were determined using a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 200 nM single-nucleotide gapped DNA, and various dNTP concentrations.

Reactions were initiated with 0.5 nM enzyme at room temperature and stopped with EDTA mixed with formamide dye. For steady-state reactions, incubation times were chosen to insure that less than 20% of the primer was extended. The radiolabeled 15-mer primer substrate and 16-mer product were resolved by denaturing polyacrylamide gel electrophoresis [$31 \times 38.5 \times 0.04$ cm, 15% (v/v) polyacrylamide (19:1 acrylamide:bis-acrylamide), 8 M urea gels run at 70–80 W, 1 h 20 min - 1 h 40 min] and quantified in the dried gels by phosphorimager.

Initial DNA polymerase inhibition studies on α,β -CF₂ 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% incorporable contaminant (Figure 1A). Comparable absence of background synthesis was evident in the assay using α,β -CF₂ 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** (supplemental information).

$$k_{\text{obs}} = \frac{(k_{\text{cat}} \times S)}{(S + K_m (1 + \frac{1}{K_i}))}$$
Equation 1

Steady-state kinetic parameters (k_{cat} , K_m) were determined by fitting the rate data to the Michaelis equation (Equation 1). Inhibition of dNTP insertion by various α,β modified dNTPs was determined at various concentrations of inhibitor (I) and sub-saturating dNTP concentration (S). The inhibitor equilibrium binding constant, K_i , was determined by fitting the inhibition data to Equation 1 for competitive inhibition by nonlinear regression methods. Equilibrium binding constants for the natural incoming dNTPs were taken from previously published data [dATP³³; dCTP³⁴; dTTP³⁵]. The K_i s for **10**, **11** and **12** were reported previously^{6,36}.

Inhibition of natural (O α,β) nucleotide insertion by the respective analogues was determined at various concentrations of the analog and sub-saturating concentrations of the natural nucleotide. The inhibition constant, K_i ($=K_d$, dissociation constant), was determined by Dixon analysis. The CF₂-analogues readily inhibit pol β single-nucleotide insertion (Figure 2). However, their binding affinities are at least an order of magnitude lower than those of the natural nucleotides. Interestingly, the -NH- and -CH₂- analog exhibited the tightest binding (~10-fold tighter than natural dNTPs), despite polarity differences between the α,β -NH and α,β -CH₂ groups. This pattern contrasts with the results obtained by O'Hagan for NADH-dependent glycerol 3-phosphate dehydrogenase with -CH₂- and -CF₂- phosphonate analogues of *sn*-glycerol-3-phosphate,³⁷ which respectively had the same and 3.5-fold greater K_m values than the natural substrate. In a study by Berkowitz on substrate mimics for glucose 6-phosphate dehydrogenase, the K_m of the α -CF₂ analogue was an order of magnitude greater than the K_m of glucose 6-phosphate, but that of the α -CH₂ 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 analog **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 CF₂ 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 terminus³⁹ 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 -CF₂ 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 analog, **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 indicates that CXY modification in the P α -O-P β bridging position of dNTPs where X,Y = F,F or H,H can modulate binding properties over a wide range of K_d while substantially retaining a dNTP-like configuration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Figure 1.

Gapped DNA synthesis assay with DNA pol β for dNTPs (dCTP, dATP, dTTP) and α,β -CXY analogues. The purity of the analogues was assessed from their failure to be inserted into a gapped DNA substrate. 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 vs. **6**; **B:** **3** vs. **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 μ M dNTP.

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 -CH₂ analogues of dATP and dGTP (**11**, **12**), and the α,β -CF₂ 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-CF₂-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.

Scheme 1. Synthesis of α,β -methylene dNTP analogues

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.]