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The 6-thioguanine/5-methyl-2-pyrimidinone base pair

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ABSTRACT

As part of a program to determine the physical possibility of expanding the number of types of base pairs in DNA, the pairing stabilities of the analog bases 6-thioguanine (G^S) and 5-methyl-2-pyrimidinone (T^B) in oligodeoxynucleotides were measured. Procedures were developed to synthesize oligodeoxynucleotides with the analog bases. The sequences of the synthesized oligomers were T-C-G-A-C-G-G-X-Y-C-C-G. An enzymatic procedure was developed to measure relative association constants of oligomer pairs with the self complementary reference oligomer, X=A and Y=T, K(T/A)=K. The results were $K(C/G)=(5\pm.5)K$, $K(T^B/G^S)=K/(1\pm.5)$, $K(T/G)=K/(9\pm3)$, $K(T^B/G)=K/(25\pm5)$, $K(C/G^S)<K/30$, $K(T^B/A)<K/40$, $K(T/G^S)<K/40$, $K(C/A)<K/40$. The results with the standard bases are consistent with other methods of measurement. The stability of the base pair G^S/T^B is approximately the same as the standard base pair A/T.

INTRODUCTION

A number of physical and biological investigations of mismatch base pairs (1-11) and investigations of base pair interactions where one base is an analog of the standard bases (12-18) have been reported. The question of whether base pairs with both bases being analogs can have the stability properties of the standard base pairs when incorporated into DNA has not been addressed in the literature. A suitable model for comparing the stabilities of the various combinations of base pairs is a comparison of the association constants between pairs of oligonucleotides (1). Each pair of oligomers contains standard base pairs at all positions along the duplex molecule except one, where the different combinations of base pairs are present.

The determination of association constants between oligomers using either the optical density transition as a function of temperature (1) or NMR measurements (19) requires substantial amounts of material. The availability of a simple procedure for measuring relative association constants using small amounts of oligomer and minimal equipment would be very useful. It would be particularly advantageous for an initial investigation of the pairing stabilities of deoxynucleoside analogs that are expensive and laborous to synthesize.

Because there were no published procedures for the synthesis of oligodeoxynucleotides containing 6-thioguanine, a detailed

investigation of chemical procedures was conducted. There is one account of the synthesis of an oligodeoxynucleotide containing 2-pyrimidinone (20). Because yields and purity of oligomers containing 5-methyl-2-pyrimidinone were important in the present investigation, chemical procedures for the synthesis of oligomers containing 5-methyl-2-pyrimidinone were investigated.

The three objectives of the experimental investigation were: 1. the determination of chemical methods for the synthesis of oligodexoynucleotides containing the bases 6-thioguanine and 5-methyl-2-pyrimidinone; 2. the development of an enzymatic procedure for the measurement of relative association constants between oligomer pairs at very low concentrations; and 3. the evaluation of the stability of the analog base pair 6-thioguanine/5-methyl-2-pyrimidinone, figure 1A, and the stabilities of the analog bases paired with the standard bases.

RESULTS

Synthesis of deoxynucleosides and protected deoxynucleosides

The synthesis of the β-deoxynucleoside of 6-thioguanine used a published method (21). Preliminary experiments indicated that among the derivatives N-acetyl, N-isobutyryl, and N-benzoyl of deoxy-6-thioguanosine only the N-benzoyl derivative had the stability to allow a significant yield of the N-protected nucleoside after removal of the protecting group from the deoxyribose.

The published method for the synthesis of the deoxynucleoside of 5-methyl-2-pyrimidinone (22) gave very erratic yields according to the authors. The major source of variability was the reduction of 4-thiothymidine with Raney nickel. To follow the extend of the reduction reaction and to determine when to terminate the reaction a spectrophotometric method was employed. The peak of absorption of 4-thiothymidine is 334 nanometers and the peak for 5-methyl-2-pyrimidinone is 322 nanometers in .1 N HCl. A series of reactions was carried out for various times. The highest consistent yields occured when the optical density at 322 nanometers was equal to or slightly greater than the optical density at 334 nanometers. The decrease in optical density at 334 nanometers from the initial solution was about a factor of 6. After work up the deoxynucleoside of 5-methyl-2-pyrimidinone was crystallized from ethyl alcohol at -20 C. Overall yields of 25 to 30% were obtained.

Oligodeoxynucleotide synthesis

The phosphotriester method (23) was employed for the synthesis of oligodeoxynucleotides containing 5-methyl-2-pyrimidinone or 6-thioguanine. Both the phosphite (24) and phosphotriester methods were used for the synthesis of oligodeoxynucleotides having standard bases only.

The chemical properties of 6-thioguanine and 5-methyl-2-pyrimidinone required some modifications and additions to standard procedures. Model experiments with deoxy-6-thioguanosine demonstrated that the mesitylene sulfonyl reagent reacted rapidly with the thioketo group. The thioketo group was regenerated completely from the mesitylene sulfonyl adduct with 1M benzenethiol in pyridine. Regeneration with mercaptoethanol was very slow and incomplete.

Preliminary experiments showed that the aqueous alkaline labilities of 6-thioguanine and 5-methyl-2-pyrimidinone percluded

the use of the standard aqueous methods of deprotecting the oligodeoxynucleotide. Model experiments demonstrated that the deoxy-3'-O-succinate bond to the solid support and the o-chlorophenylphosphate bond were cleaved slowly with syn-2-nitro-benzaldoximate in anhydrous pyridine with no base modification. The isobutyryl and benzoyl groups were removed from the bases by ammonolysis in anhydrous methanol without modification of the bases.

A quanitative study (not shown) of the rate and yield of the ammonolysis of the protecting groups of the oligomers containing 6-thiguanine and 5-methyl-2-pyrimidinone, demonstrated the importance of keeping the oligomers in solution during deprotection. With cetyltrimethylammonium as the counterion for the oligomers quanitative deprotection was accomplished in 7 days at room temperature. Without a counter ion to maintain solubility yields were very variable and never quanitative.

Purification and characterization of oligodeoxynucleotides

Table I lists the oligodeoxynucleotides synthesized. Because this was the first time 6-thioguanine has been incorported synthetically into an oligodeoxynucleotide and only the second time for the incorporation of a 2-pyrimidinone (20), it was essential to verify the presence of the new bases in the oligonucleotides. In addition it was necessary to establish that the purity of the oligomers were sufficient for the method of measurement of the relative association constants. After purification of the oligonucleotides with preparative acrylamide electrophoresis the purity was estimated in two ways. The oligomers were examined by reverse phase HPLC (24) and only one major peak with about 90% of the optical density at 254 nanometers was found. However the recovery of oligomers from reverse phase HPLC is substantially below 100% and thus it is useful to have a second method that recovers 100 % of the oligomer even if its selectivity is less than reverse phase HPLC. The second method was acrylamide gel electrophoresis with ^{32}P phosphorylated oligomers. Over 90 % of the radioactivity was found in one band. Other bands were estimated to each have less than 2% of the total radioactivity. Although neither method was conclusive, together they indicated a purity of at least 90% for each of the oligomers.

The composition of each oligonucleotide was verified by a quanitative digestion with S1 nuclease and HPLC with a weak anion

TABLE I: Oligonucleotides synthesized

Designation	Sequence (5'->3')
A-T	T-C-G-A-C-G-G-A-T-C-C-G
A-T^{m}	T-C-G-A-C-G-G-A-T^{m}-C-C-G
G^{s}-T	T-C-G-A-C-G-G-G^{s}-T-C-C-G
G-T	T-C-G-A-C-G-G-G-T-C-C-G
A-C	T-C-G-A-C-G-G-A-C-C-C-G

Legend: The bases 6-thioguanine and 5-methyl-2-pyrimidinone are designated G^{s} and T^{m}.

exchange resin. The reason for not using snake venom phosphodiesterase was the very slow hydrolysis that occured when the 6-thioguanine base was incountered. Because the measurement of the areas of the peaks was uncertain to 15% for the standard bases and 6-thioguanine, and was much greater for 5-methyl-2-pyrimidinone because of its low adsorption, the number of nucleotides in an oligomer was counted by a direct method. The oligonucleotides were ^{32}P phosphorylated at the 5' end and then degraded with snake venom phosphodiesterase. The products were analyzed at various times by acrylamide gel electrophoresis and visualized by radioautography. The number of products separated by electrophoresis was 12 for all the oligomers analyzed.

Relative association constants

The traditional method of measuring the association constant of oligonucleotide pairs is to determine the optical density transition curves as a function of temperature for a range of concentrations (1). For oligonucleotides with short runs of complementary sequences the transition curve is broad and the location of the temperature of the mid point requires very good data and flat base lines. In addition there is some theoretical disagreement about interpretation (1,8). NMR measurements require substantial amounts of the oligomers (19). Because the amounts of the oligonucleotides synthesized with the analog bases were limited and preliminary experiments demonstrated significant problems with the determination of the base lines for the optical density transition method, an enzymatic method was developed to measure association constants. T4 ligase can join two duplex oligonucleotides together when the ends of each duplex have an appropriate structure. The concentration of the duplex structure of a pair of oligonucleotides was determined by the amount of ligation of the duplex to a carrier duplex. The carrier duplex was constructed of standard base pairs and it was present in a high enough concentration so that a large amount of ligation between carrier molecules took place. The concentration of the duplex of interest was kept so low that only ligation to the carrier molecules took place. The amount of the duplex ligated to the carrier was followed by labeling one of the oligonucleotides of the duplex at the 5' end with ^{32}P phosphate. The carrier was phosphorylated at the 5' end with cold phosphate. Both the carrier duplex and the duplex of interest had the single stranded sequence T-C-G-A at both ends which allowed four base pairs to form between the two duplex molecules and allowed efficient joining by T4 ligase. Because single stranded oligonucleotides can be ligated to the carrier duplex, it was necessay to determine the extent of this reaction. The oligonucleotide ligated to the carrier molecule was separated from the unligated oligonucleotide by electrophoresis in an acrylamide gel.

With the assumptions listed in appendex A the concentration of unligated oligonucleotide 1 as a function of time, C(1,t), is:

$$\text{I.} \qquad \ln[C(1,t)/C(1,0)] = -[k_S(1)+\varepsilon K_A(1,2)\ C_A(2)]F(t)$$

where $K_A(1,2)$ is the association constant between oligomers 1 and 2, $k_S(1)$ is the relative rate of ligation of single stranded oligomer 1 to the carrier molecule compared to the rate of liga-

tion of the duplex molecule, $C_A(2)$ is an average value of the concentration of oligomer 2 during the time of the reaction, ε is 1 when components 1 and 2 are different oligomers and 2 when the oligomers are the same, and F(t) is a function of time which depends on the amounts of enzyme and carrier molecule but does not depend on the amounts of oligomers 1 and 2.

The ligation assay can be run in two modes. The first uses an initial rate measurement. The second mode allows the ligation of carrier molecules to go to completion. Experiments performed at temperatures of interest demonstrated significantly higher levels of variability in the initial rate mode compared to the completion mode. The reason was that the high concentration of carrier molecules required for significant ligation gave high ligation rates and the amount of ligation was very sensitive to the times used. The completion mode was very insensitive to the times used after the time most of the ligation had taken place.

The concentration of carrier molecule to use was investigated. The two conditions that the concentration of carrier molecule should meet are: 1. There is sufficient duplex carrier so that small amounts of the ^{32}P labeled duplex can be ligated to it. 2. There is enough carrier duplex so that self ligation of the duplex of interest does not take place and the progress of the ligation of the carrier molecules to themselves is not significantly changed by the concentration of the duplex of interest. The latter condition is meet by using low concentrations of the complementary oligomers whose association constant is to be measured. The former condition was established empirically. For initial concentrations of carrier molecule above .5μM the measured relative association constants did not change so long as the final concentration of the oligomers being ligated to the carrier was not reduced to less than about 25% of the initial concentration.

Table II contains data from a typical experiment. Reaction 4 of Table II used the self complementary oligonucleotide A-T at a concentration which allowed only single stranded ligation. The region of single stranded ligation was the concentration range where the fraction of oligomer ligated was independent of concentration. Equation I becomes

$$\text{II.} \qquad k_S(A\text{-}T)\,F(t) = -\ln[C(A\text{-}T,t)/C(A\text{-}T,0)] = -\ln[6592/13361]$$

Reaction 3 of Table II has a concentration of A-T where both duplex and single stranded molecules were ligated and equation I becomes

$$\text{III.} \quad k_S(A\text{-}T)\,F(t) + 2\,K_A(T/A)\,C_A(A\text{-}T)\,F(t) = -\ln[57240/126053]$$

The $k_S(A\text{-}T)\,F(t)$ term is known from equation II and using equation IIA of appendex A, $C_A(A\text{-}T) = .6\times10^{-7}$ M. Combining these results

$$\text{IV} \qquad K_A(T/A)\,F(t) = 7\times10^5$$

Using reaction 2 of Table II for the measure of the single stranded ligation of $A\text{-}T^{\blacksquare}$ to carrier and reaction 1 for both

Table II: Ligation of oligodeoxynucleotide to carrier

Reaction #	composition	unligated oligonucleotide cpm	ligated oligonucleotide cpm
1	$A-T^{H}$*(.4x10^{-7}M) $G^{S}-T$(.7x10^{-7}M)	10577	1350
2	$A-T^{H}$*(.4x10^{-7}M)	11632	875
3	A-T*(.85x10^{-7}M)	57240	68813
4	A-T*(.13x10^{-7}M)	6592	6769

Legend: The indicated oligonucleotides were ligated to the carrier molecule T-C-G-A-C-C-C-G-G-G. The asterisk denotes the oligonucleotide that was ^{32}P phosphorylated. The ligation reaction was composed of the indicated oligonucleotides and .06M tris-HCl, pH 7.5, 5mM dithiothreitol, 0.5 mM ATP, 5mM $MgCl_2$, 10^{-6}M 5'-phosphorylated carrier oligonucleotide, and 0.15 Weiss units of T4 DNA ligase in 10 µl. The temperature was 19 C and the reaction time was 60 minutes. An aloquot of the reaction was diluted 1 to 4 with 80% formamide and 2 µl samples were run on a 10% acrylamide, 7M urea gel at 50 C. After autoradiography to locate the oligonucleotides the appropriate regions of the gel were excised and counted.

single stranded ligation and duplex ligation of $A-T^{H}$ with $G^{S}-T$ to carrier

V. $K_{(}T^{H}/G^{S}) = 7.1x10^{5}$

and

VI. $K_{A}(T/A) = 1\ K_{A}(T^{H}/G^{S})$

Table III contains a summary of all of the results.

The measurements were performed at two different concentrations of enzyme and the concentrations of all of the oligonucleotides were varied. Relative association constants were not affected.

DISCUSSION

The major considerations that suggested the choice of the base pair were: 1. The hydrogen bond between the 3-nitrogen of the pyrimidine and the 1-nitrogen of the purine was retained for proper alignment in the base pairing. 2. Spectroscopy (26) in the gas phase suggested that the hydrogen bond force constant of sulfur is one half that of oxygen and that the most stable angle between the X-H axis and the symmetry axis of the sulfur bond is 90 degrees instead of the 45 degrees of oxygen. 3. X-ray analysis of 6-thioguanosine showed the predominate form to be the thioketo tautomer (27). Figure 1A shows the base pair 5-methyl-2-pyrimidinone/6-thioguanine. Figure 1B shows the graphics representation of the stereochemistry of replacing a cytosine/

Table III: Relative association constants

Oligomer pair	Association constant	Value relative to K(T/A)
(A-C/T-G)	K(C/G)	5±2
(A-T^{m}/T-G^{s})	K(T^{m}/G^{s})	1±.5
(A-T/T-G)	K(T/G)	1/(9±3)
(A-T^{m}/T-G)	K(T^{m}/G)	1/(25±5)
(A-C/T-G^{s})	K(C/G^{s})	<1/30
(A-T^{m}/T-A)	K(T^{m}/A)	<1/40
(A-C/T-A)	K(C/A)	<1/40
(A-T/T-G^{s})	K(T/G^{s})	<1/40

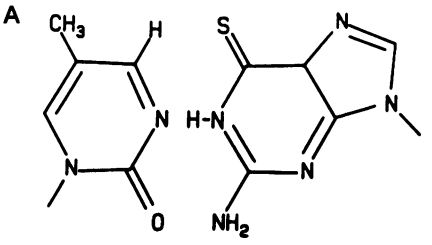
Legend: The first oligomer in each expression is written 5'to 3', the second 3' to 5'. The "<" indicates that the amount of duplex ligation was within the experimental uncertainty of the amount found in the absence of the complementary oligodeoxynucleotide.

guanine base pair with a 5-methyl-2-pyrimidinone/6-thioguanine base pair in an oligodeoxynucleotide duplex (28). There is no significant steric interference between the thioketo group and the hydrogen which would affect the hydrogen bonding of the two bases.

The extreme variability in the yield of 1-(2-deoxyribosyl)-5-methyl-2-pyrimidinone reported by Wrightman and Holy (22) was eliminated by monitoring spectrophotometrically the course of the reduction of 4-thiothymidine with Raney nickel. Because there are reactions taking place with the desired product during the reduction, it was empirically established that the highest consistent yields of the 5-methyl-2-pyrimidinone deoxynucleoside occured when the optical density at 322 nanometers was equal to or slightly greater than the optical density at 334 nanometers. This occured when there was still a significant amount of 4-thiothymidine present.

The phosphotriester method was initially selected instead of the phosphite method for the synthesis of the oligonucleotides with 6-thioguanine because of the repeated oxidation steps with iodine in the latter method. At the begining of the investigation it was not obvious how to protect the thioketo with an easily removable group that was not alkaline labile. It is now clear that a good protecting group is mesitylene sulfonyl which can be quanitatively removed with M benzenethiol. With this protection of the thio the phosphite method should be applicable.

The composition of each oligomer was verified by a quanitative S1 nuclease digestion and analysis by HPLC with a weak anion exchange resin. Because of the significant uncertainty in the amount of the 5-methyl-2-pyrimidinone nucleotide due to its low adsorption, the number of nucleotide residues was



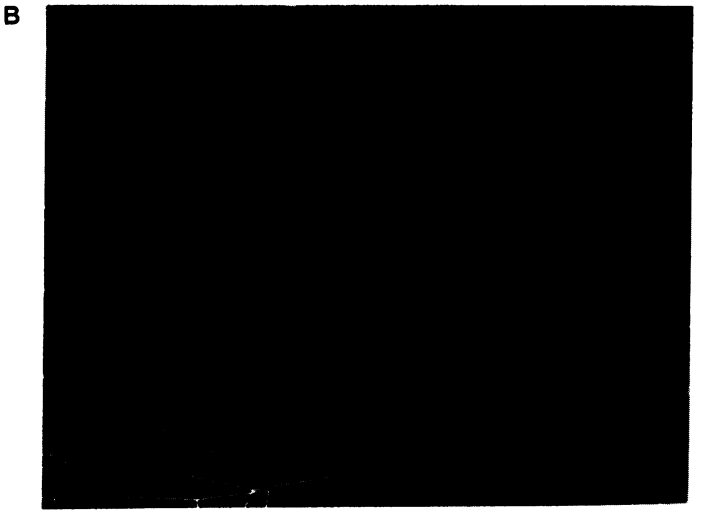


Figure I:
(A) A schematic representation of the base pair 5-methyl-2-pyrimidinone, left base, and 6-thioguanine, right base. (B) The photograph shows the base pair 5-methyl-2-pyrimidinone/6-thioguanine derived from a cytosine/guanine base pair. The cytosine/guanine base pair is three base pairs from the end of an oligodeoxynucleotide duplex determined by X-ray crystallography (28). The amino group of cytosine, the left base, was replaced with a hydrogen, bond length 1.09 A. The 5-methyl group is not shown. The oxygen of guanine, the right base, was replaced with a sulfur, bond length 1.7 A. No other changes were made. The dots indicate the extent of the van der Waals radii for sulfur, 1.8 A, and hydrogen , 1.2 A.

determined. The 5'- ^{32}P phosphorylated oligonucleotide was digested with snake venon phosphodiesterase for various times and the products separated by acrylamide gel electrophoresis. 12 products were found, confirming the composition.

Literature values of the ratio of the association constants of oligonucleotides that have a G/C pair substituted for an A/T

pair range from 1 to 10 (1) depending on the sequence. The precision of the data give an uncertainty of 50% or more in the ratios. The value from the enzymatic method was 5±2, Table III. A direct NMR measurement at low ionic strength and 15 C of the ratio of the association constants between 7-mers with a G/T substituted for a G/C gave a value of 1/25 (19). The equivalent ratio from Table III is 1/45 at 19 C.

A concern with the enzymatic method was the possibility that a significant amount of ligation would take place with partially paired duplexes. Thus it was essential to verify that oligomers with only one substantial mismatch pairing would in fact show very little ligation. Aboul-ela et al (1) have shown that the duplex formed from 9-mers with the mismatch base pair C/A have an association constant orders of magnitude less than a duplex with a T/A base pair. The enzymatic method, Table III, (A-C/T-A), was unable to detect any duplex ligation. The result demonstrated that ligation was not occuring at a significant level with partial base paired duplexes. The reason was that the mismatch base pair was only three base pairs from the end of the oligomer that was ligated.

The values of the relative association constants determined by the enzymatic method are consistent with the values obtained by both the optical density transition and NMR measurements. The base pair G^{S}/T^{H} has essentially the same stability as the standard base pair A/T. The stability of base pairing of the analog bases with the standard bases is less than the mismatched base pair G/T. The results suggest that the class of base pairs with a thioketo group opposite a hydrogen can provide a source of complementary base pairs.

The biologically important question of whether the analog base pair can be replicated with an acceptable level of fidelity by present day polymerases has not been determined in this investigation. However one experimental observation is relevant. Charczuk et al (29) have presented evidence that there is a very strong discrimination against standard bases being incorporated across from 2-pyrimidinone when the Klenow fragment of DNA polymerase I is used for in vitro replication.

EXPERIMENTAL SECTION

Materials

The chemicals employed and their sources were:β-deoxynucleosides (Sigma), benzenethiol (Eastman), cetyltrimethylammonium bromide (Aldrich), 2-chlorophenyl-dichlorophosphate (Aldrich), 4,4'-dimethoxytrityl chloride (Aldrich), long chain alkylamine controlled pore glass (Pierce), 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (Aldrich), mercury (II) cyanide (Aldrich), 1-methylimidazole (Aldrich), 2-nitrobenzaldoxime (Aldrich),p-nitrophenyl actate (Sigma), silver carbonate (Aldrich), silica Woelm TSC (INC Nutritional Biochemical). All solvents were redistilled and stored under appropriate anhydrous conditions (23).

Enzymes employed and their sources were: polynucleotide kinase (Bethesda Research Lab., New England Biolabs., U.S. Biochemical Corp., Pharmacia), snake venom phosphodiesterase (Sigma) T4 DNA ligase (Bethesda) Research Lab., New England Biolabs.), Sal I (New England Biolabs.), S1 nuclease (Bethesda Research Lab).

Synthesis of deoxynucleosides

1-(2-deoxy-β-D-ribofuranosyl)-5-methyl-2-pyrimidinone

4-thiothymidine was prepared by a published procedure (30). Deoxyribosyl-5-methyl-2-pyrimidinone was prepared from 4-thiothymidine by reduction with Raney nickel. 5.9 gm (22 mmole) of 4-thiothymidine was added to 180 ml of distilled water and 60 ml of ethyl alcohol in a flask with reflux condenser. 24 gm of Raney nickel was added and the solution heated to reflux. For the highest yields a 4-thiothymidine solution was titrated with each Raney nickel preparation and the optical density of a sample in 0.1 M HCl was determined at 322 and 334 nanometers. The optical density decreased at the two wavelengths. The reaction was terminated when the optical density at 322 nanometers was equal to or greater than the optical density at 334 nanometers. The decrease in optical density at 334 nanometers from the initial solution was about a factor of 6 for the maximum yield. The reaction was followed with silica gel t.l.c. using isopropanol. A blue fluorescent spot, characteristic of the compound, appeared with an Rf of .35. The initial greenish solution became a very light yellow by the end of the reaction. Because other products were formed the reaction was not run until all the 4-thiothymidine was used. The suspension was filtered hot and the Raney nickel boiled with 150 ml of water. The solution was filtered hot. The combined filtrates were evaporated. Deoxyribosyl-5-methyl-2-pyrimidinone was purified by dry column chromatography with Woelm silica III. 10 ml of methanol was added to the solid from the Raney nickel reduction. 3.7 ml of the clear solution was added to 3.7 gm of Woelm silica III and air dried. The dried silica with the deoxyribosyl-5-methyl-2-pyrimidinone was added to the top of a column of dry Woelm silica, 20 inches long by 1 inch diameter, contained in nylon tubing. Isopropanol was the solvent. The solvent front was allowed to reach the bottom of the silica column before terminating the chromatography. The nylon tubing was cut into 1 inch sections and the silica in each section was extracted with 10 ml of methanol. Samples from each section were run on silica gel t.l.c. with isopropanol. The methanol extracts were pooled from sections that showed only the fluorescent spot with Rf .35. The pooled silica from the appropriate sections was extracted again with methanol and pooled with the first extraction. After filtration the methanol was evaporated to a small volume and the remaining fine particles of silica were removed by centrifugation. The remaining methanol was evaporated and the residue dissolved in 10 ml of hot ethyl alcohol. The solution was placed at -20 C. Crystals formed overnight. The yellow supernatant was decanted and the crystals washed in cold ethanol. The volume was reduced to 4 ml and placed at -20 C. More crystals formed. Silica gel t.l.c. revealed a contamination of less than 5%. The ultraviolet-visible spectrum of a solution of the crystals was equivalent to the literature spectrum (31). The overall yield from 4-thiothymidine to the final produce was about 25%.

N-benzoyl-deoxy-6-thiguanosine

To 0.5 gm (1.7 mmole) of deoxy-6-thioguanosine, that had been repeatedly dried by evaporation of anhydrous pyridine, was added 3.3 ml of anhydrous pyridine and 6.6 ml of redistilled chloroform. At 4 C, 4.7 ml of redistilled chloroform containing 1.35 ml (12 mmoles) of benzoyl chloride was added dropwise with

stirring in a flask with a $CaCl_2$ drying tube. After the addition of the benzoyl chloride all of the deoxy-6-thioguanosine went into solution and the solution was yellow. The solution was allowed to come to room temperature and was stirred for three hours Silica gel t.l.c. of a sample of the reaction with methanol/chloroform (.5:9.5 v/v) showed one spot with UV absorption that turned dark brown on exposure to acid and heat. The spot was at the solvent front. The reaction solution was poured into 60 ml of ice. After melting 10 ml of chloroform was shaken with the aqueous emulsion. After separation of the phases, the organic phase contained all the yellow color. The organic phase was washed three times with 20 ml of water. The organic solution was dried with sodium sulfate and evaporated to an oil.

19 ml of pyridine was added to the oil. A clear solution was obtained. 1.8 ml of water was added and then 19 ml of methanol was added. The solution was placed at 4 C and 2N NaOH was added slowly until a pH reading of 12.4-12.5 was reached. The pH was maintained around 12.4 by the addition of 2N NaOH. The hydrolysis was followed with silica gel t.l.c. using methanol/chloroform (1.5:8.5 v/v). A majority of the UV absorbing material became one spot with an Rf of 0.3. The Rf of deoxy-6-thioguanosine was 0.19. The reaction was stopped by lowering the pH to a reading of 7.8 with 20% acetic acid. A considerable loss was sustained if an exchange resin was used for the neutralization. The solution was evaporated to an oil. 600 ml of water was added to the oil and heated to 70 C with stirring. Liquid was decanted from the insoluble material and placed at 4 C. A precipitate formed and was recovered by filtration. The aqueous solution was evaporated to 250 ml and a second precipitate recovered by filtration. Silica gel t.l.c. of the combined precipitates showed more than 90% was the N-benzoyl derivative.

Triethylammonium (5'-O-4,4'-dimethoxytrityl-protected-deoxynucleoside-3'-O-2-chlorophenyl phosphate))

All the compounds were prepared by standard methods (33). It was essential to check all suspectible solvents for peroxides when carrying out the procedures with deoxy-6-thioguanosine.

Cleavage, Deprotection, and Regeneration

The glass support with the oligodeoxynucleotide was dried in vacuum over P_2O_5 and KOH. 0.45 ml of anhydrous pyridine with 55 µl of benzenethiol (1 M) was added to the glass under dry nitrogen gas. After 8 hours at room temperature the solution was removed and the glass support was washed four times with 1 ml of dichloromethane. The remaining dichloromethane was removed by vacuum and the glass support dried over P_2O_5 and KOH.

16.6 mg (100 µmole) of syn-2-nitrobenzaldoxime was dried by repeated evaporation of anhydrous pyridine. 200 µl of anhydrous pyridine was added in a nitrogen atmosphere to the dry nitrobenzaldoxime and then 36 µl of dry tetramethylguanidine was added. The solution was added to the dry glass support. The solution and glass support were sealed in a vial under nitrogen and kept at room temperature for 5 days. The extent of the release of the oligodeoxynucleotide into the solution was followed by assaying 1 µl of the solution for the dimethoxytrityl group. 27 mg of p-nitrophenylacetate was added under nitrogen to the nitrobenzaldoximate solution to use up the remaining oximate ions. After three hours 1 ml of pyridine containing 10 µl of 20% acetic acid was added. The pH was measured by paper to make sure the pH was

between 7 and 8. The solution was removed from the glass support and 1 ml of 50% aqueous pyridine was added to the glass support and the suspension shaken for 30 minutes. The solution was removed and combined with the initial pyridine solution. The solution was evaporated to dryness.

1 ml of dry methanol containing 9 mg (25 mmole) of cetyltrimethylammonium bromide was added to the dried N-protected oligodeoxynucleotide. The solution was saturated with NH_3 gas at 4 C. The test tube was stoppered and placed at room temperature in the dark. After 7 days the test tube was opened at 4 C and the solution evaporated at room temperature with dry nitrogen. The residue was dissolved in 1 ml of 80% acetic acid to release the dimethoxytrityl group. After 20 minutes 1 ml of water was added and the aqueous solution was extracted five times with 2 ml of water saturated diethylether. The aqueous phase was evaporated to 200 µl. The solution was yellow. The solution was placed on a Dowex AG-50Wx2 column, 1.5x3 cm, that had been washed with 1 M NH_4HCO_3 and then washed with distilled water. The column was eluted with distilled water and 0.5 ml fractions were collected. The fractions were monitored at 260 nanometers for the oligodeoxynucleotide and the appropriate fractions were pooled. The pooled fractions were evaporated to a small volome. The oligonucleotide was placed on a Waters Associates C_{18} cartridge and after washing with water eluted with 6:4 methanol:water. The solution was evaporated to dryness and stored at -20 C.

Extinction Coefficients

The extinction coefficients at 260 nanometers of the oligodeoxynucleotides listed in Table I were calculated as follows: 1. An extinction coefficient of 10 x $10^4 m^{-1}$ cm for A-T was calculated from the data listed by Soher (32). 2. The extinction coefficient of the G^s-T oligodeoxynucleotide was taken to be 10 x $10^4 M^{-1}$ cm since the nucleoside of 6-thioguanine has an extinction coefficient at 260 nanometers of 8 x $10^3 M^{-1}$ cm essentially independent of pH. 3. The extinction coefficient of the oligodeoxynucleotide containing 5-methyl-2-pyrimidinone was taken to be 9.2 x $10^4 M^{-1}$ cm because the deoxynucleotide of 5-methyl-2-pyrimidinone has essentially no absorption at 260 nanometers (31).

Ligation Assay

Labeled oligodeoxynucleotide concentrations varied between .1 and .01 µM. The carrier oligodeoxynucleotide was T-C-G-A-C-C-C-G-G-G and its concentration was 1 to 2 µM. The other components were 0.06M Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, .5 mM ATP, and .06 to .005 Weiss units of T4 ligase per µl. The temperature was 19 C. The buffer, $MgCl_2$, dithiothreitol, and oligodeoxynucleotides were combined and subjected to the following temperature cycle: 60-65 C for 5 minutes, room temperature for 15 minutes, and put on ice for 15 minutes. The 0.5 ml polypropylene test tubes were centrifuged to return all the water to the bottom before addition of ATP and the ligase.

HPLC analysis

The nucleotides were analyzed on a weak anion exchange resin, Syn Chropak AX100, 250x4.6 mm, SynChrom Inc, Linden, IN., with a mobile phase of .05F KH_2PO_4, pH 4.5. The nucleosides were analyzed on an octadecyl-silica column, 250x4.6 mm, with a mobile phase of 2.5% methanol and .02F KH_2PO_4, pH 5.5. Methanol was changed to 10% for deoxyadenosine.

APPENDIX A

The assumptions for the derivation of an equation describing the time course of the concentration of oligomer 1 when it is ligated to a duplex carrier molecule either as a single strand or as a duplex with oligomer 2 are: 1. There is a sufficiently high concentration of the carrier molecule so that the time course of the self ligation of the carrier molecule is not significantly perturbed by the amount of incorporation of the oligomers 1 and 2. 2. The concentration of the duplex of oligomer 1 and 2 is low compared to the K_M so that the rate of ligation is proportional to the concentration of the duplex. 3. The concentration of the duplex of oligomers 1 and 2 is sufficiently low that self ligation is insignificant compared to ligation with the carrier molecule. 4. The equilibrium condition is maintained between the oligomers and the duplex during the course of the ligation. With these assumptions the differential equation for the loss of oligomer 1 due to ligation to the carrier molecule is:

$$\text{IA.} \qquad \frac{dC(1)}{dt} = -\{\epsilon K_A(1,2)C(1)C(2) + k_s(1)C(1)\}g(t)$$

where the function g(t) characterizes the amount of enzyme-carrier complex available for the ligation reaction with oligomer 1 as a function of time and the other symbols are defined in the text. It is assumption 1 that allows g(t) to be independent of oligomers 1 and 2, and assumptions 2 and 4 that allows the equation to be written as a product of g(t) and the concentrations of the oligomers. An experimentally useful solution to equation IA is obtained by considering C(2) to be a constant $C_A(2)$, an average that has a value between the initial and final concentration of component 2. The solution is equation I of the text, where

$$F(t) = \int_0^t g(\tau)\, d\tau.$$

When a large fraction of the oligomer 2 is ligated as a single strand to the carrier molecule, it is necessary to solve an equation equivalent to IA for component 2, and then to use this solution in equation IA for C(2). The result is:

$$\text{IIA.} \qquad C_A(2) = \frac{C(2,0) - C(2,t)}{\ln[C(2,0)/C(2,t)]}$$

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