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γ-Radiolysis DNA Products: Synthesis of 6-(α-Thyminyl)-5,6-dihydro-4-thiothymine Derivatives

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Received March 22, 2004

Far-UV photolysis of 4-thiothymidylyl(3′−5′)thymidine led to the formation of three stable derivatives: one resulting from a combination between a 3′-end methylene radical and a 5′-end C_4 radical [4-(α-thyminyl) derivative] and two formed after a combination between a 3′-end methylene radical and a 5′-end C_6 radical [6-(α-thyminyl) derivative]. In the latter series, two stereochemical pathways took place during the reaction between the methylene and C_6 radicals. The major pathway occurred when the 5′-base glycosidic bond had an anti conformation leading to an *S* configuration of the C_6 Tp-end. The minor pathway, which had never been reported before in this series, involved a 5′-base in a syn conformation leading consequently to the *R* configuration at the C_6 Tp-end. The 5,6-dihydrothymine moiety of these two adducts presented a 5,6-trans diaxial substitution that resulted from the epimerization, at the 5,6-dihydropyrimidine 5-position, of a less stable cis-disubstituted intermediate.

Introduction

Among the dimeric thymine products resulting from the reaction between one methyl thymine and another thymine [α-thyminyl thymine (α-TT) adducts], the 5-(α-thyminyl)-5,6-dihydrothymine derivative is the best known and studied adduct.[1] Called spore photoproduct because of its occurrence in bacterial spore DNA after UV treatment,[1c] this compound is also formed under the direct effect of γ-radiation on thymidine (Figure 1).[2] A less studied α-TT adduct is the 6-(α-thyminyl)-5,6-dihydrothymine adduct (Figure 1), the major product formed under the direct effect of γ-radiation on thymidine.[2] Although this compound has not yet been isolated from γ-irradiated DNA, its formation cannot be excluded. Therefore, this class of thymine dimeric products deserves full consideration, particularly when a permanent manned station on the moon and a manned mission to Mars are currently being envisaged.

To investigate the biophysical and biological properties of DNA models containing at a specific site a 6-(α-thyminyl)-5,6-dihydrothymine derivative, we faced the crucial need to efficiently prepare this type of adduct. We herein report on the synthesis of two 6-(α-thyminyl)-5,6-dihydrothymine diastereomer derivatives.

α-TT adduct analogues have been synthetically obtained using the peculiar 4-thiouracil photochemistry, and we have previously reported that the 6-(α-thyminyl)-5,6-dihydro-4-thiouracil) adduct **2** can be prepared by photolysis of ds^{4}UpT **1** (Scheme 1).[3,4] Consequently, we decided to explore the photochemistry of the known compound s^{4}TpT **3**[5] in view of an easy access to 6-(α-thyminyl)-5,6-dihydrothymine derivatives.

Results and Discussion

Photolysis of an oxygen-free aqueous solution of **3** at 366 nm led to its complete disappearance with the concomitant formation of three products **4**−**6** (in a 2.5/6/1 ratio, respectively) as revealed by the examination of the ^{1}H NMR spectrum of the crude irradiation mixture.

Structural Determination of Compounds 4−6. Compound **4**, isolated in 21% yield by reversed-phase chromatography, was rapidly identified as the 4-(α-thyminyl)-5-methyl-2-pyrimidone derivative from its spectral data. Its UV spectrum displayed two maxima (λ_{max}

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10.1021/jo0401570 CCC: $27.50
Published on Web 06/16/2004

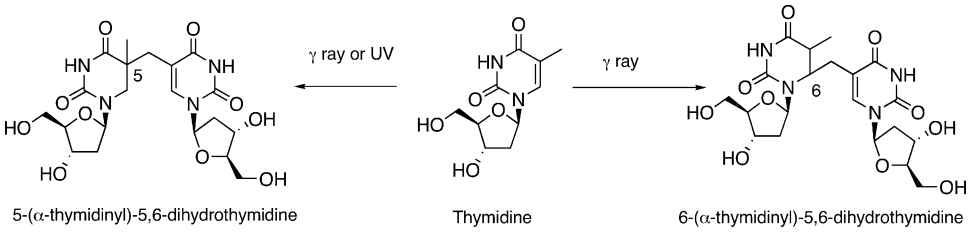
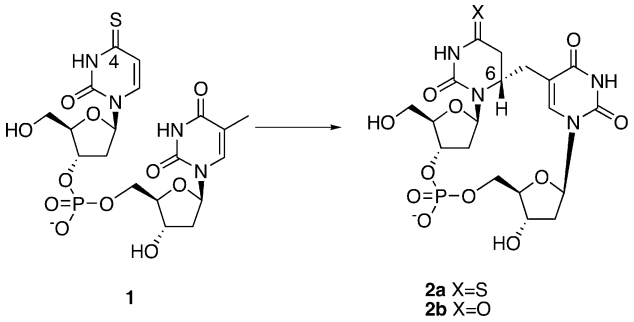


FIGURE 1. Structure of α-TT adducts derived from thymidine under irradiation.

SCHEME 1



270 and 312 nm) in accordance with the presence of a thymine and a 5-methyl-2-pyrimidone chromophore within its structure. Its HR mass spectrum [*m*/*z* 551.1155 $(M + Na)^+$] indicated the molecular formula $C_{20}H_{25}N_4O_{11}PNa$ (calcd 551.1164) evidencing, compared to **3**, the loss of H_2S. The key ^1H NMR arguments confirming the 4-(α-thyminyl)-5-methyl-2-pyrimidone structure were the presence of the two characteristic H_6 proton signals at δ 8.74 and 6.65, of only one methyl signal (δ 2.13), and of one set of methylenic protons (δ 4.03 and 3.51).[4]

Compounds **5** and **6** were isolated by reversed-phase chromatography in 33 and 11% yield, respectively. Examination of the ^1H NMR data of **5** and **6** rapidly demonstrated their belonging to the 6-(α-thyminyl) series. Of particular interest was the presence, in the ^1H NMR spectrum of **5** and **6**, of a methyl doublet at δ 1.46 and the presence of only one olefinic proton (δ 7.64 for **5** and δ 7.82 for **6**). However, the ^1H NMR spectrum of **5** and **6** revealed, for each compound, the presence of a minor product (**7** and **8**, respectively), presenting an ^1H NMR profile closely related to that of the major component. As in the case of **5** and **6**, the ^1H NMR spectrum of **7** and **8** exhibited also a shielded methyl doublet (δ 1.33 for **7** and δ 1.38 for **8**) and one olefinic proton (δ 7.68 for **7** and δ 7.96 for **8**). Upon storage in D_2O, the methyl doublet signal of **5** and **6** collapsed to a singlet while a one-proton signal [quintet at δ 3.14 (J = 6.7 Hz) for **5** and δ 3.28 (J = 6.8 Hz) for **6**] concomitantly disappeared. Consequently, the signal at δ 3.14 (**5**) and δ 3.28 (**6**) was attributed to the exchangeable H_5 5,6-dihydropyrimidine proton. Its coupling constant value with H6 (J = 6.7 Hz for **5** and J = 6.8 Hz for **6**) evidenced, in each case, a cis relationship between H_5 and H_6. Upon storage in D_2O, the methyl doublet of **7** and **8** also collapsed to a singlet although at a much slower rate. Interestingly, we also observed that, in aqueous solution, **5** and **6** were moderately stable and gave rise to **7** and **8**, respectively, at a slower rate than the isotopic exchange. Furthermore, we observed that in concentrated aqueous ammonia, the transformation of **5** into **7** and **6** into **8** occurred instantly and quantitatively. As the differences between **5** and **7**, as well as between **6** and **8**, concerned exclusively the 5,6-dihydropyrimidine moiety and since **7** and **8** were found to be stable, we therefore primarily carried out the identification of these two latter derivatives.

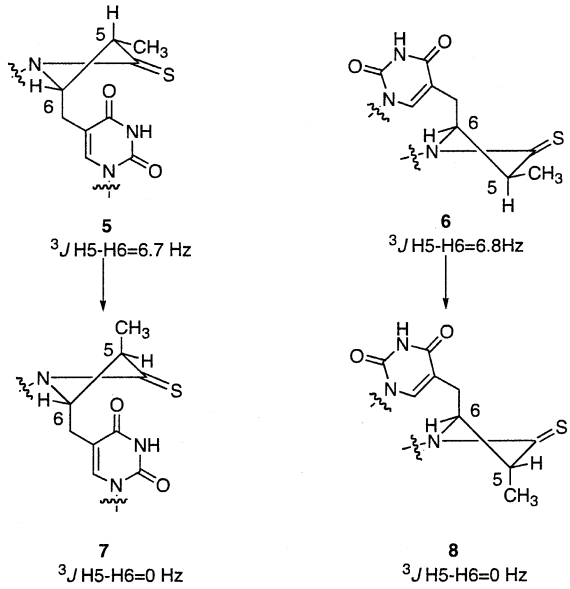
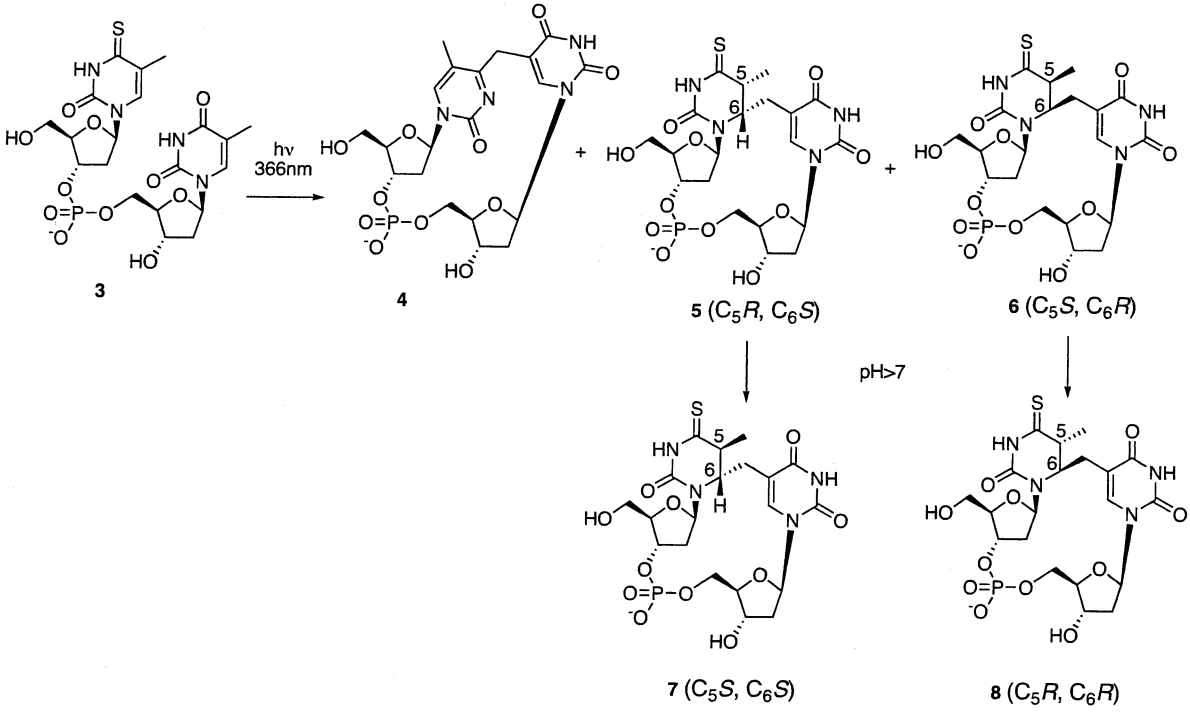


FIGURE 2. Schematic representation of the dihydropyrimidine moiety conformation of compounds **5**−**8**.

The UV spectrum of **7** and **8** (ca. λ_{max} 278 nm) was in full accordance with those observed in the 6-(α-thyminyl)-5,6-dihydro-4-thiouracil series.[3,4] Their mass data [*m*/*z* 561 $(M - H)^+$ in each case] evidenced that they were isomers of **5** and **6** and hence that **7** and **8** were not simply the C_4-oxygenated derivatives of **5** and **6**. All the proton resonances of **7** and **8** were assigned from their COSY spectrum. In the ^1H NMR spectrum of **7** and **8**, the H_5 Tp- resonance appeared as a quartet in both cases. The lack of coupling between Tp- H_6 and H_5 in **7** and **8** evidenced that, conversely to **5** and **6**, these protons experienced a trans diequatorial orientation, a relationship in full accordance with the observed NOE (NOESY experiment) between H_6 Tp- and Me Tp-. Therefore, in **7** and **8**, the dihydropyrimidine substituents at C_5 and C_6 are trans diaxially oriented (Figure 2). Interestingly, the interresidue NOE observed, only in the case of compound **7**, between H1′ Tp- and H6 -pT permitted us to ascertain, for this compound, a (6*S*) Tp- configuration and, since the 5,6-substituents are trans diaxial, a similar configuration at C_5. Therefore, to satisfy the trans diaxial position of the substituents, **8** had to be the other half chair conformer with the C_5 and C_6 atoms having an *R* configuration.

NOEs in **7** and **8** were also highly informative to determine the relative orientation of the pyrimidone, with

SCHEME 2



respect of their glycosidic bond. NOEs observed between H_6 Tp- and $H_{1'}$ Tp-, $H_{2'}$ Tp-, and $H_{3'}$ Tp- revealed a certain degree of freedom of the Tp- unit of **7** and **8** around the glycosidic bond. Concerning the -pT unit, in the case of **7**, an anti/syn equilibrium was observed (NOE between H_6 -pT and $H_{1'}$ and $H_{2'}$ of its sugar). In contrast, for **8**, H_6 -pT evidenced only an NOE with $H_{2'}$ of its sugar signing an anti glycosidic bond conformation for the -pT sugar.

The acidic character of H_5 in **5** and **6** clearly demonstrated that, in alkaline solution, epimerization could only occur at this position and hence that **5** and **6** were the C_5 epimers of **7** and **8**, respectively. Consequently, the (5*R*,6*S*) configuration was attributed to **5** and the (5*S*,6*R*) configuration to **6** (Scheme 2). Such configurations are fully consistent with the observed ^3J couplings (Figure 2).

Mechanistic Considerations. Compounds **4**−**6** are likely to derive from an hydrogen abstraction from the thymine methyl group by the excited thiocarbonyl group. This generates a biradical that undergoes direct coupling or rearranges prior coupling. In case of direct coupling, the intermediate formed after combination between the methylene and the C_4 position undergoes H_2S elimination and gives rise to **4**. Migration of the C_4 initially formed radical to the C_6 position and coupling with the methylene radical gives the 6-(α-thyminyl) adducts **5** and **6**, depending on the glycosidic conformation of the Tp- base. Compound **5** results from a major pathway in which the combination between the thyminyl radical and the C6-thyminyl radical occurs when in the 5′-nucleoside an anti glycosidic bond conformation is present. In contrast, compound **6** results from a minor pathway in which the 5′-thymine base is in a syn glycosidic bond conformation (Figure 3).

If in the cyclobutane[6] and in the N^3-methylthietane series[7] trapping of the 5′-base syn conformer has already been observed, it has never been reported in the 6-(α-thyminyl)-5,6-dihydrothymine photoproduct series. The alternative pathway being minor (less than 15%), we decided to revisit the photochemistry of ds^{4}UpT (**1**) to

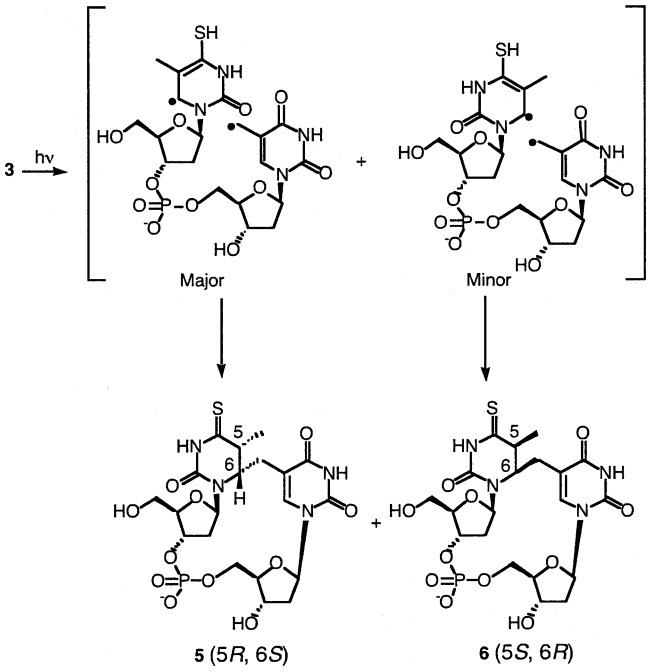


FIGURE 3. Representation of the major (5′-anti conformation) and minor (5′-syn conformation) biradical intermediates of **3** leading to **5** and **6**.

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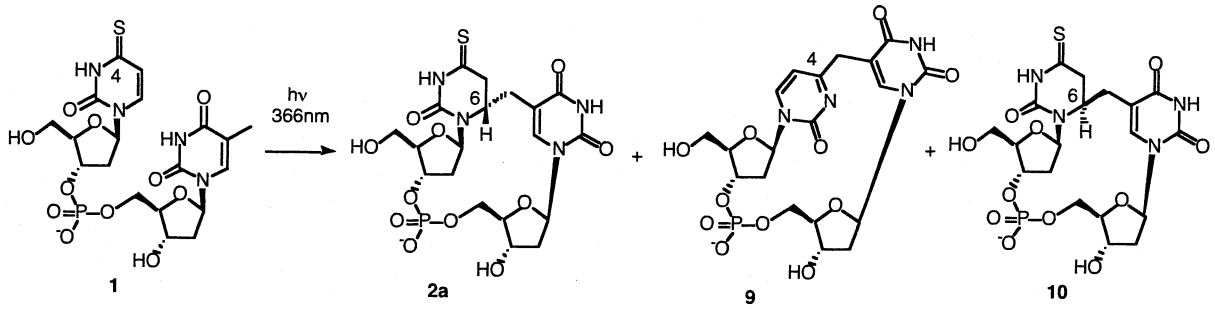
SCHEME 3



determine if this behavior was specific to s[4]TpT. Analysis of the 6.5 to 9 ppm region of the ^{1}H NMR spectrum of the crude irradiation mixture of **1** at 366 nm showed the formation of three products **9**, **2a** and **10** (2/4/1 ratio, respectively). Two of them were the already reported 4- and 6-(α-thyminyl) adducts[3,4] (**9** and **2a**, respectively). The ^{1}H NMR spectrum of the third product (**10**) displayed a deshielded signal (singlet at δ 7.90) easily attributed to H_6 -pT and whose chemical shift was reminiscent of that observed for the similar proton in **6** and **8**. HPLC-mass analysis of the crude irradiation mixture indicated for **10** a UV (λ_{max}=280 nm) and MS (*m*/*z* 547 $(M-H)^+$) confirming its appertaining to the 6-(α-thyminyl) series. Consequently, **10** was identified as the C_6 epimer of **2a** and this study confirmed that, as observed for **3**, irradiation of **1** at 366 nm leads also to two 6-(α-thyminyl) derivatives, epimers at the C_6 position: a major isomer **2a** (6*S* configuration) and a minor isomer **10** (6*R* configuration) (Scheme 3). Thus, the photochemical behavior of **1** is similar to that of **3** meaning that the combination of the methylene and C_6 radicals, produced by UV-excitation of both anti and syn Tp- conformers can be achieved in this series. The possibility to independently obtain two isomers at position C6 should allow to determine the importance of the C_6-stereochemistry on the biological properties of these adducts.

Also noteworthy is the fact that, once the two radicals have reacted together, the resultant thioenolate equilibrates with its thiocarbonyl form by protonation on the C_5 position exclusively from the less hindered side of the 5,6-dihydropyrimidine (side opposite to the thyminyl substituent) to afford the kinetic adducts (**5** or **6**). Upon standing in alkaline aqueous solution, these two compounds quantitatively convert into the thermodynamic adducts **7** and **8**, respectively, leading to a trans diaxially 5,6-disubstituted-5,6-dihydropyrimidine. Interestingly, the transient thioenolate stereospecific protonation/C_5 epimerization sequence rigorously follows the rules that we have previously established in the h[5](6−4) photoproduct series.[8,9] However, since the series was different, we decided to calculate, by ab initio, the free energy difference (ΔG) in the gas phase between the two C_5 epimers of the major adduct (**5** and **7**). Using the strategy applied in the h[5](6−4) photoproduct series,[9] the free energy of **5** was found to be 2.47 kcal mol^{-1} (theory level HF/6-31G*// HF/6-31G); 1.48 kcal mol^{-1} (theory level MP2/6-31G*// HF/6-31G) higher than that of **7**. Consequently, as demonstrated in the h[5](6−4) photoproduct series, it is likely that in the 6-(α-thyminyl)-5,6-dihydrothymine series, van der Waals interactions between the N_1- and C_6-5,6-dihydropyrimidine substituents also force the 5,6-dihydropyrimidine in the half-chair conformation in which the C_6 substituent has an axial position (reduced N_1- and C_6-substituents interaction). Then an energy minimum is reached when steric interaction between the C_5-substituting methyl group and the bulky C_6-substituent is reduced, a situation only obtained when the C_5-methyl adopts an axial orientation (Figure 2).

Conclusion

If this study provides a nice extension of our rules predicting the most stable conformation of 5,6-dihydropyrimidines together with their C_5 and C_6 configuration,[9] it principally demonstrates that among the four 6-(α-thyminyl) diastereomers, only the two *trans*-diaxially substituted are stable over a long period of time. These two compounds, epimers at their Tp- 5- and 6-position, can be efficiently prepared by photolysis of s[4]TpT, the major stable epimer presenting a (5*S*,6*S*) configuration. Oligonucleotides containing this kind of adduct present a great interest, from a biological standpoint, since they are relevant to γ-radiation induced DNA-damaged products. The modified oligonucleotides could be used to probe the mechanism of translesion DNA polymerases and repair enzymes. As witnessed by the isolation of **2b** during the initial study concerning the photolysis of **1**,[3] the hydrolysis of the thiocarbonyl function of **7** and **8** can be easily envisaged prior to its incorporation into oligonucleotides. However, it is highly likely that modified solid-phase synthesis protocols should permit the incorporation of **7** or **8** into oligonucleotides to afford either 6-(α-thyminyl)-5,6-dihydro-4-thiothymine-containing oligonucleotides or 6-(α-thyminyl)-5,6-dihydrothymine-containing oligonucleotides after thiocarbonyl hydrolysis during the final deprotection step. The determination of the conditions that would make **7** and **8** two valuable and versatile adducts is currently being investigated in our laboratory.

Experimental Section

Photolysis Procedure. An argon-purged 0.3 mM aqueous solution (100 mL) of **3** was irradiated for 30 min with an Original Hanau Quartzlampen Fluotest-Forte. This procedure was repeated five times. Photolysates were concentrated, and the concentrates purified by reversed-phase chromatography (LiChroprep RP-18) using a gradient of CH_3CN in H_2O (0−10%).

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4-(α-Thyminyl)-5-methyl-2-pyrimidone derivative (4): yield 21%; UV (λ_{max} H_2O) 270, 312 nm; ^{1}H NMR (D_2O; 400 MHz) δ 8.74 (1H, s), 6.65 (1H, s), 6.21 (1H, d, ^3J(H,H) = 5.9 Hz), 6.11 (1H, dd, ^3J(H,H) = 5.2, 9.0 Hz), 4.48 (1H, br d, ^3J(H,H) = 5.8 Hz), 4.28 (1H, m), 4.20−4.11 (3H, m), 4.03 (1H, br d, ^3J(H,H) = 15.7 Hz), 3.98 (1H, dd, ^3J(H,H) = 3.5, 14.0 Hz), 3.91 (1H, ddd, ^3J(H,H) = 4.9, 6.8, 10.2 Hz), 3.72 (1H, td, ^3J(H,H) = 7.3, 10.2 Hz), 3.51 (1H, br d, ^3J(H,H) = 15.7 Hz), 2.80 (1H, ddd, ^3J(H,H) = 5.9, 11.6, 13.2 Hz), 2.57 (1H, dd, ^3J(H,H) = 5.7, 13.2 Hz), 2.50 (1H, ddd, ^3J(H,H) = 1.5, 5.2, 14.3 Hz), 2.13 (3H, s), 1.95 (1H, ddd, ^3J(H,H) = 5.8, 9.0, 14.3 Hz); ^{13}C NMR (D_2O; 62.8 MHz) δ 177.1, 166.7, 156.9, 152.5, 144.7, 135.8, 116.4, 112.1, 87.8, 87.2, 86.3, 85.3, 72.1, 68.8, 66.6, 59.4, 40.0, 39.7, 33.1, 14.6; HRMS (ES) $(M + Na)^+$ calcd for $C_{20}H_{25}N_4O_{11}PNa$ 551.1164, found 551.1155.

6-(α-Thyminyl)-(5*R*,6*S*)-dihydro-4-thiothymine derivative (5): yield 33%; UV (λ_{max} H_2O) 280 nm; ^{1}H NMR (D_2O; 300 MHz) δ 7.64 (1H, s), 6.32 (1H, t, ^3J(H,H) = 6.5 Hz), 5.92 (1H, t, ^3J(H,H) = 6.8 Hz), 4.63 (1H, m), 4.18−3.97 (5H, m), 3.72 (2H, m), 3.14 (1H, qu, ^3J(H,H) = 6.7 Hz), 2.78−2.30 (6H, m), 1.46 (3H, d, ^3J(H,H) = 6.7 Hz); HRMS (ESI) $(M + Na)^+$ calcd for $C_{20}H_{27}N_4O_{11}PNaS$ 585.1032, found 585.1036.

6-(α-Thyminyl)-(5*S*,6*R*)-dihydro-4-thiothymine derivative (6): yield 11%; UV (λ_{max} H_2O) 279 nm; ^{1}H NMR (D_2O; 300 MHz) δ 7.82 (1H, s), 6.36 (1H, dd, ^3J(H,H) = 6.2, 8.1 Hz), 5.36 (1H, br d, ^3J(H,H) = 8.0 Hz), 4.59 (1H, td, ^3J(H,H) = 3.7, 7.3 Hz), 4.30−3.73 (7H, m), 3.28 (1H, qu, ^3J(H,H) = 6.8 Hz), 2.80 (2H, m), 2.60−2.30 (4H, m); 1.46 (3H, d, ^3J(H,H) = 6.8 Hz); HRMS (ESI) $(M - H)^-$ calcd for $C_{20}H_{26}N_4O_{11}PS$ 561.1056, found 561.1044.

6-(α-Thyminyl)-(5*S*,6*S*)-dihydro-4-thiothymine derivative (7): UV (λ_{max} H_2O) 281 nm; ^{1}H NMR (D_2O; 250 MHz) δ 7.68 (1H, s), 6.29 (1H, t, ^3J(H,H) = 6.9 Hz), 6.08 (1H, t, ^3J(H,H) = 6.0 Hz), 4.73−4.59 (2H, m), 4.21 (1H, m), 4.18−4.09 (2H, m), 4.0 (1H, m), 3.89−3.79 (2H, m), 3.72 (1H,dd, ^3J(H,H) = 4.4, 12.4 Hz), 3.26 (1H, q, ^3J(H,H) = 7.1 Hz), 2.74 (2H, m), 2.53 (2H, t, ^3J(H,H) = 6.5 Hz), 2.43 (2H, dd, ^3J(H,H) = 5.0, 7.0 Hz); 1.33 (3H, d, ^3J(H,H) = 7.1 Hz); ^{13}C NMR (D_2O; 62.8 MHz) δ 212.6, 166.6, 152.6, 151.2, 141.1, 111.5, 86.6, 86.4, 85.5, 83.9, 74.8, 71.8, 66.4, 61.7, 56.8, 49.3, 39.9, 36.3, 30.3, 20.4; MS (ESI) $(M - H)^+$ 561.

6-(α-Thyminyl)-(5*R*, 6*R*)-dihydro-4-thiothymine derivative (8): UV (λ_{max} H_2O) 281 nm; ^{1}H NMR (D_2O; 400 MHz) δ 7.96 (1H, s), 6.53 (1H, dd, ^3J(H,H) = 5.9, 8.7 Hz), 6.21 (1H, t, ^3J(H,H) = 5.9 Hz), 4.75 (1H, m), 4.59 (1H, td, ^3J(H,H) = 3.0, 6.3 Hz), 4.29−4.25 (2H, m), 4.08−4.01 (2H, m), 3.92−3.86 (2H, m), 3.49 (1H, br dd, ^3J(H,H) = 4.2, 10.2 Hz), 3.14 (1H,br q, ^3J(H,H) = 7.0 Hz), 3.00 (1H, dd, ^3J(H,H) = 4.2, 14.0 Hz), 2.82 (1H, dd, ^3J(H,H) = 10.2, 14.0 Hz), 2.62 (2H, dd, ^3J(H,H) = 5.9, 8.8 Hz), 2.41 (1H, ddd, ^3J(H,H) = 3.0, 5.9, 14.2 Hz), 2.30 (1H, ddd, ^3J(H,H) = 6.6, 8.7, 14.2 Hz), 1.38 (3H, d, ^3J(H,H) = 7.0 Hz); ^{13}C NMR (D_2O; 75.4 MHz) δ 212.3, 166.6, 152.7, 150.9, 141.3, 112.4, 86.2, 85.8, 85.3, 83.2, 71.7, 71.0, 65.5, 59.5, 59.2, 48.2, 39.2, 36.3, 29.8, 19.6; MS (ESI) $(M - H)^+$ 561.

Computational Methods. Gas-phase geometry minimization of **5** and **7** and their corresponding frequency analysis were achieved at the HF level using the 6-31G basis set. Single-point energy calculations were performed using the HF/6-31G*//HF/6-31G and MP2/6-31G*//HF/6-31G theory levels.[10] Computational calculations were carried out on a cluster of dual-athlons using software GAMESS,[11] and free energy differences, ΔG, were calculated as previously reported.[9]

Acknowledgment. We thank the CNRS (PCV 2000) and the Université de Picardie Jules Verne (Cluster of computers) for financial support. We are also grateful to CONACYT (Mexico) for a doctoral fellowship to J.U.O.M.

Abbreviations: ds^{4}UpT, 2′-deoxy-4-thiouridylyl(3′−5′)thymidine; s^{4}TpT, 4-thiothymidylyl(3′−5′)thymidine; for the sake of simplification, Tp- refers to the 5′ terminal thymidinyl residue and -pT to the 3′ terminal thymidinyl residue.

Supporting Information Available: General methods; ^{1}H NMR spectra of **4**−**8**; Cartesian coordinates of optimized structures **5** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0401570

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