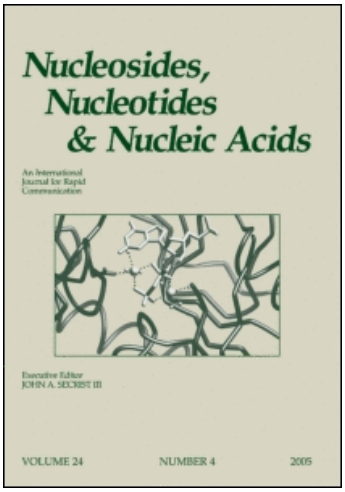

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SEQUENCING REACTIONS USING 6-THIO-2'-DEOXYGUANOSINE-5'-TRIPHOSPHATE

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ABSTRACT : 6-Thio-2'-Deoxyguanosine-5'-Triphosphate (6SdGTP) was employed as a substrate replacing dGTP in sequencing reactions with Sequenase™ and Thermo Sequenase™.

The antineoplastic drug 6-thioguanine can be metabolized *in vivo* as a substrate for DNA polymerases and when incorporated into cellular DNA causes cytotoxicity [1]. The phosphorylated derivative 6-Thio-2'-Deoxyguanosine-5'-Triphosphate (6SdGTP) is an active substrate for many purified DNA polymerases, including human [2]. Previous studies have shown that 6SdGTP is utilized by E. Coli DNA polymerase I at a rate of approximately 1/3 that of dGTP [3]. Here we report the performance of chemically synthesized[4] 6SdGTP in a series of sequencing reactions using Sequenase™ and Thermo Sequenase™ .

Initially, we undertook a primer extension study without ddNTP's to appraise 6SdGTP as a substrate for Thermo Sequenase™ DNA polymerase. As in all of this work, elongation occurred on single stranded M13mp18, starting at the -40 primer, using α^{35}S-dATP to introduce labels in newly-synthesized DNA. In labeling step reactions with all four native dNTP's extension occurs up to ~40 bases using 0.5 pmol of primer template and 3 pmol of each dNTP. Replacing dGTP with 6SdGTP revealed an elongation up to the first GG sequence at ~20 bases. Increasing the amount of 6SdGTP to 10 or 20 pmol allows normal extension distance, but with a different termination pattern.

A set of experiments were then performed varying the concentration ratio of ddGTP to 6SdGTP from 1:1 to 1:200. Thermo Sequenase™ reaction mixtures were run at pH = 9.5[5] and 65°C and Sequenase™ T7 DNA polymerase at pH = 7.5 [6] and 37°C. The results

are shown in FIG. 1. When the ratio of dd/6Sd is 1:1, both polymerases display satisfactory sequence ladder up to about 40 bases, the end of extension. At higher concentrations of 6SdGTP, Thermo Sequenase™ exhibits extensions up to 260 bases but a pattern of non-specific terminations (stops) was seen. These stops were predominant at multiple G sites, namely at the triple G site at 40 bases (CCC in the template strand) and at the quadruple G sites at 150 and 260 bases. In many experiments, extension would not proceed past the GGGG at 150. False terminations at A sites also occurred. Some false termination sites, such as GGAAG (128) and GAGTGAG (161), were at runs of purines. Sequenase™ T7 showed a much better pattern of band uniformity at a ratio of 1:10 (dd : 6Sd). A stop was still observed at 150 which is intensified at ratios of 100 and 200 to 1. However, many more false terminations are observed in the G lanes at A, T, and C sites than observed with Thermo Sequenase™ .

Does pH effect the band uniformity ? If the pKa of 6SdGTP is lower than the pH of the reaction mixture, perhaps the stops at multiple incorporation sites are caused by the addition of consecutive negatively-charged bases. The pKa of 6SdGTP was determined by UV titration at 250 nm and curve fitting. An inflection point was observed at pH = 8.43. A second series of sequencing experiments was then undertaken from pH = 7.5 to pH = 10 with Thermo Sequenase™ polymerase. The ratio of dd to 6Sd was 1:100 in all reactions. The pH of the reaction mixture had very little effect on the length of extension. Bands were somewhat more uniform at pH = 7.5 than at higher pH where again noticeable stops at multiple G incorporation sites were seen. The pH had very little effect on non-specific terminations.

The modified substrate 6SdGTP exhibits sequence specific differentiation in rate when in competition with ddGTP, slowing down in areas of multiple incorporation. A number of false termination patterns are also observed with this nucleotide. One explanation for these observations is the poor annealing property of the 6SG:C base pair in the enlarging duplex. Distortion of a double helix may cause the polymerase to refuse a weakly annealing substrate or to prematurely reject the growing primer altogether. To begin to answer some of these questions we turned to computational chemistry. *Ab initio* single point calculations were done on neighboring (same strand) G-G, 6SG-G, and the enolate form 6SHG-G bases in the geometry that they occur in double stranded DNA. These calculations look at the base stacking energies. All other atoms in the duplex were removed for the calculation. Initial runs were done using the restricted Hartree-Fock STO-3G basis set. The total energy and the electronic kinetic energies were determined for these cases. A comparison shows the total energy of the 6SG-G destabilized substantially over that of the G-G pair and the energy for 6SHG-G increases to an even greater extent. Electronic kinetic energy of both 6SG-G and 6SHG-G pairs increased by the same extent

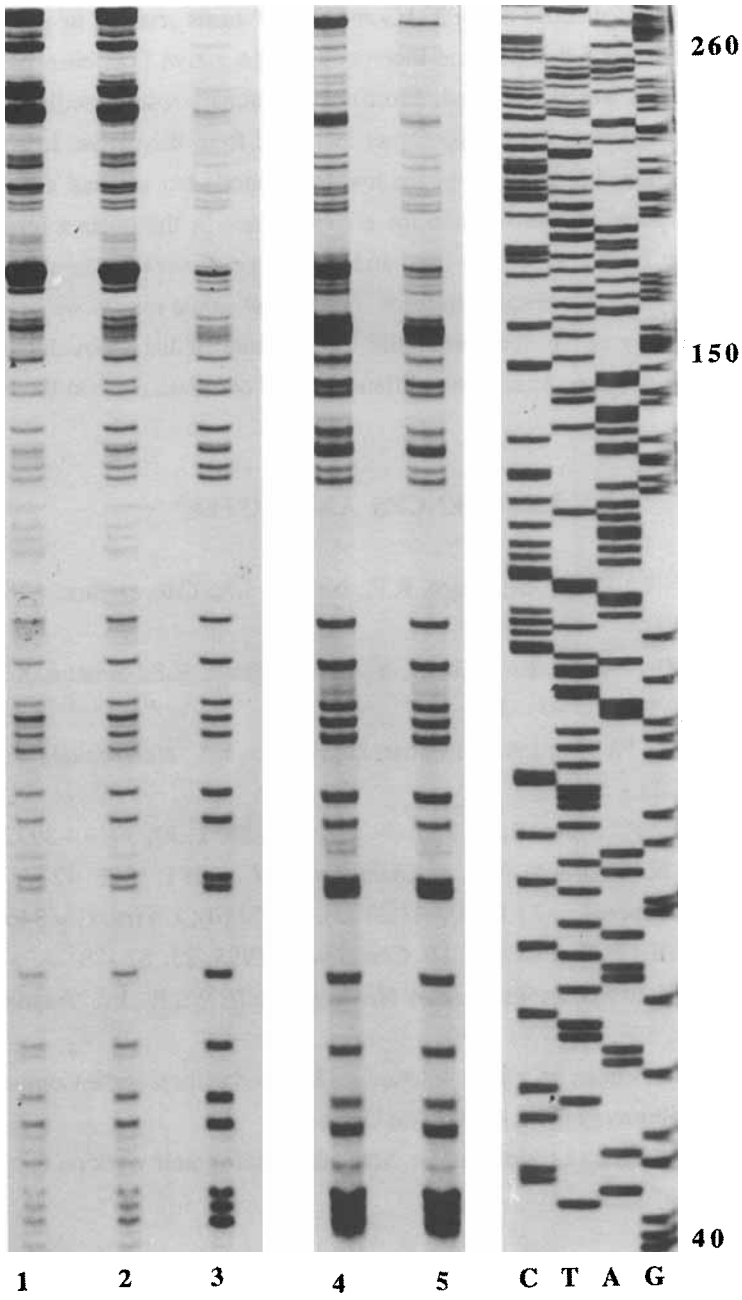

Figure 1: All lanes (from ~40 to 260 shown here) represent sequencing termination reactions using single stranded M13mp18 as template, the -40 universal cycle primer, and internal labelling α^{35}S-dATP. Lanes 1 through 3 were run with Sequenase™ T7 at pH = 7.5 at 37°C and lanes 4, 5, C, T, A, and G use Thermo Sequenase™ at pH = 9.5 at 65°C. All numbered lanes represent G lanes using 6SdGTP to ddGTP in the following ratios: lane 1 (200:1); lane 2 (100:1); lane 3 (10:1); lane 4 (200:1), lane 5 (100:1).

over G-G. Further calculations using 321G and 631G* basis sets are in progress.[7] The geometry of a modified 6-thio-guanine incorporated into a five G:C base pair stretch of double stranded DNA was also studied. From our preliminary results we find that all three hydrogen bonding distances in the 6SG:C pair increased from the native. In a normal G:C pair the bases are aligned in the plane of the hydrogen bonds but we find a skewing of ~5 degrees from the plane of the bases in the 6SG:C pair. In the enolate form of 6-thio-guanine, hydrogen bonds (now only two) and the degree of skewing in the plane of the bases increased beyond that of the keto form. From these initial results we believe that the modified base 6-thio-guanine does destabilize the structure of the DNA duplex. Further work is in progress to better define these differences and correlate them to the experimental results.

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7. This work was done on a Silicon Graphics Iris workstation at the Computational Biology Laboratory at the Ohio State University.
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