

6-Thio-2'-Deoxyinosine: Synthesis, Incorporation, and Evaluation as a Postsynthetically Modifiable Base in Oligonucleotides

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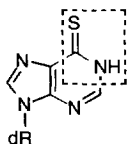
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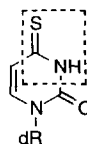
Abstract: The synthesis of 6-thio-2'-deoxyinosine (d^{S6I}) in a form suitably protected for solid-phase oligonucleotide synthesis is reported. This thionucleic acid was incorporated in high yield into short oligodeoxynucleotides, and the thiocarbonyl group could be modified by S-alkylation with complete chemoselectivity. The quantitation of incorporation and facile post-synthetic modification was demonstrated by enzymatic digestion and HPLC analysis, and the effect of covalent alkylation was determined by ΔT_m measurements of the corresponding duplex oligonucleotides with dC as the complementary base. © 1997 Elsevier Science Ltd.

Introduction

Since the 1950's, 6-mercaptopurine (6-MP) has been used for the treatment of acute leukemia and as an immunosuppressive agent.¹ It is generally accepted that the cytotoxicity of 6-mercaptopurine is due to *in vivo* conversion to 6-thioguanosine-5'-triphosphate via the purine salvage pathway, and the subsequent incorporation of significant levels of 6-thioguanosine into DNA and RNA.² The introduction of 6-thioguanosine into DNA may induce damage such as single strand cleavage, DNA-protein cross-links, interstrand cross-links, and sister chromatid exchanges. However, our interest in thionucleic acids was not the investigation of 6-mercaptopurine metabolism or biological activity, but rather the use of the reactive thiocarbonyl group as a easily manipulable site for the chemoselective introduction of reporter groups and cross-linking tethers into duplex oligonucleotides.



6-thio-2'-deoxyinosine (d^{S6I}, **1**)



4-thio-2'-deoxyuridine (d^{S4U})

In concert with our goal of template-directed covalent cross-linking of oligonucleotides,³ we have examined various modified nucleic acids as platforms upon which to tether chemically reactive functionality. Herein, we report the results of our studies on the incorporation of 6-thio-2'-deoxyinosine (d^{S6I}, **1**) into

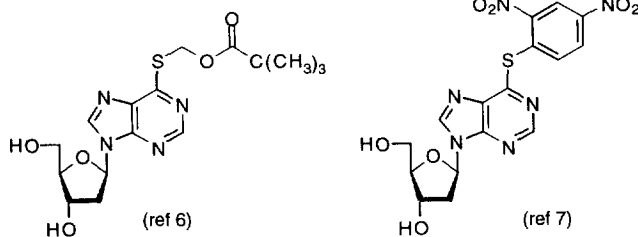
Dedicated with respect and admiration to Professor Samuel J. Danishefsky.

synthetic oligonucleotides, and we demonstrate the suitability of this nucleoside as a purine-based platform upon which to append tethered functionality within the major groove of duplex DNA. We demonstrate the quantitative postsynthetic modification of the thiocarbonyl group with alkylating agents, and the quantitation of modified bases by enzymatic digestion.

The impetus to investigate 6-thio-2'-deoxyinosine (**1**) as a potential pendent site stems from our previously successful studies on the postsynthetic modification^{4,5} and covalent cross-linking³ of 4-thio-2'-deoxyuridine. 6-Thio-2'-deoxyinosine has a thiocarbonyl group that can be manipulated chemoselectively to attach tethered electrophiles within synthetic strands of oligonucleotides. Molecular models of a 6-thio-2'-deoxyinosine base within the 9-mer 5'-d(ACGA^{S6}ICCAC) indicated that tethers attached via the C6 sulfur of the purine analog would be ideally suited to position chemically interesting functional groups within the major groove of duplex DNA, appropriately positioned to interact with the complementary strand(s) of DNA.

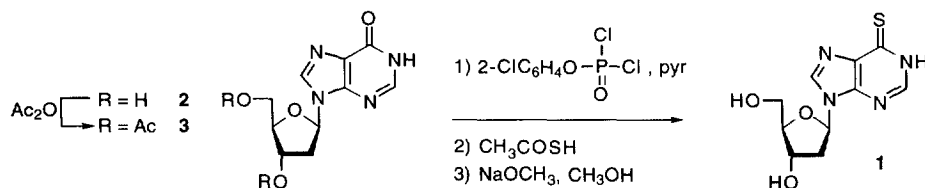
Synthesis of 6-Thio-2'-Deoxyinosine

The synthesis and incorporation of 6-thio-2'-deoxyinosine into synthetic oligonucleotides has been described previously. In a report by Clivio and co-workers,⁶ this thionucleoside was incorporated into an oligonucleotide of unspecified sequence using an *S*-pivaloyloxymethyl protecting group, which was removed by treatment with conc. NH₄OH at room temperature. The extent of incorporation of the thionucleic acid was not reported. In a report by Xu and co-workers,⁷ the thiocarbonyl group was "protected" with a 2,4-dinitrophenyl group, which could be displaced with a sulfur nucleophile at the completion of the oligomer synthesis to reintroduce the thiocarbonyl group. The yields for thionucleoside incorporation and post-synthetic derivatization were not reported. More recently, Xu reported on the incorporation of 6-(methylthio)purines into DNA.⁸

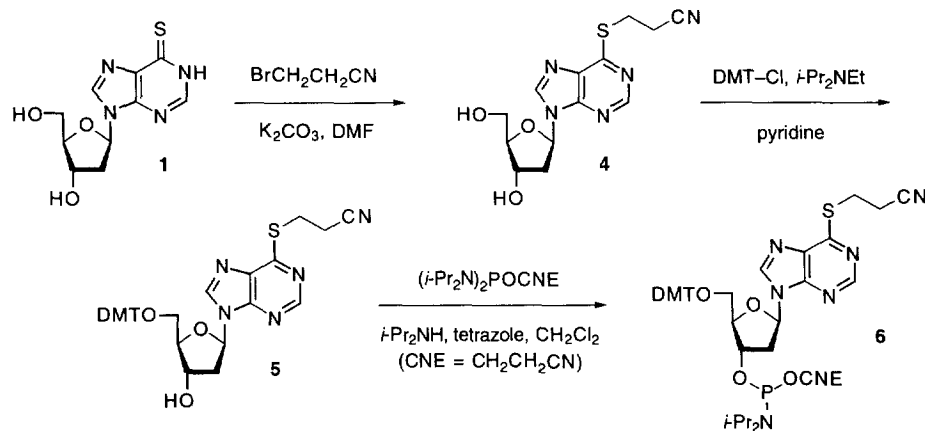


The synthesis described herein utilizes the highly effective *S*-cyanoethyl ether to protect the thiocarbonyl group of d^{S6}I. We first described the use of the *S*-cyanoethyl group for sulfur protection of 4-thio-2'-deoxyuridine during oligonucleotide synthesis in 1991,⁹ and it has been used successfully by a number of other workers for incorporation of thionucleic acids into DNA and RNA.¹⁰ Acylation of 2'-deoxyinosine (**2**) gave bis-*O*-acetyl inosine (**3**) in quantitative yield. Methods for conversion of amide carbonyl groups to the corresponding thiocarbonyl have historically used P₂S₅,^{11,12} but we found **3** to be inert to thionation with the more easily handled Lawesson's reagent (2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide).¹³ Activation of the C6 oxygen as the corresponding 6-pyridyl derivative was reported by Adamiak and

co-workers,¹⁴ and this method proved to be effective in this and other studies for the synthesis of thionucleosides. Following the basic procedure of Xu, *et al.*,⁷ treatment of **3** with 2-chlorophenyl dichlorophosphate in dry pyridine formed the corresponding C6-pyridinium salt. Nucleophilic displacement of pyridine with thiolacetic acid formed 3',5'-bis-*O*-acetyl-6-thio-2'-deoxyinosine after an aqueous work-up. Methanolysis of the *O*-acetyl esters afforded 6-thio-2'-deoxyinosine (dS⁶I, **1**) in 68% overall yield.



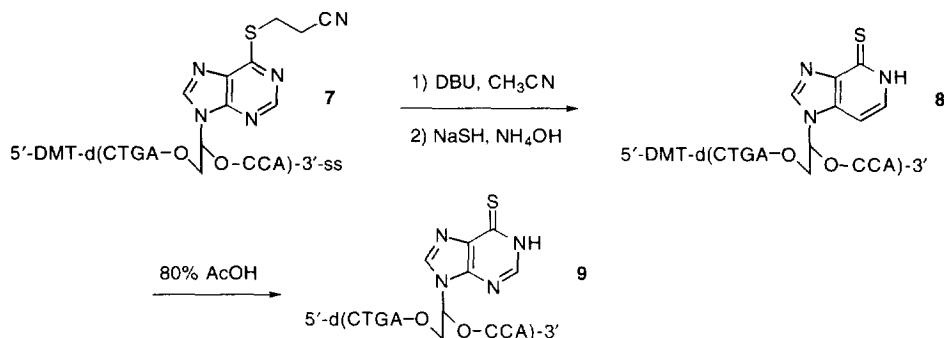
Alkylation of the thiocarbonyl of **1** with 3-bromopropionitrile (K₂CO₃, DMF) afforded the *S*-cyanoethyl ether **4** in 85% yield.¹⁵ Treatment of **4** with dimethoxytrityl chloride (*i*-Pr₂NEt, pyridine)¹⁶ gave the 5'-DMT ether (**5**), and subsequent phosphitylation [(*i*-Pr₂N)₂POCNE, tetrazole, *i*-Pr₂NH, CH₂Cl₂] of the remaining free 3'-hydroxyl group under standard conditions¹⁷ afforded phosphoramidite **6** in 69% yield.



Incorporation of 6-Thio-2'-Deoxyinosine into a Synthetic Oligonucleotide

The phosphoramidite **6** was incorporated using standard techniques¹⁸ into the 8-mer oligonucleotide 5'-d(CTGA^{S6}ICCA) (**9**). In previous studies on thionucleic acids, we⁵ and others¹⁹ have found that ammonolysis of a thioimidate can lead to nucleophilic displacement by NH₃, to afford the corresponding cytidine residue. To circumvent this ammonolysis, the support-linked oligonucleotide **7** was first treated with DBU (1 M in CH₃CN) to remove the *S*- and *O*-cyanoethyl groups prior to cleavage from the solid support. The oligomer was liberated with conc. NH₄OH in the presence of NaSH²⁰ (50 mM) to afford the 5'-DMT ether **8**. Purification of **8** by RP-HPLC (gradient elution: 95-50% A over 40 min; A = 0.1 M triethylammonium acetate, pH = 6.5; B = 100% CH₃CN) removed failure sequences and other by-products. Removal of the DMT-ether

(80% acetic acid, 25 °C) followed by a second RP-HPLC (92-75% A over 30 min) purification afforded the 8-mer 5'-d(CTGA^{S6}ICCA) (**9**). Levels of incorporation of d^{S6}I phosphoramidite **6** into oligonucleotide **7** as measured by trityl cation release were variable, but typically exceeded 80%.



The incorporation of d^{S6}I (**1**) into the oligonucleotide **9** was evident by a peak in the UV absorbance spectrum of **9** at $\lambda = 324$ nm corresponding to the absorbance of 6-thio-2'-deoxyinosine (upper trace), which was not present in the spectrum of normal DNA (lower trace).

Enzymatic digestion of the purified 8-mer 5'-d(CTGA^{S6}ICCA) with snake venom phosphodiesterase and calf intestinal alkaline phosphatase²¹ (10 mM MgCl₂, 10 mM HPO₄²⁻, pH 7.0) gave the expected ratio of nucleosides by RP-HPLC analysis (Table 1).

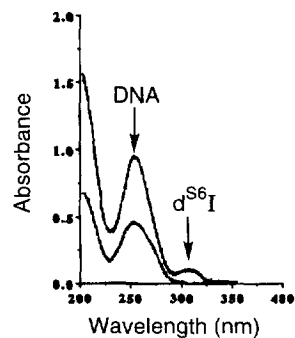


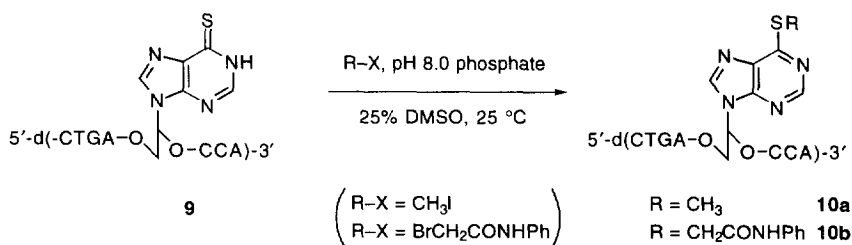
Table 1 – Enzymatic Digestion/HPLC Integration Ratios for Oligomer 9

Nucleoside	RT (min) ^a	Theoretical	Found
dC	3.8	3	3.3
dG	6.9	1	1.0
T	8.9	1	1.2
d ^{S6} I	9.8	1	0.9
dA	11.0	2	2.2

^aGradient elution: 98-88% A, 12 min; 88-40% A, 8 min; 40-25% A, 5 min; A = 0.1 M triethylammonium acetate, pH = 6.5; B = 100% CH₃CN

Post Synthetic Modification

A goal of this project was to modify the oligonucleotide postsynthetically at the thiocarbonyl group of the 6-thio-2'-deoxyinosine base in a *chemoselective* fashion. Confident that d^{S6}I had been synthetically incorporated into a strand of DNA, we examined the modification of the oligonucleotide by alkylation of the thiocarbonyl with simple alkylating agents. The alkylation products were then characterized and quantified to determine the chemoselectivity of these reactions.



The 8-mer 5'-d(CTGA^{S6}ICCA) (**9**) contains each of the four natural nucleosides found in DNA, in addition to 6-thio-2'-deoxyinosine. The shorter length of the oligonucleotide was chosen so that upon alkylation of the thiocarbonyl, maximal change in retention time would be observed by RP-HPLC analysis. Treatment of **9** (RT = 10.0 min) with iodomethane in phosphate buffer (0.1 M HPO₄²⁻, pH 8.0) and 25% DMSO as co-solvent afforded postsynthetically modified oligonucleotide **10a** with complete consumption of **9** after 30 min. Treatment of **9** with *N*-(phenyl)- α -bromoacetamide under identical conditions afforded **10b** (see Table 2 for changes in oligonucleotide retention times).

Table 2 – Retention Times of Alkylated Oligomer 10

Entry	Oligonucleotide	R	RT min ^a
1	d(CTGA ^{S6} ICCA) 9	–	10.0
2	d(CTGA ^{RS} ICCA) 10a	CH ₃	12.2
3	d(CTGA ^{RS} ICCA) 10b	CH ₂ CONHPh	17.2

^aRP-HPLC, gradient 92-75% A, 30 min; A = 0.1 M triethylammonium acetate, pH 6.5; B = 100% CH₃CN

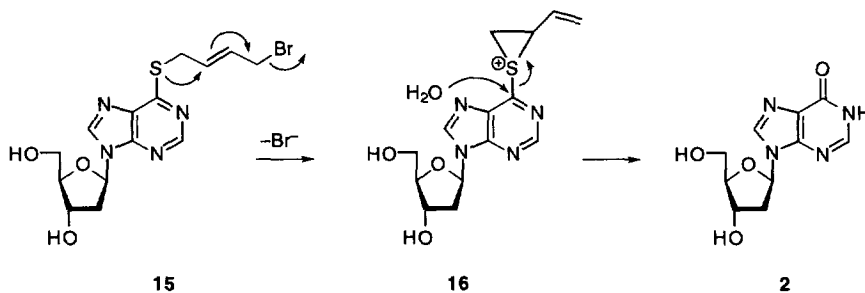
The alkylated 8-mers (**10a**, **10b**) were digested enzymatically to their constituent 2'-deoxynucleosides for quantitation of postsynthetic modification of the thiocarbonyl group. The oligonucleotides (0.5 OD₂₆₀) were treated with snake venom phosphodiesterase and calf intestine alkaline phosphatase in standard assay buffer for \geq 12 h. Longer reaction times were required for oligonucleotides containing modified thioinosine residues. The reaction mixtures were microfiltered and analyzed by RP-HPLC. The expected ratios of the deoxynucleosides were observed (Table 3).²² No covalent modification of other bases was evident by RP-HPLC, and no unreacted 6-thio-2'-deoxyinosine was present.

Table 3 – Enzymatic Digest Data for Alkylated Oligomer 10

Entry	Nucleoside	RT min ^{a,b}		Theoretical Ratio	Found ^b		ϵ_{254}^c ($\times 10^{-3}$)
		10a	10b		10a	10b	
1	dC	4.1	4.0	3	3.2	2.8	6.0
2	dG	7.7	7.8	1	1.0	0.93	13.5
3	T	9.1	9.1	1	1.0	1.0	7.0
4	dA	11.4	11.4	2	2.1	2.1	14.3
5	RS6I	19.5	–	1	0.9	–	28.7 ^d
6	RS6I	–	21.5	1	–	1.1	26.0 ^e

^aRP-HPLC, gradient 98-88% A, 12 min; 88-40% 8 min; 40-25% 5 min; A = 0.1 M triethylammonium acetate, pH = 6.5; B = 100% CH₃CN. ^b10a (R = CH₃), 10b (R = CH₂CONHPh); ^cvalues taken from reference 23; ^dmeasured experimentally as ϵ_{283} ; ^emeasured experimentally as ϵ_{280} .

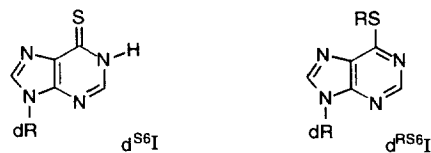
In the course of looking at alkyl groups containing additional chemically reactive functionality, we examined a number of bis-alkyl halides in the alkylation of d^{S6}I within oligonucleotides. Alkylation reactions of **1** with *trans*-1,4-dibromo-2-butene took an undesirable pathway, which was elucidated by ¹NMR. In deuterated phosphate buffer (DPO₄²⁻, pD 7.5, 50% d₆-DMSO) the reaction mixture showed several products, principally 2'-deoxyinosine (**2**). Presumably, the formation of **2** begins with the initial formation of the alkyl sulfide **15**. The sulfide proved sufficiently nucleophilic to attack the electrophilic tether to form a presumed intermediate thiiranium ion **16**. Displacement of thiirane by water led to the formation of 2'-deoxyinosine. (This type of rearrangement/hydrolysis was also observed with 1,4-dibromobutane and also in analogous alkylation studies with 4-thio-2'-deoxyuridine.)²⁴



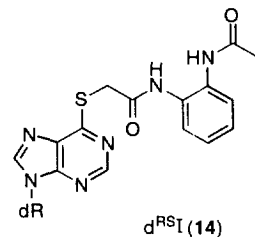
Duplex Stability Studies

Incorporation of 6-thio-2'-deoxyinosine (**1**) into DNA was expected to alter the stability of the corresponding duplex oligonucleotides, and changing the tautomeric form of 6-thio-2'-deoxyinosine (d^{S6}I) to the

S-alkyl thioimidate (d^{RSI}) would further alter the base-pairing characteristics of the nucleoside. This mismatch that would be created was expected to moderately destabilize double-helical DNA oligomers, depending upon the identity of the complementary base-pairing nucleoside and the chemical nature of R.



It has been demonstrated by many workers that single mismatches do not seriously destabilize duplex structure.²⁵ This expectation was confirmed experimentally in the present studies by thermodynamic characterization using ΔT_m measurements.²⁶ For these studies, we used a longer 17 base oligonucleotide in order to provide more stable duplex structures with easily measurable melting transitions. Using the above synthesis and postsynthetic modification protocols, 6-thio-2'-deoxyinosine (d^{S6I} , **1**), *S*-methyl 6-thio-2'-deoxyinosine (d^{MeS6I} , **12**) and *S*-(2-(*N*-(2-(*N*-acetyl)amino)phenyl)acetamido)-6-thio-2'-deoxyinosine (d^{RSI} , **14**)²⁷ were incorporated within the 17 base-pair T7 RNA polymerase promoter sequence 5'-d(TAATACGAXCCACTATA)-3' (X = d^{S6I} , d^{MeS6I} , or d^{RSI}) and were annealed with the complementary strand 3'-d(ATTATGCTCGGTGATAT)-5'.²⁸ These experiments provided a direct measure of the duplex destabilization caused by the presence of the non-natural bases within an oligonucleotide.



ΔT_m Results

The d^{S6I} (**1**) containing oligonucleotide and its complementary strand in SSC buffer (2.5 M sodium chloride, 1.5 mM sodium citrate, pH 7.2, 0.5 mL) were heated to 90 °C and allowed slowly to re-cool to 25 °C overnight. The melting temperature (T_m) was measured as 65 °C. The T_m of the *S*-alkylated oligonucleotides containing *S*-methyl-6-thio-2'-deoxyinosine (d^{MeS6I}) **12** or *S*-(2-(*N*-(2-(*N*-acetyl)amino)phenyl)acetamido)-6-thio-2'-deoxyinosine (d^{RSI}) **14** were found to be 55 °C and 60 °C, respectively in SSC buffer. These results are consistent with those observed by Xu.⁷ Thus, the addition of an *S*-alkyl group moderately destabilized the duplex, although the phenylenediamine derivative **14** formed a more stable duplex than the simple *S*-methyl oligomer, possibly due to hydrogen bonding between the amides on the *o*-phenylenediamine and the exocyclic amine on the complementary dC or C6-carbonyl group on the adjacent dG residues.³

Conclusion

The results presented herein demonstrate three important objectives in the project for covalent cross-linking of DNA:³ (1) synthesis of the thionucleic acid 6-thio-2'-deoxyinosine, protected as the *S*-cyanoethyl ether,⁹ for use on an automated DNA synthesizer; (2) the effective incorporation of this modified base into a synthetic oligonucleotide, the identification of the modified base by UV spectroscopy, and quantification of 6-thio-2'-deoxyinosine in the enzymatic digest; and, (3) the quantitative and chemoselective postsynthetic alkylation of the thiocarbonyl group⁵ within the 8-mer 5'-d(CTGAS⁶ICCA) and the identification and quantification of the alkylated nucleoside by enzymatic digestion.

EXPERIMENTAL SECTION

Tetrazole was sublimed *in vacuo* and stored over desiccant. Bis(4-methoxyphenyl)phenylmethyl chloride was recrystallized from 2,2,4-trimethylpentane and stored over desiccant. Synthetic oligonucleotides were synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer. All HPLC solvents were filtered through a 0.45 μm FP Vericel membrane filter and degassed with helium prior to use. Acetic acid was distilled prior to use for HPLC buffers. Deuteriochloroform (CDCl_3) was passed through a small plug of Al_2O_3 immediately prior to use. Reverse-phase high performance liquid chromatography (RP-HPLC) was performed using a Perkin-Elmer system consisting of a Model 250 Biocompatible LC Pump, an LC-290 Spectrophotometric Detector, and an LCI-100 Laboratory Computing Integrator. All chromatography was performed using a Hamilton PRP-1 reverse-phase column, 10 μm particle size, 4.1 \times 250 mm. Mobile phases: A = 0.1 M triethylammonium acetate, pH 6.5; B = CH_3CN .

S-(2-Cyanoethyl)-6-Thio-2'-deoxyinosine (4). A solution of **1** (0.30 g, 1.1 mmol) in dry DMF (5 mL) was treated with 3-bromopropionitrile (0.19 mL, 2.2 mmol, 2 equiv) and K_2CO_3 (0.31 g, 2.2 mmol, 2 equiv) under N_2 at 27 $^\circ\text{C}$. After 12 h, the reaction mixture was filtered through Celite[®] and concentrated *in vacuo* (2 \times 5 mL xylenes). The residue was purified by flash chromatography (2 \times 10 cm silica, 50-100% CH_2Cl_2 -hexanes then 0-10% EtOH/ CH_2Cl_2) to afford **4** (0.32 g, 0.36 g theor., 90%) as an oil: ^1H NMR (300 MHz, DMSO-d_6) δ 8.73 (s, 1 H, C2-H), 8.69 (s, 1 H, C8-H), 6.41 (t, $J = 7.5$ Hz, 1 H, C1'-H), 5.34 (d, $J = 3.0$ Hz, 1 H, C3'-OH), 4.96 (t, $J = 6.0$ Hz, 1 H, C5'-OH), 4.43 (m, 1 H, C3'-H), 3.87 (m, 1 H, C4'-H), 3.59 (t, $J = 6.0$ Hz, 2 H, $\text{SCH}_2\text{CH}_2\text{CN}$), 3.55 (m, 1 H, C5'-H), 3.48 (m, 1 H, C5'-H), 3.02 (t, $J = 6.0$ Hz, 2 H, $\text{SCH}_2\text{CH}_2\text{CN}$), 2.72 (m, 1 H, C2'-H), 2.33 (ddd, $J = 12.0, 6.0, 3.0$ Hz, 1 H, C2'-H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.2, 156.7, 153.3, 148.7, 136.4, 124.4, 93.2, 89.1, 75.8, 66.7, 44.6, 28.8, 23.2; IR (neat) ν_{max} 3420, 2894, 2240, 1663, 1432, 1275, 1181, 1050, 883, 752 cm^{-1} .

S-(2-Cyanoethyl)-5'-O-(bis(4-methoxyphenyl)phenylmethyl)-6-thio-2'-deoxyinosine (5). A solution of **4** (0.28 g, 0.88 mmol), co-evaporated with pyridine (2 \times 5 mL), in dry pyridine (5 mL) was treated with diisopropylethylamine (150 μL , 0.88 mmol, 1 equiv) and bis(4-methoxyphenyl)phenylmethyl chloride (0.45 g, 1.3 mmol, 1.5 equiv) under N_2 at 25 $^\circ\text{C}$. After 2 h, the reaction mixture was diluted with satd. aqueous NaHCO_3 (15 mL) and extracted with CH_2Cl_2 (3 \times 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated *in vacuo*, and the residue was purified by flash chromatography (4 \times 10 cm Et_3N deactivated silica, 0-100% CH_2Cl_2 /hexanes then 5% MeOH/ CH_2Cl_2) to afford **5** (0.31 g, 0.55 g theor., 55%) as a foam: ^1H NMR (500 MHz, CDCl_3) δ 8.61 (s, 1 H, C2-H), 8.10 (s, 1 H, C8-H), 7.34 (dd, $J = 8.3, 1.7$ Hz, 2 H, ArH), 7.23 (m, 7 H, ArH), 6.75 (d, $J = 2.1$ Hz, 4 H, ArH), 6.44 (app t, $J = 6.4$ Hz, 1 H, C1'-H), 4.63 (m, 1 H, C3'-H), 4.14 (m, 1 H, C4'-H), 3.74 (s, 6 H, OCH_3), 3.56 (t, $J = 6.9$ Hz, 2 H, $\text{SCH}_2\text{CH}_2\text{CN}$), 3.35 (d, $J = 7.1$ Hz, 2 H, C5'-H), 2.89 (t, $J = 6.9$ Hz, 2 H, $\text{SCH}_2\text{CH}_2\text{CN}$), 2.89 (ddd, $J = 13.2, 6.3, 3.0$ Hz, 1 H, C2'-H), 2.52 (ddd, $J = 13.2, 5.9, 3.1$ Hz, 1 H, C2'-H); ^{13}C NMR (125 MHz, CDCl_3) δ 159.0, 152.6, 152.2, 144.0, 142.1, 136.0, 130.4, 128.4, 128.3, 127.6, 113.6, 88.6, 87.0, 84.9, 73.0, 55.6, 46.6, 40.6, 24.8, 19.1; IR (neat) ν_{max}

3394, 2933, 2256, 1600, 1564, 1507, 1251 cm^{-1} ; FABMS (relative intensity) m/z 624 ($M + H^+$, 5), 303 (60), 258 (10), 206 (10), 154 (base); HRMS, m/z calcd for $C_{34}H_{34}N_5O_5S$: 624.2281, found: 624.2252.

S-(2-Cyanoethyl)-5'-O-(bis(4-methoxyphenyl)phenylmethyl)-6-thio-2'-deoxyinosine 3'-O-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (6). A solution of **5** (0.20 g, 0.33 mmol) in anhydrous CH_2Cl_2 (4.0 mL) was treated sequentially with tetrazole (12 mg, 0.17 mmol, 0.5 equiv), dry diisopropylamine (24 μL , 0.17 mmol, 0.5 equiv), and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (0.13 mL, 0.40 mmol, 1.2 equiv) under N_2 at 27 °C. After 3 h, the reaction mixture was diluted with CH_2Cl_2 (30 mL), washed with satd. aqueous NaHCO_3 (10 mL), and the organic extracts were dried (Na_2SO_4). The solvent was removed *in vacuo* and the residue was purified by flash chromatography (1 \times 16 cm Et_3N deactivated silica, 20-100% CH_2Cl_2 /hexanes) to afford a diastereomeric mixture of **6** (0.19 g, 0.28 g theor., 69%) as a white foam: ^1H NMR (400 MHz, CDCl_3) δ 8.61 (d, $J = 1.3$ Hz, 1 H, C2-H), 8.16 (d, $J = 7.2$ Hz, 1 H, C8-H), 7.35 (m, 2 H, ArH), 7.23 (m, 7 H, ArH), 6.75 (m, 4 H, ArH), 6.44 (m, 1 H, C1'-H), 4.75 (m, 1 H, C3'-H), 4.30 (m, 1 H, C4'-H), 3.75 (s, 3 H, OCH_3), 3.74 (s, 3 H, OCH_3), 3.68 (m, 2 H, $\text{NCH}(\text{CH}_3)_2$), 3.57 (t, $J = 7.0$ Hz, 2 H, $\text{SCH}_2\text{CH}_2\text{CN}$), 3.34 (m, 2 H, C5'-H), 2.90 (t, $J = 6.3$ Hz, 2 H, $\text{OCH}_2\text{CH}_2\text{CN}$), 2.74 (m, 1 H, C2'-H), 2.60 (m, 1 H, C2'-H), 2.59 (t, $J = 7.0$ Hz, 2 H, $\text{SCH}_2\text{CH}_2\text{CN}$), 2.44 (t, $J = 6.3$ Hz, 2 H, $\text{OCH}_2\text{CH}_2\text{CN}$), 1.22 (d, $J = 6.8$ Hz, 6 H, $\text{CH}(\text{CH}_3)_2$), 1.16 (d, $J = 6.8$ Hz, 6 H, $\text{CH}(\text{CH}_3)_2$); ^{13}C NMR (100 MHz, CDCl_3) δ 158.8, 158.7, 158.5, 151.7, 148.4, 144.5, 141.2, 135.6, 131.9, 131.2, 130.1, 130.0, 128.2, 128.1, 128.0, 127.8, 126.9, 126.8, 118.2, 117.6, 117.4, 113.2, 86.5, 86.1, 86.0, 85.9, 85.8, 84.8, 77.4, 77.1, 76.8, 74.2, 74.0, 73.5, 73.4, 63.4, 63.2, 58.4, 58.3, 58.2, 58.1, 55.2, 46.3, 45.9, 43.3, 43.2, 39.5, 39.4, 24.7, 24.6, 24.5, 24.4, 20.4, 20.3, 20.2, 18.7; ^{31}P NMR (121 MHz, CDCl_3) δ 148.96, 148.85; EIMS (relative intensity) m/z 824 ($M + H^+$, 5), 401 (15), 303 (base), 284 (50), 272 (40), 244 (40), 226 (55).

General Method for ΔT_m Studies. The oligonucleotides 5'-d(TAATACGA S6 ICCACTATA)-3' (0.5 OD $_{260}$) and its complement 3'-d(ATTATGCTCGGTGATAT)-5' (0.5 OD $_{260}$) were mixed in SSC buffer (500 μL , 2.5 M NaCl, 1.5 mM sodium citrate, pH 7.2) at 90 °C and allowed to slowly cool to 25 °C overnight. The hybridization solution was placed in a 1 mL cuvette and the absorbance was measured with a UV/vis spectrometer equipped with a temperature controlled thermal bath. The absorbance was measured at $\lambda = 260$ nm and the temperature was increased in 5 °C increments from 25 to 85 °C. When the bath reached the desired temperature, the solution was allowed to equilibrate for 25 min prior to the next absorbance measurement.

General Method for the Synthesis of Oligonucleotides. Phosphoramidite chemistry using β -cyanoethyl protecting groups and standard solid-phase synthesis protocols were used. Phosphoramidite **6** was used to incorporate 6-thio-2'-deoxyinosine ($d^{S6}\text{I}$) residues. Standard exocyclic amine protecting groups were used (isobutryl for dG, benzoyl for dA and dC). For **6**, coupling time was increased to 8 min.

Oligonucleotide 5'-d(CTGA S6 ICCA) (**9**) was synthesized on a 1.0 μmol scale. β -Cyanoethyl groups were removed with 1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in CH_3CN (25 °C, 3 h) while the oligonucleotide was still attached to the solid support. Cleavage from the support and base deprotection was accom-

plished using conc. NH_4OH containing 50 mM NaSH (16 h, 25 °C). Following RP-HPLC purification of the 5'-DMT material (95-50% A over 40 min), the product fraction was lyophilized and the trityl ether was removed with 80% acetic acid (200 μL , 1 h, 25 °C). The solution was concentrated to dryness, taken up in buffer A (1 mL), passed through a 0.2 μm membrane filter (Gelman 0.2 μm Acrodisc LC13), and the final d^{S6}I -containing product was isolated by RP-HPLC (92-75% A over 30 min).

General Method for Postsynthetic Modification. The oligonucleotide **9** ($\approx 1.0 \text{ OD}_{260}$) in phosphate buffer (0.1 M HPO_4^{2-} , pH 8.0, 100 μL) was treated with iodomethane (33 mM in DMF, 40 μL) at 25 °C. After 30 min, the reaction mixtures were diluted with H_2O (400 μL) and extracted with ether ($3 \times 500 \mu\text{L}$). The residual ether was removed under a stream of nitrogen and the modified oligomer was isolated by RP-HPLC (92-75% A over 30 min) to afford a solution **10a**. The solvent was removed under a stream of nitrogen.

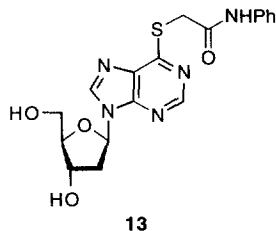
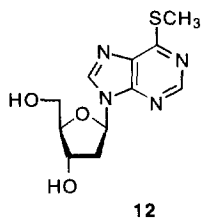
General Method for Enzymatic Digestion of Oligonucleotides. Enzyme digestion of oligonucleotides was performed with snake venom phosphodiesterase from *Crotalus durissus* (2 mg/mL, approximately 1.5 U/mg) and alkaline phosphatase from calf intestine (1 U/ μL), obtained from Boehringer Mannheim. Treatment of a solution of oligonucleotide ($\approx 0.5 \text{ OD}_{260}$) in 10 mM potassium phosphate buffer (100 μL , pH 7) containing 10 mM MgCl_2 with snake venom phosphodiesterase (5 μL , 7.5 U) and alkaline phosphatase (5 μL , 5 U). The mixture was incubated for 3 h at 37 °C (overnight for the modified oligonucleotides), passed through a 0.2 μm membrane filter (Gelman 0.2 μm Acrodisc LC13), and analyzed by RP-HPLC (1 mL/min; linear gradient: 98-88% A, 12 min; 88-40% 8 min; 40-25%; 5 min; A: 0.1 M triethylammonium acetate pH = 6.5; B: 100% CH_3CN), detection at 254 nm initially, changed to 332 nm at 9 min for detection of d^{S6}I , changed to 254 nm at 10 min for the detection of dA, and changed to the λ_{max} of the S-alkylated bases at 12 min. Extinction coefficients of the natural bases were as reported by Connolly,²³ and those of the non-natural bases were determined experimentally. Peaks on the HPLC were identified by retention time comparison with authentic samples. Extinction coefficients for oligonucleotides containing non-natural bases, ϵ -values were calculated using published procedures,²⁵ first by calculating the natural bases and then adding on the experimentally determined extinction coefficient of the non-natural nucleoside.

ACKNOWLEDGMENT. This work was supported by a grant from the National Institutes of Health (GM-47991). We thank American Cyanamid, Pfizer, and Bristol-Myers Squibb for their support. RSC is the recipient of a Camille and Henry Dreyfus Foundation Distinguished New Faculty Award (1989-94), an American Cancer Society Junior Faculty Research Award (1990-93), the American Cyanamid Young Faculty Award (1993-96), and an Alfred P. Sloan Foundation Research Fellowship (1995-97). JCA was the recipient of a Department of Education Graduate Fellowship (1991-92). We thank Regina G. York (University of South Carolina) for performing oligonucleotide syntheses.

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(Received 6 September 1996; accepted 6 November 1996)