4′-Acylated Thymidines: A New Class of DNA Chain Terminators and Photocleavable DNA Building Blocks

Andreas Marx,[†] Maria P. MacWilliams,[‡] Thomas A. Bickle,[‡] Urs Schwitter,[†] and Bernd Giese*,[†]

Department of Chemistry, University of Basel St. Johanns-Ring 19, CH-4056 Basel, Switzerland Department of Microbiology, Biozentrum University of Basel, Klingelbergstrasse 70 CH-4056 Basel, Switzerland

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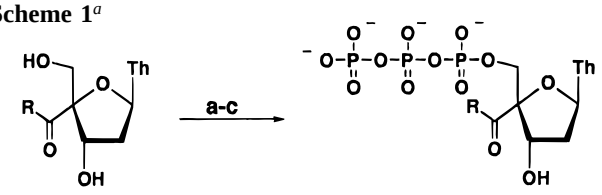
Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) has been widely targeted to combat AIDS. A large part of the research has been directed toward the development of new chain-terminating nucleoside analogues. Their common feature is either the lack of a 3′-hydroxy functionality as found in the 2′,3′-dideoxyribonucleosides (like ddI, ddC, or 2′,3′-didehydro-2′,3′-dideoxyribonucleosides) or its substitution by another functionality (like 3′-azidothymidine, AZT).[1] One nucleoside triphosphate with a remaining 3′-hydroxyl group is 4′-azidothymidine. Its action on HIV-1 RT has been studied extensively, and it has been shown that its incorporation does not lead to chain termination.[2] Only the incorporation of two consecutive 4′-azidothymidines leads to termination of DNA chain elongation. This termination action has been ascribed to the *C3′-endo* conformation of the sugar pucker leading to pertubation of the nascent DNA strand.[3]

We have developed a new class of chain-terminating nucleotides (**2**), which are acylated in the 4′-position and adopt the same conformation (*C3′-exo*) as the unmodified nucleotides in a DNA double strand (B-form DNA[4]) (see Scheme 1). The conformation of the sugar pucker of the 4′-acylated nucleotides **2a**−**c** was assigned by means of NMR data analysis[5] in D_2O and CD_3OD. They strongly prefer the *C3′-exo* conformation, as shown by the large coupling constant between H1′ and $H2'_{pro\text{-}S}$ (8.5−10.2 Hz) as well as the small coupling constant between $H2'_{pro\text{-}R}$ and H3′ (<0.2 Hz). This is due to the strong equatorial preference of the 4′-acyl substituent. The 4′-modified nucleotide triphosphates **2a**−**c** were synthesized by phosphorylation[6] of the 4′-acylated nucleosides **1a**−**c**.[7] Now we have found that, depending on the size of the substituent, 4′-acylated thymidines can act as substrates for HIV-1 RT as well as for other DNA polymerases and reverse transcriptases but inhibit further DNA strand elongation by a new mechanism.

To study the mechanism of action of the 4′-acylated triphosphates **2a**−**c**, we designed an assay where a 40mer DNA template demands the incorporation of a thymidine analogue opposite to an adenosine residue at position 30.[8] When HIV-1 RT was used to promote strand elongation in the presence of the methyl ketone **2a**, the formation of 30mer, 31mer, and full-length product was observed by polyacrylamide gel electrophoresis after incubation for 2h (Figure 1, lane 3). The band pattern, obtained in the autoradiogram, indicates that HIV-1 RT is able to elongate the DNA strand after incorporation of **2a**. Further studies revealed that the incorporation of methyl ketone was complete after 20 min. The formation of 30mer and 31mer products demonstrates that after incorporation of **2a** the elongation takes place at a slower rate for at least the next two nucleotides. Subsequently, the polymerase resumed a more efficient rate of nucleotide incorporation to complete the DNA strand without yielding any products shorter than a 40mer. When the incubation was continued for an additional 3 h, the complete formation of 40mer full-length product was observed (Figure 1, lane 4).[9] All of the other DNA polymerases and reverse transcriptases we tested (Klenow fragment of *Escherichia coli* DNA polymerase I, modified T7 DNA polymerase, AMV RT, M-MuLV RT) incorporated the methyl ketone **2a** but were unable to promote further strand elongation. With these enzymes, nucleotide **2a** behaved like a chain terminator in spite of its free 3′-hydroxyl group and the *C3′-exo* conformation. The fact that only HIV-1 RT is able to elongate the DNA strand can be explained by its broad substrate tolerance and error-prone properties.[10]

As we increased the bulk of the 4′-substituent to the Et ketone **2b**, we observed only the formation of a 30mer reaction product (Figure 1, lanes 5, 6), even when HIV-1 RT was used. Reaction times up to 24 h did not lead to the formation of detectable amounts of longer DNA oligonucleotides. This shows that HIV-1 RT is unable to promote further DNA strand elongation after incorporation of the Et ketone **2b**. The same results were

Scheme 1[a]



1 a: R = Me , b: R = Et, c: R = Bu^t 2

[a] Conditions: (a) $POCl_3$, 1,8-bis(dimethylamino)naphthalene, $OP(OMe)_3$, 0 °C; (b) $[n\text{-}Bu_3NH]_2H_2P_2O_7$, $n\text{-}Bu_3N$, DMF, 0 °C; (c) H_2O, $[Et_3NH]HCO_3$, 0 to 25 °C, 48% (**2a**), 43% (**2b**), 52% (**2c**).

[†] Department of Chemistry.

[‡] Department of Microbiology.

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(8) The primer extension reaction was performed as described in the following: To anneal primer and template, a solution of ^{32}P-end-labeled 20mer DNA primer (0.8 pmol, 5′-d(GTGGTGCGAATTCTGTGGAT)) and 40mer DNA template (3.0 pmol, 5′-d(TCGGTCGTTCATCCTTGCT-GATCCACAGAATTCGCACCAC)) in 80 μL reaction buffer was heated to 80 °C and subsequently allowed to cool down to 25 °C within 1 h. Polymerase, diluted in the reaction buffer, was added up to a final volume of 90 μL. To 6 μL aliquots of this solution we added 4 μL of a buffered solution comprised of dATP, dGTP, dCTP, and one of the 4′-acylated thymidine triphosphates **2a**−**c**. The solutions were incubated at 37 °C for various time periods (1−5 h). The reactions were stopped by addition of 5 μL of 98% formamide containing 10 mM EDTA (pH 8.0). The products were analyzed by polyacrylamide gel electrophoresis (PAGE, 19% polyacrylamide, 7 M urea). After electrophoresis, the gel was tranferred to filter paper, dried, and autoradiographed at −60 °C.

(9) The use of a 40mer DNA template which calls for incorporation of two thymidine analogues opposite two consecutive adenosine residues at positions 30 and 31 resulted in chain termination. Even after incubation for 8 h or incubation with an increased amount of HIV-1 RT, no formation of 40mer full length product was observed. A 30mer was the main reaction product with a 31mer as a minor product. This indicates that the steric strain of the incorporated methyl ketone **2a** slowed down the incorporation rate of the incoming second methyl ketone to a much greater extent than it does for a nonmodified nucleotide.

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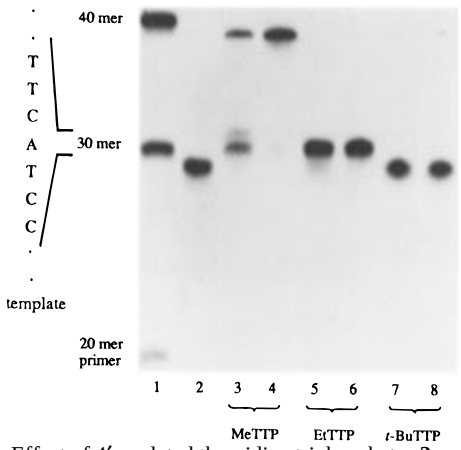

Figure 1. Effect of 4′-acylated thymidine triphosphates **2a**−**c** on DNA strand synthesis catalyzed with HIV-1 RT. Shown is an autoradiogram of a 19% denaturing PAGE gel. Lane 1: line marker. Lane 2: ^{32}P-5′-end-labeled primer (0.05 pmol), template (0.20 pmol), dATP, dGTP, dCTP (1.0 μM); incubated at 37 °C for 5 h in a buffer containing 20 mM Tris−HCl (pH 7.5), 6 mM $MgCl_2$, 40 mM KCl, 0.5 DTT, and 0.15 units HIV-1 RT. Lanes 3, 4: as in lane 2, but with **2a** (MeTTP, 100 μM) incubated for 2 and for 5 h. Lanes 5, 6: as in lane 2, but with **2b** (EtTTP, 100 μM) incubated for 2 and for 5 h. Lanes 7, 8: as in lane 2, but with **2c** (*t*-BuTTP, 100 μM) incubated for 2 and 5 h.

obtained with all other DNA polymerases and reverse transcriptases tested. Thus, an increase in the bulk of the 4′-substituent by one methylene group, which does not lead to any significant conformational change of the sugar pucker, ensures complete inhibition of further DNA synthesis after incorporation. Steric shielding by the 4′-substituent of the incorporated Et ketone **2b** may be responsible for the prohibition of the phosphorodiester bond formation at the secondary 3′-OH function.[11] The bulkiest 4′-acylated thymidine, the *t*-Bu ketone **2c**, is even not accepted as a substrate for either HIV-1 RT or all the other DNA polymerases tested, as indicated by the formation of a 29mer reaction product (Figure 1, lanes 7, 8).

4′-Acylated nucleotides **2a** and **2b** are the first examples of a new class of nucleotide analogues which are DNA chain terminators in spite of the fact that they adopt the *C3′-exo* conformation and that a 3′-hydroxyl functionality is present. This makes this class of nucleosides potential antiviral agents and tools for study of DNA polymerase/DNA interactions.

In addition, it has been shown that 4′-acylated DNA can be site-specifically cleaved upon irradiation with light.[12] Since HIV-1 RT is able to incorporate the methyl ketone **2a** and elongate the DNA strand, this method can be used to enzymatically synthesize photolabile DNA. Irradiation with light ($h\nu$ > 305 nm) of the enzymatically synthesized DNA containing the 4′-acetylated thymidine **2a** (Figure 2, lane 2) led to the formation of a faster moving band on a polyacrylamide electrophoresis gel indicating a site-specific cleavage of the DNA (Figure 2, lanes 3−6).[13]

Analogue **2a** is the first nucleotide which can be incorporated into DNA enzymatically and cleaved site-specifically with light of a wavelength longer than 305 nm. The phototriggered DNA bond cleavage may be useful to study the topologies and dynamics of nucleic acid structure.[14] The products released from DNA cleavage by irradiation were identified as a 29mer with 3′-phosphate and 3′-phosphoglycolate as 3′-DNA termini and a 10mer with a 5′-phosphate 5′-DNA terminus.[15] They are similar to the main cleavage products generated from 4′-DNA radicals under oxidative conditions using Fe^{2+} complexes of bleomycin,[16] methidiumpropyl−ethylenediaminetetraacetic acid[17] or γ-ray radiation.[18] Since reactions of **2a** mimics the damage produced by γ-ray radiation, it may be useful for the study of DNA strand break repair.[19] It could also be applied in photocross-linking studies of protein−DNA interactions, where the photoactive species are often incorporated enzymatically by

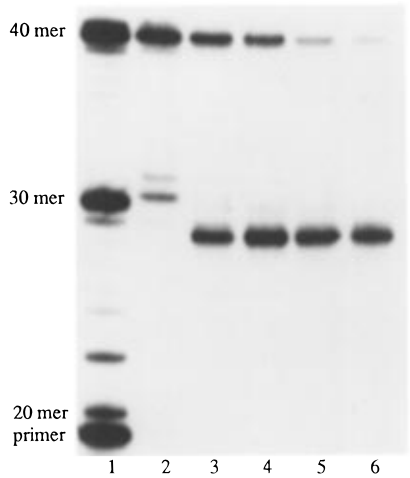

Figure 2. Irradiation of enzymatically synthesized 4′-acetylated DNA. Shown is an autoradiogram of a 12% denaturing PAGE gel. Lane 1: line marker. Lane 2: 4′-acetylated DNA synthesized with HIV-1 RT in the presence of **2a** as described in Figure 1. Lanes 3−6: same as in lane 2, but after irradiation for 0.5 h, 1 h, 1.5 h, and 2 h, respectively.

DNA polymerases.[20] In conclusion, our observation that nucleotides which adopt the *C3′-exo* conformation and are still carrying a 3′-hydroxy function can act as chain terminators is new. This finding makes **2a** and **2b** potential tools in antiviral applications. The fact that 4′-acylated nucleotides **2a,b** can be incorporated enzymatically and **2a** even elongated may be useful for the study of DNA strand repair and protein−DNA interactions.

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Supporting Information Available: Analytical data of 4′-acylated thymidine triphosphates **2a**−**c**, selected MALDI-TOF spectra (7 pages). See any current masthead page for ordering instructions.

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(11) Another explanation for this action may be that the steric interference of the analogue **2b** with the enzyme results in the release of the primer/template complex from the enzyme. It is also possible that the primer/template complex cannot be translocated in the polymerase cleft to ensure the next nucleotide incorporation event.

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(13) Irradiation of 4′-acetylated DNA: The DNA was enzymatically synthesized with HIV-1 RT as described in ref 7 and Figure 1, precipitated with ethanol, and resuspended in 100 μL of buffer containing 20 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, and 40 mM KCl. The solution was heated to 80 °C and subsequently allowed to cool to 25 °C within 1 h. Irradiation (Osram 500W, 305 nm filter) was performed at 10 °C. Aliquots (15 μL) were removed at various time points (0.5−2 h), precipitated with ethanol, resuspended in formamide, and analyzed by PAGE.

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