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Enhanced Base Pairing and Replication Efficiency of Thiothymidines, Expanded-size Variants of Thymidine

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Steric effects have become widely cited as important factors in recognition and replication of DNA. Because the DNA backbone is relatively rigid, selectivity in hybridization against mismatches having different sizes and geometry is significant. However, the pairing fidelity observed in the enzymatic replication of DNA is markedly higher.[1] It is hypothesized that polymerases achieve this by tightly surrounding the new base pairs being synthesized, thus providing an even stricter steric environment.[2]

To test these proposed effects, it would be useful to evaluate polymerase activities with DNA base analogues having varied sizes. In one recent test of halogen-substituted nonpolar DNA base analogues with gradually increasing sizes,[3] the replication enzymes of *Escherichia coli* were highly sensitive to even sub-angstrom alterations of sterics. Surprisingly, it was observed that DNA polymerase I prefers analogues that are larger than natural DNA bases. However, this apparently enlarged steric preference has not yet been tested with hydrogen-bonding nucleotide analogues. Thus we turned to the thiocarbonyl group as a way to increase size by a small increment. This substitution, 0.45 Å longer than the carbonyl group,[4] has previously been used on several nucleoside analogues but has not yet been generally studied for its effects on DNA base pairing and replication. Here we report the results of base-pairing studies and of polymerase kinetics studies of 2-thio- and 4-thiothymidine.[5] We find that the larger size can lead to increased efficiency and selectivity in pairing and replication.

Both the 2-thio- and 4-thiothymidine deoxyribosides (**2S** and **4S**, Figure 1) are known[5] and have been employed especially for the unique chemistry of the thiocarbonyl group, including nucleophilic reactivity[6] and photo-cross-linking.[7] Surprisingly, although a few preliminary tests of their hybridization have been carried out,[8] their pairing selectivity and quantitative replication properties are largely unknown. In light of their possible utility in evaluating steric effects on nucleic acid systems, we undertook a general and quantitative analysis of base-pairing and replication properties of these two analogues. We carried out a base-pairing study of both thiothymidine isomers in a 12-base-pair duplex. Thermal denaturation experiments confirmed[8a] that **4S** was slightly destabilizing to the duplex compared to natural thymidine, while the **2S** isomer was more stabilizing than the natural congener in this sequence context, increasing T_m by 1.4 °C with one substitution (Figure 2; Table S1, Supporting Information). The pairing selectivity of the thio analogues was also evaluated; results showed that the **2S** isomer gave higher pairing specificity than thymine itself, with a 12.0 °C decrease in T_m for the most stable mismatch (**2S**-G) compared to a 7.5 °C drop for the natural T-G mismatch.

Interestingly, the **4S** isomer showed very different behavior, forming a **4S**-G pair with T_m even higher than that of a natural T-A base pair. Thus, in summary, **2S** forms pairs with high stability and selectivity, while **4S** is slightly destabilizing in this context and shows dual pairing ability with A and G. Models suggest that **4S** should have somewhat unfavorable steric interactions with the amino group of adenine in a **4S**-A base pair (see Figure S1, Supporting Information), and gas-phase AM1 calculations suggest that **4S** can exist in a relatively stable enethiol tautomer that could adopt a triply hydrogen-bonded Watson−Crick pair geometry with G (Figure S2, Supporting Information). It is possible that the enhanced pairing properties of **2S**-A and **4S**-G arise from stronger stacking of the larger and more polarizable species[9] or from altered solvation and/or cation interactions; more studies will be needed to test these effects.

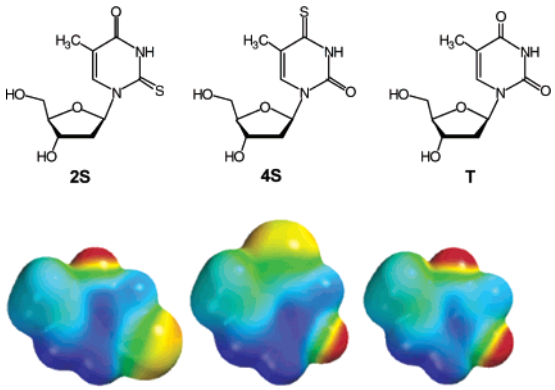

Figure 1. Structures of 2-thiothymidine (**2S**) and 4-thiothymidine (**4S**), with unmodified thymidine shown at the right for comparison. Space-filling models of the bases alone are shown below each structure.

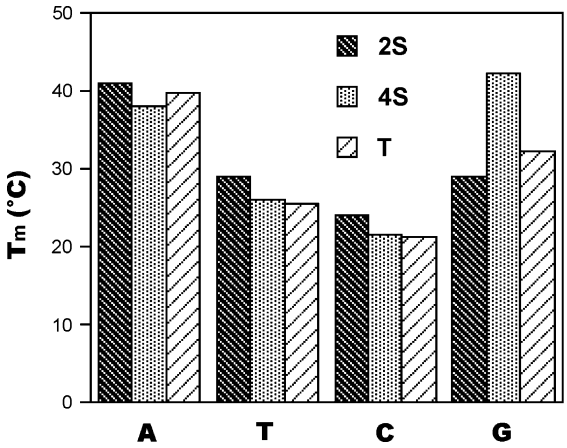

Figure 2. Histogram showing pairing stability (as T_m) and selectivity of **2S** and **4S** placed opposite each of the natural bases in a 12-base-pair DNA duplex. The sequence is dAAGAAXGAAAAG·dCTTTTCYTTCTT, where X is **2S**, **4S**, or T, and Y is each of the four natural bases as shown. Conditions: 2 μM DNA, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM phosphate (pH 7.0). Uncertainty in T_m values is less than ±0.5 °C.

Few pairing data for **2S** and **4S** exist in the literature for comparison to the current results. Thermal denaturation studies have been reported for both compounds paired opposite adenine,[8a] generally showing some stabilization (relative to T) by **2S** and weak

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Table 1. Steady-State Kinetics Data for Processing of Base Pairs and Mismatches Involving Thiothymidine Nucleotides by Kf Pol I (exo-)[a]

nucleoside triphosphate	X[b]	K_m (μM)	V_{max} (% min^{-1})[c]	efficiency (V_{max}/K_m)	relative efficiency
2S	A	3.7(0.8)	12.9(2.9)	3.5×10^6	2.2
4S	A	2.0(0.1)	11.8(0.1)	5.9×10^6	3.7
T	A	7.0(1.8)	11.0(2.3)	1.6×10^6	1
2S	T	103(18)	0.802(0.051)	7.9×10^3	4.9×10^{-3}
4S	T	104(27)	0.203(0.042)	2.0×10^3	1.3×10^{-3}
T	T	80(13)	0.015(0.005)	1.8×10^2	1.1×10^{-4}
2S	C	75(22)	0.022(0.007)	3.0×10^2	1.9×10^{-4}
4S	C	226(20)	0.138(0.013)	6.1×10^2	3.8×10^{-4}
T	C	279(6)	0.018(0.001)	6.6×10^1	4.1×10^{-5}
2S	G	15(7)	0.006(0.002)	4.0×10^2	2.5×10^{-4}
4S	G	59(13)	0.246(0.048)	5.9×10^3	3.7×10^{-3}
T	G	136(60)	0.139(0.049)	1.1×10^3	6.9×10^{-4}

[a] Conditions: 200 nM *2*3mer/28mer primer−template duplex and varied polymerase concentrations in a buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 50 μg/mL BSA, and 1 mM dithiothreitol, incubated at 37 °C in a reaction volume of 10 μL. Standard deviations are given in parentheses. [b] Template base. See Table S1 for data with **2S** and **4S** as the template base. [c] Normalized for the lowest enzyme concentration used.

destabilization by **4S**, consistent with the current results. Studies were not previously carried out with the three different mismatches of each compound; however, there does exist one previous experiment for the **4S**-G mismatch,[8b] which showed strong destabilization, in marked contrast to the current results. This difference might be due to a difference in sequence context or to **4S** degradation under some DNA synthesis conditions.[10]

To test the effects of the added size on DNA replication, we carried out steady-state kinetics experiments on replication of the thio analogues by the Klenow fragment of *E. coli* DNA polymerase I (Kf exo-). The larger-than-natural analogues were tested both as template bases and as incoming dNTP analogues (see Table 1 and Table S2, Supporting Information). When replicated opposite adenine, both thio analogues showed high efficiency compared to natural thymidine. This is especially the case where the analogues were used as triphosphate derivatives, where **2S**-triphosphate was 2.2-fold more efficient than dTTP and **4S**-triphosphate was 3.7-fold more efficient. In the template strand, the analogues had the same efficiency as thymidine when paired with dATP. Thus, the added size of the 2-thio and 4-thio groups had no deleterious effects on efficiency of this polymerase, and indeed, the increased bulk was associated with generally increased efficiency for incoming nucleotides, consistent with the recent observation that the Kf enzyme generally shows a kinetic preference for base analogues larger than the natural ones.[3]

Although the enzymatic incorporation of thiothymidines into DNA has been observed previously,[11] we are aware of only one previous kinetics study of either isomer. It was reported[12] that the **4S** dNTP derivative was incorporated opposite adenine with efficiency 1.3-fold higher than for incorporation of dTTP by the Kf enzyme; that is generally consistent with the current result. To our knowledge, no previous kinetics data exist for **2S** as the dNTP derivative nor for either isomer in a template strand of DNA.

We assessed the effects of the larger thio groups on replication selectivity by carrying out studies with all mismatched partners. Our data show that, consistent with its effects in hybridization, the analogue **4S** gave increased efficiency of **4S**-G mismatched pair synthesis, whether in a template strand or as a dNTP analogue. With the **4S** dNTP analogue, the overall fidelity of base pair synthesis was the same as for the natural nucleotide as a result of the fact that both the **4S**-A and **4S**-G pairs were equally increased in efficiency (Figure S3, Supporting Information). With **4S** in the template, however, the pair fidelity was decreased by the (possibly tautomeric) G-**4S** pair efficiency. The **2S** isomer behaved differently than **4S**; as a dNTP analogue, **2S** showed a high efficiency for being mispaired opposite T, leading to a lowered selectivity compared to that of natural thymidine. However, in a template strand, **2S** displayed higher fidelity than natural T, since the **2S**-G mispairing was suppressed significantly.

In summary, we find that the increased sizes of the thio groups at the 2- and 4-positions have significant effects on the hybridization properties of DNAs containing them, and on DNA replication as well. Some of the effects, including the increased stability and hybridization selectivity of **2S** and the high efficiency of polymerase replication of both isomers, may prove useful in a number of applications and may have relevance to the biological activity of other thionucleosides (thiopurines) in the treatment of leukemia.[13] Thio substitution of guanine also is known to affect hybridization and replication properties.[14] It remains to be seen whether other enzymes would respond similarly to such steric effects.[15]

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Supporting Information Available: Details of DNA synthesis and characterization, thermal denaturation, and enzyme kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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