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Two Scaffolds from Two Flips: (α,β)/(β,γ) CH_2/NH "Met-Im" Analogues of dTTP

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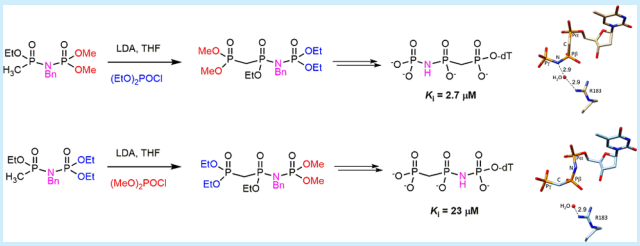
Supporting Information

ABSTRACT: Novel α,β-CH_2 and β,γ-NH (**1a**) or α,β-NH and β,γ-CH_2 (**1b**) "Met-Im" dTTPs were synthesized via monodemethylation of triethyl-dimethyl phosphorimido-bisphosphonate synthons (**4a**, **4b**), formed via a base-induced [1,3]-rearrangement of precursors (**3a**, **3b**) in a reaction with dimethyl or diethyl phosphochloridate. Anomerization during final bromotrimethylsilane (BTMS) deprotection after Mitsunobu conjugation with dT was avoided by microwave conditions. **1a** was 9-fold more potent in inhibiting DNA polymerase β, attributed to an NH-group interaction with R183 in the active site.



DNA polymerase (pol) β fills the short gaps in DNA during base excision repair (BER) of damaged DNA.[1] Pol β is tightly regulated in normal cells, but is often mutated and/or overexpressed in cancer cells.[2] Selective inhibition of pol β to prevent repair of drug-damaged DNA is therefore a promising approach for cancer therapy.[3]

dNTP analogues are critically important tools to study the mechanism and fidelity of DNA processing enzymes such as DNA polymerases and also are a basis for drug design targeting these enzymes in viral or cancer cells. Modifications of natural dNTP substrates in their triphosphate moiety have been recently utilized to probe the fidelity and catalytic mechanism of pol β.[4] Typically, when modification is carried out at the β,γ-bridge position, the resulting analogue is a substrate of pol β with altered enzyme affinity and leaving group properties. By varying the stereoelectronic properties of a substituted β,γ-methylene bridge (Figure 1A), correlations of log k_{pol} with leaving group pK_{a4} were used to identify the rate-determining step (RDS) in the catalysis of nucleotide insertion by the enzyme.[5] Alternatively, if CH_2, CXY, or NH replaces the P_α–O–P_β bridging oxygen, insertion catalyzed by pol β is prevented, and the analogue is an inhibitor, but not a substrate (Figure 1B).[6]

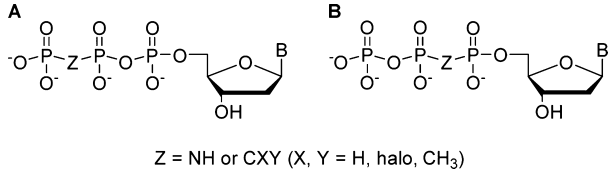


Figure 1. Singly bridge-modified dNTP analogues as (A) substrates and (B) inhibitors of pol β.[5,6]

The bond lengths and angles of the P–N–P moiety more closely resemble those of the natural P–O–P link in comparison with the P–C–P parameters,[7] which are significantly different. Thus, using CH_2 as both the α,β and β,γ linkage may perturb the geometry of the ternary complex with the enzyme.[8] A further concern with a doubled CH_2 linkage is the divergence in polarity of methylene from oxygen.[9] One might consider a double CF_2 linkage to address this problem; however, in an analogue wherein a CF_2 group replaced both oxygen bridges in dTTP, the pol β affinity was drastically decreased.[10] Analysis of crystal structures of the ternary complexes of (α,β)- or (β,γ)-CH_2-dNTP with pol β and DNA[11] suggests that the structural perturbations introduced by a single CH_2 linkage can be accommodated.[6a,b] Substituting both the (α,β)- and (β,γ)-linking oxygens with imido linkages limits modification relative to the more synthetically versatile methylene group. We therefore considered two new scaffolds to explore selective inhibition of pol β, in which the P_α–O–P_β and P_β–O–P_γ oxygens are replaced by either a methylene (CH_2) or an imido (NH) group in alternation: "Met-Im" nucleotides (Figure 2).

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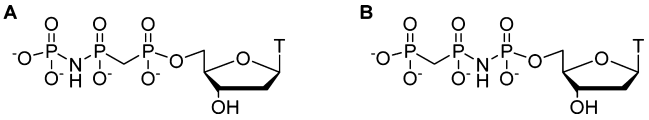
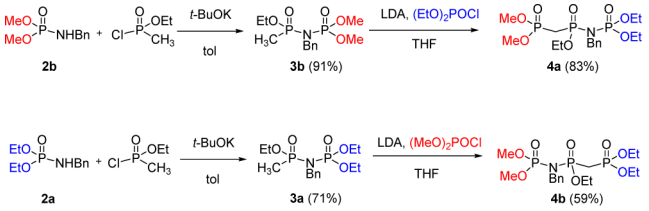


Figure 2. (A) (α,β)-CH_2-(β,γ)-NH-dTTP (**1a**) and (B) (α,β)-NH-(β,γ)-CH_2-dTTP (**1b**) "Met-Im" nucleotides.

Our approach to a general pairwise synthesis of "Met-Im" nucleotides centered on construction of the isomeric pentaalkyl phosphorimido-bisphosphonate synthons **4a** and **4b** (Scheme 1) which have terminal $(EtO)_2P(O)$ or $(MeO)_2P(O)$ groups

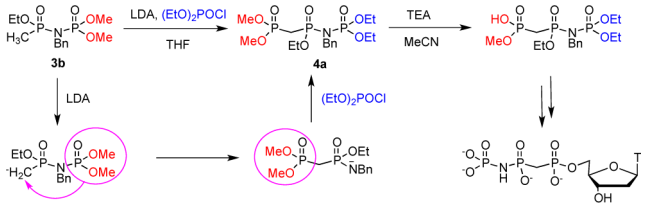
Scheme 1. Flip Synthesis of 4a/b



joined via a NBn or CH_2 bridge to a central EtOP(O). Regioselective removal of one terminal methyl would expose a free P(O)OH, which would become the P_α group in the final nucleotide analogue product (**1a** or **1b**) after coupling to dT and deprotection.

To synthesize **4b**, we initially prepared precursor **3b** from dimethyl benzylphosphoramidate **2b**[12] and ethyl methylphosphonochloridate[13] in the presence of *t*-BuOK (Scheme 1). We envisioned next converting the lithium salt of **3b** into **4b** by reaction with diethyl chlorophosphate. In the event, the LDA-promoted reaction of **3b** proceeded with the formation of the isomeric precursor **4a** (Scheme 1), apparently via an anionic 1,3-rearrangement[14] of the initially formed carbanion. The driving force of this N → C rearrangement[14a−e] is likely formation of the **3b** carbanion, followed by the rapid migration of the dimethoxyphosphoryl group to the anionic carbon and then reaction of the resulting amido anion with diethyl chlorophosphate, yielding the "flipped" product **4a** (Scheme 2).

Scheme 2. [1,3] N → C Migration Mechanism



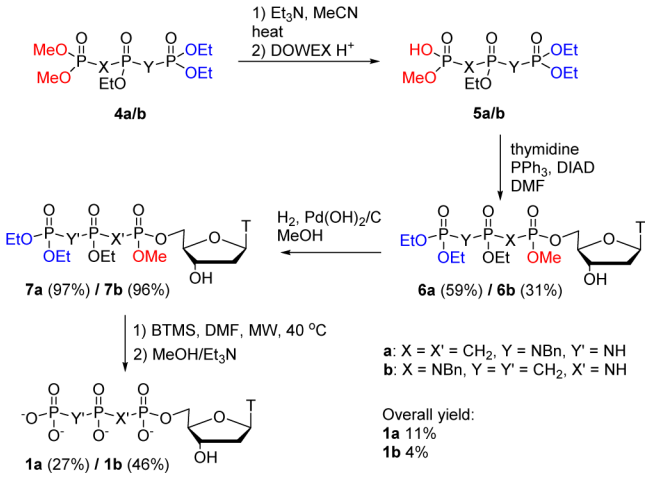
This result suggested that the original intermediate target, **4b**, should be accessible via the same "flip" route simply by swapping the ethyl groups in $(EtO)_2P(O)Cl$ with the methyl groups in **3b**, resulting in **3a** which indeed reacted with $(MeO)_2P(O)Cl$ under similar conditions forming **4b** (Scheme 1).

The isomers **4a** and **4b** were characterized by their ^1H, ^{31}P, and ^{13}C NMR spectra and by MS. The ^{31}P resonance at δ 22.7 ppm is assigned to a $(MeO)_2P(O)CH_2$ in **4a** ($\Delta\delta$ +3 ppm relative to $(EtO)_2P(O)CH_2$ in **4b**), both coupled (J_{PP} = 4 Hz) to the same phosphorus at δ ~23 ppm, assigned to the central P(O)OEt. The most upfield resonance (δ 3 ppm) is then $(EtO)_2P(O)NBn$ in **4a**, with the $(MeO)_2P(O)NBn$ resonance in **4b** again observed 3 ppm downfield (both with J_{PP} = 20 Hz to the central phosphorus nucleus).

The structures of **4a** and **4b** were further established by $^1H-^{31}P$ gHMBC NMR. For **4a** (Figure S14) a strong cross-peak is observed for the protons of two OCH_3 groups (3.84 and 3.79 ppm) and the phosphonate ^{31}P resonance (22.7 ppm), while no cross-peak is observed for the terminal phosphorimide ^{31}P resonance (2.79 ppm). In contrast, the $^1H-^{31}P$ gHMBC NMR of **4b** (Figure S18) reveals a strong cross-peak for the OCH_3 protons (3.70 and 3.54 ppm) and phosphorimide ^{31}P resonance (6 ppm), while no cross-peak is observed for these protons and the terminal phosphonate ^{31}P resonance (19.65 ppm).

The same procedure was utilized for the synthesis of **1a** and **1b** (Scheme 3). After selective removal of a single methyl group

Scheme 3. Synthesis of "Met-Im" Nucleotides 1a/b



from **4a** or **4b** with TEA,[15] the resulting salt was converted by DOWEX H^+ to the corresponding acid (**5a** or **5b**) which was coupled to thymidine under Mitsunobu conditions[16] to form **6a** or **6b**. Debenzylation by catalytic hydrogenolysis then gave **7a** or **7b** (Scheme 3).[5e]

The final deprotection step, simultaneous removal of the remaining methyl and three ethyl groups, was effected by silyldealkylation with BTMS followed by neutral hydrolysis.[17] The major product had the anticipated MS ($[M - H]^-$ = 478), but in a preliminary pol β dTTP incorporation assay exhibited very low (K_i > 1 mM) inhibitory potency. Careful analysis of the NMR (Figure S28) suggested that the major product had undergone extensive anomerization (the undesired α-anomer is usually not the main product when β-ribonucleotides are treated with BTMS;[18] however, anomerization has been observed for some β-deoxyribonucleotides).[19]

Fortunately, we discovered that microwave (MW) acceleration[20] significantly reduced anomerization: treatment of **7a** with microwave irradiation for 30 min at 40 °C followed by hydrolysis gave 80% of the desired β-anomer **1a** (Scheme 4), which was purified using two-stage preparative (strong anion exchange (SAX) followed by C18) HPLC.[5b]

Similarly, deprotection of **7b** was achieved in 7 min at 40 °C, with minor α-anomer formation, and the pure β-product **1b** could again be isolated by preparative HPLC.

Inhibition of pol β by **1a** or **1b** at variable concentrations for a constant concentration of dTTP was determined using a

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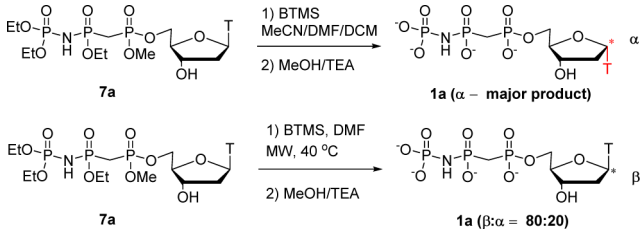
Scheme 4. Microwave Reaction Abated Anomerization of Nucleotides 1 during BTMS Reaction



standard gel assay (Figure 3A). For each inhibitor concentration, an observed rate was determined and then plotted, and

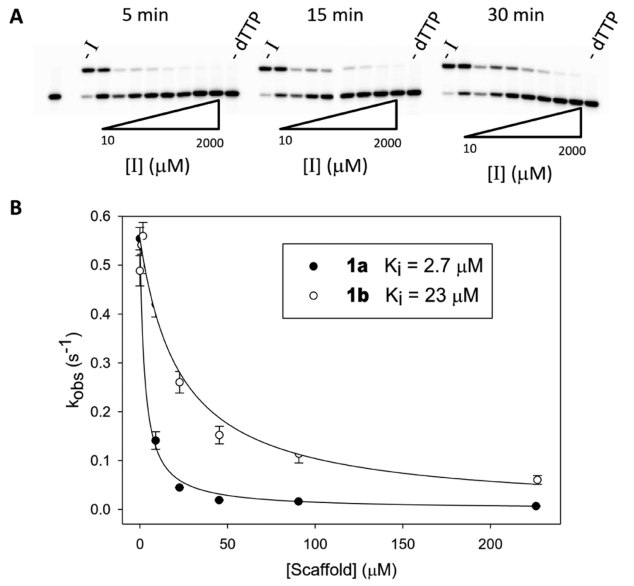


Figure 3. Inhibition of pol β by "Met-Im" dTTP analogues. (A) Representative gels for inhibition by **1a**. Aliquots were quenched at 5, 15, and 30 min, and the DNA was run on denaturing polyacrylamide gels to separate unextended from extended primer. The first lane corresponds to reaction without inhibitor. The final lane in each set is the reaction without dTTP, showing that the inhibitor is not incorporated into DNA by pol β. (B) Hyperbolic decay fits of data from inhibition reactions. Filled circles represent **1a**, and open circles, **1b**. Error bars represent σ for three independent experiments.

the data were fit to a hyperbolic decay curve from which the K_i was calculated (Figure 3; see Supporting Information for details).

The K_i values for the two inhibitors differed by 9-fold (**1a**, 2.7 μM; **1b**, 23 μM).

The X-ray crystal structures of **1a** and **1b** in ternary complexes with pol β and DNA (Table S1) reveal that both bound inhibitors assume conformations similar to those of the natural nucleotide (Figure 4). However, the inhibitor having its NH group at the β,γ-bridging position (**1a**) has a water-mediated H-bonding interaction between its NH and R183 in the active site. This interaction is unavailable for **1b** which has a β,γ-CH_2 at the β,γ-bridge, suggesting that some or all of the observed K_i difference could be attributed to it (the α,β-NH in **1b** has no apparent interactions with proximal active site residues).

In summary, we have described the synthesis of two novel (α,β):(β,γ)-CH_2/NH-dTTP isomers that will permit exploration of active site interactions of DNA polymerases both near

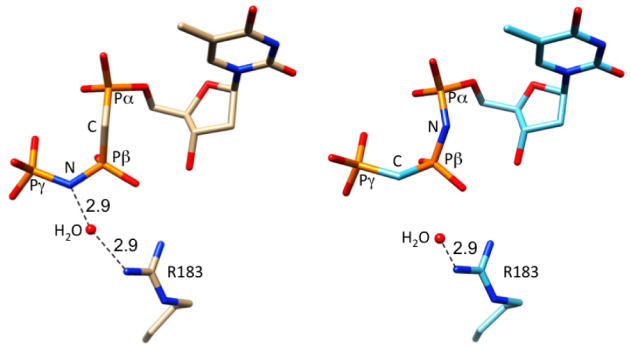


Figure 4. **1a** (left) and **1b** (right) in ternary complex with pol β and DNA. R183 makes a water-mediated H-bond with the β,γ-NH in **1a**. A corresponding interaction between R183 and the α,β-NH of **1b** is not possible.

the β,γ-bridge atom of the enzyme bound nucleotide and in a more interior region of the active site adjacent to the locus of catalysis. Furthermore, they constitute isomeric scaffolds for the future introduction of stereodefined substituents[5e] at either CH_2 position to modulate inhibitor activity.

X-ray crystallographic studies of the inhibitor-enzyme ternary complex (with bound DNA primer and template) reveal that (β,γ)-NH is uniquely capable of a structural water-mediated H-bond interaction with Arg183, which is a promising "anchor" to bind selective inhibitors of DNA polymerase β.[5,6]

The synthetic strategy devised to prepare **1a** and **1b** should be broadly applicable to the synthesis of other "Met-Im" nucleotides by simply coupling the appropriate nucleoside with synthons **4a** and **4b**. Their synthesis takes advantage of a [1,3] N → C P(O)(OR_2) migration, which neatly provides access to both compounds by a simple swap of terminal alkyl ester groups. The observed suppression of unwanted anomerization by microwave acceleration in the BTMS-mediated deprotection of **7a** and **7b** may also be of general utility and further extends the scope of BTMS for preparation of phosphonic acids incorporating sensitive functional groups.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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