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Prevention of guanine modification and chain cleavage during the solid phase synthesis of oligonucleotides using phosphoramidite derivatives

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ABSTRACT

Phosphoramidite reagents can phosphitylate guanine bases at the O^6-position during solid phase synthesis and serious chain cleavage occurs if the base phosphitylation is not eliminated before the iodine/water oxidation step. This can be accomplished by i) blocking the O^6-position with a 2-cyanoethyl protecting group for deoxyribonucleotides or with a p-nitrophenylethyl group for ribonucleotides, ii) regenerating the guanine base with water or acetate ions, or iii) using N-methylanilinium trifluoroacetate (TAMA) as the phosphoramidite activator. The effectiveness of these methods was demonstrated by both ^{31}P NMR studies and by the synthesis of $d(Gp)_{23}G$, $(Gp)_{14}G$, and $d\text{-}(Gp)_{13}rG$ sequences.

INTRODUCTION

The solid phase synthesis of oligodeoxyribonucleotides using nucleoside phosphoramidite precursors has become widely utilized[1]. More recently the phosphoramidite procedure has also been applied to the synthesis of oligoribonucleotides[2]. The procedure is fast, efficient, and many of the side reactions which caused base modification in previous "phosphate triester" approaches[3-9] are eliminated. However, our recent investigation[10] into possible depurination of oligodeoxyadenylate and oligodeoxyguanylate sequences revealed an unusual amount of chain degradation in sequences containing a large number of guanine bases. This degradation was shown to be the result of a multi-step process which involved phosphitylation of the guanine base at the O^6-position as the first step. The base phosphitylation can be observed by ^{31}P NMR and has only been detected with guanine bases.

We have speculated[10] that the observed chain degradation arises from depurination of the species formed by migration of

the phosphite group from O^6 to the N^7-position of the guanine base. This migration would be possible by means of neighbouring group attack from N^7 during the iodine/water oxidation reaction. The iodine/water oxidation reaction is a step unique to phosphite triester synthesis strategies and extensive chain cleavage has not been reported when O^6-phosphorylation or modification occurs in phosphate triester synthesis.[3-8]

There are three possible approaches to eliminate the problem of chain cleavage during phosphite condensation procedures. The first method would be to simply block the O^6-position with a suitable protecting group. The second strategy would be the use of some nucleophile to cleave the O^6-phosphite linkage and regenerate the guanine base after the coupling reaction, but before the oxidation reaction. The third approach would be the use of a phosphoramidite activator which eliminates the guanine modification during the coupling reaction. This manuscript describes the successful use of these three strategies in the synthesis of oligoribo- and oligodeoxyribonucleotides by respective use of: 1, O^6-cyanoethyl or p-nitrophenylethyl protecting groups; 2, acetate ion cleavage of O^6-phosphite linkages; or 3, phosphoramidite activation by N-methylanilinium trifluoroacetate (TAMA)[11] .

RESULTS AND DISCUSSION

The extensive chain cleavage which occurred in the synthesis of the oligodeoxyguanylate sequence, $\mathbf{d(Gp)_{23}G}$, using our previous synthesis cycle[10], could be detected by either polyacrylamide gel electrophoresis (FIGURE 1-1) or liquid chromatography (FIGURE 5-3). As expected, chain cleavage was also observed when the phosphoramidite method was applied to the synthesis of the oligoguanylate sequence, $\mathbf{(Gp)_{14}G}$ (FIGURE 1-2). In each case, the synthetic material contained only short fragments and no $\mathbf{d(Gp)_{23}G}$ nor $\mathbf{(Gp)_{14}G}$ product was detected.

The use of various protecting groups for the protection of the O^6-position of guanine has been described[12-19] by several researchers for phosphate triester synthesis using arylsulphonyl condensing reagents. We previously used the p-nitrophenylethyl (NPE) protecting group[12,13] to prevent O^6-phosphitylation and subsequent chain cleavage in the synthesis of oligodeoxyguanylate sequences[10]. However, this group is unsatisfactory for oligode-

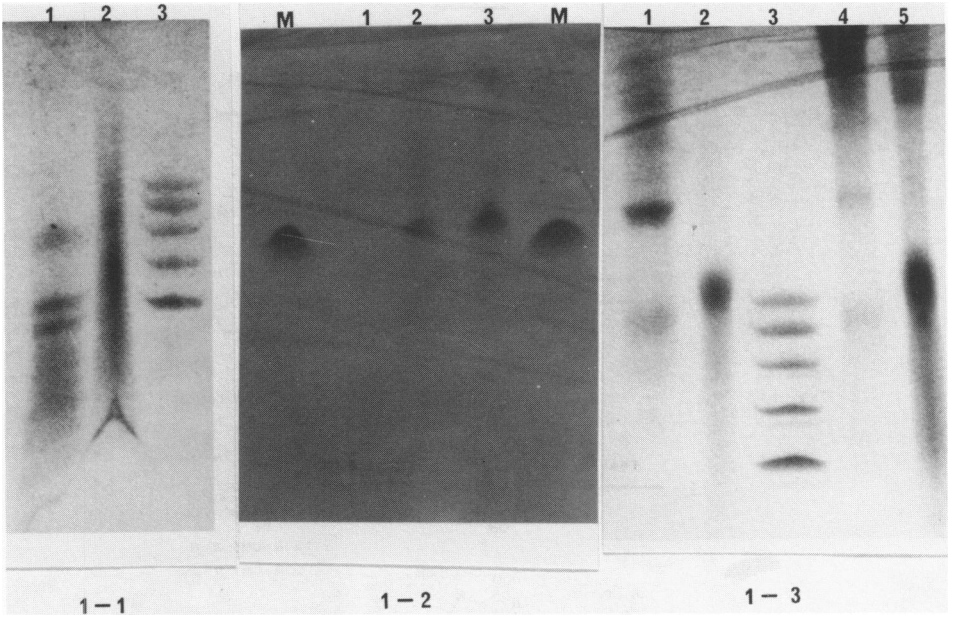
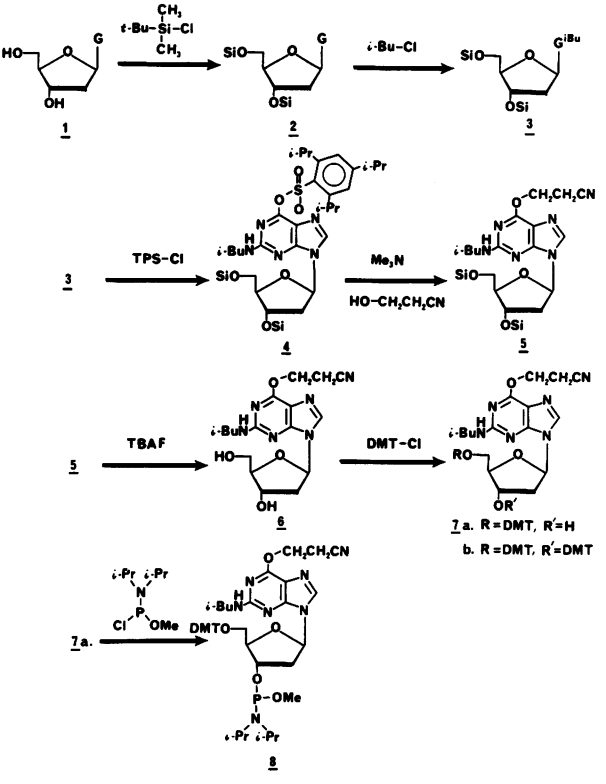


FIGURE 1: Electrophoresis of oligodeoxyguanylate and oligoguanylate sequences on 24% (1-1 and 1-3) or 20% (1-2) polyacrylamide /7M urea gels. Figure 1-1: Lane 1, crude mixture of sequences from the attempted synthesis of $d(Gp)_{23}G$ which did not eliminate guanine O^6-phosphitylation; Lane 2, the crude mixture shown in lane 1 treated with formaldehyde to remove secondary structure; Lane 3, 12, 16, 20, 24, and 28-base oligo-T markers. Figure 1-2: Lane 1, crude mixture from the synthesis of $(Gp)_{14}G$ which used neither O^6-protection nor any capping solution to eliminate O^6-phosphitylation; Lane 2, the crude mixture from the synthesis of $(Gp)_{14}G$ which used acetic anhydride/dimethylaminopyridine solution to eliminate guanine O^6-phosphitylation; Lane 3, the crude mixture from the synthesis of $(Gp)_{14}G$ which used the O^6-*p*-nitrophenylethyl protected derivative **12**; Lanes M, $d(Tp)_{17}T$ markers. Figure 1-3: Lane 1, the crude mixture from the synthesis of $d(Gp)_{23}G$ which used the O^6-cyanoethyl protected derivative **8**; Lane 2, the crude mixture shown in lane 1 treated with formaldehyde to remove secondary structure; Lane 3, 12, 16, 20, 24, and 28-base oligo-T markers; Lane 4, the crude mixture from the synthesis of $d(Gp)_{23}G$ which used acetic anhydride/dimethylaminopyridine solution to eliminate guanine O^6 -phosphitylation; Lane 5, the crude mixture shown in lane 4 but treated with formaldehyde to remove secondary structure.

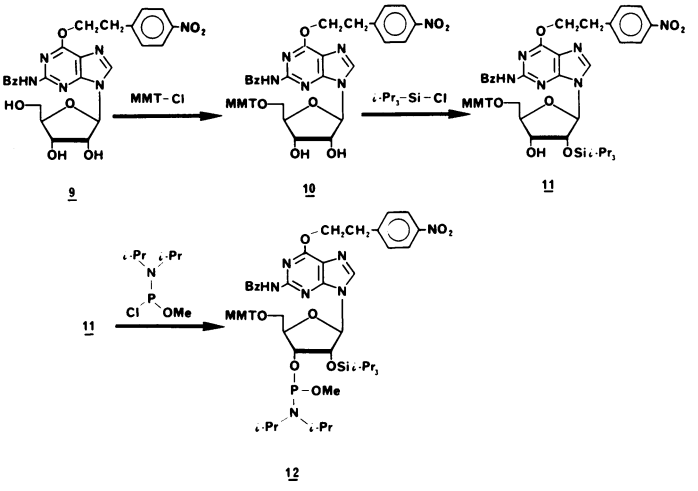
oxyribonucleotide synthesis because removal of the NPE group requires an additional deprotection step with 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) solution and we found it difficult to completely remove all of the O^6-protecting groups using DBU.

SCHEME 1



To overcome this problem, we tried the more labile 2-cyanoethyl group as an O^6-protecting group. The O^6-(2-cyanoethyl)-2'-deoxyguanosine-3'-N,N-diispropylphosphoramidite derivative, **8**, was prepared (SCHEME 1) using a synthetic strategy similar to that developed by Jones et al[17]. Most of the steps in the synthesis of compounds **2-6** proceeded in high yield. The only exception was the desilylation reaction which produced compound **6**. The large excess of tetrabutylammonium fluoride present in this reaction mixture complicated the isolation of pure **6**. Although TBAF may be removed by use of a Dowex Na^+ ion exchange resin[17], this resulted in the loss of a large amount of **6**. We therefore used an alternative procedure which did not isolate pure **6**. Instead, a partial purification step was performed by flash chromatography on silica gel and the mixture of TBAF and **6** obtained was treated

SCHEME 2



9 10 11 12

with dimethoxytrityl chloride. The tritylated nucleoside **7a** was then easily purified by silica gel chromatography because of its increased lipophilicity. Conversion of **7a** into phosphoramidite, **8**, was performed using N,N-diisopropylmethylphosphonamidic chloride and a quantitative yield of **8** was obtained.

Derivative **8** was tested in solid phase synthesis by preparing a twenty-four base long oligodeoxyguanylate sequence. An average coupling yield of 97.5% (calculated from quantitation of the released trityl cation) was obtained and the overall yield was 55%. All of the 2-cyanoethyl groups were removed using the ammonium hydroxide hydrolysis step that was part of the existing deprotection procedure and no additional deblocking step was required. The crude mixture of oligodeoxyguanylate sequences obtained at the end of the synthesis was applied to a 24% polyacrylamide gel. This gel showed that there was no longer any fast moving material indicative of chain cleavage (FIGURE 1-3). However, self association of the deoxyguanylate strands[20] did produce a large amount of material with very slow electrophoretic mobility.

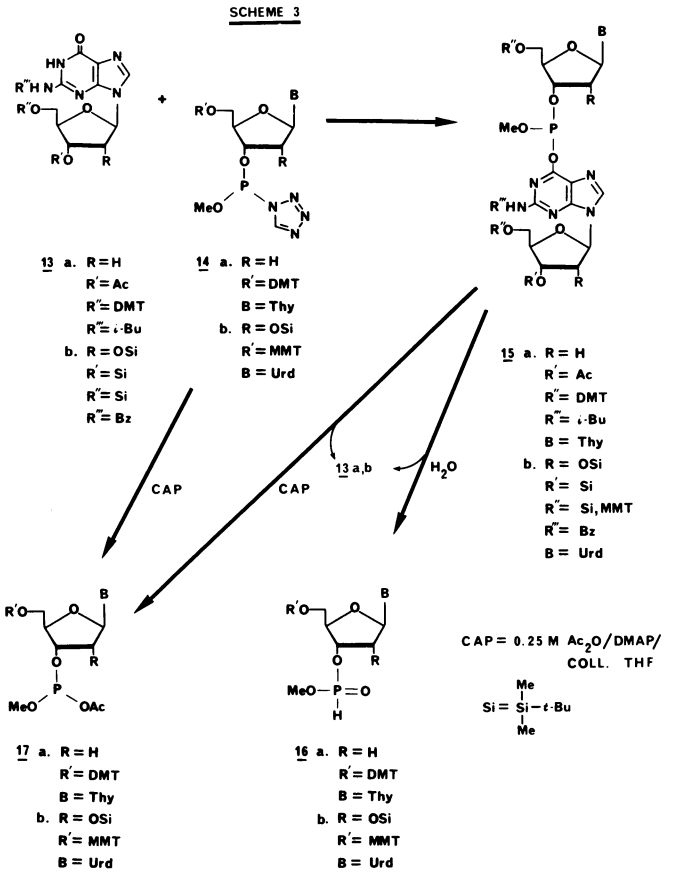
The solid phase synthesis of oligoribonucleotides was also improved considerably by using an O^6-protecting group on the guanosine nucleosides. The O^6-p-nitrophenylethyl protected guanosine phosphoramidite derivative, **12**, prepared in SCHEME 2, was

used in the synthesis of a $(Gp)_{14}G$ ribonucleotide. This synthesis had an average coupling yield of 94.4% and an overall yield of 44%. The removal of the p-nitrophenylethyl protecting groups from the ribonucleotide sequence was much easier than was the case with the deoxyribonucleotides since the NPE groups were removed[21] along with the silyl protecting groups by the TBAF reagent thus avoiding a separate deprotection step using DBU. The yield of crude oligomer was 24%. The completely deprotected $(Gp)_{14}G$ sequence appeared as an intense, well-defined band when the crude product was examined on a polyacrylamide electrophoresis gel (FIGURE 1-2, lane 3) and no sign of chain cleavage was evident. The crude product was purified by preparative gel electrophoresis followed by extraction of the gel and desalting to give pure $(Gp)_{14}G$ in an overall yield of 10%.

The synthesis of compounds **8** or **12** requires a large number of steps. We therefore sought alternative methods for the elimination of the O^6-side reactions which would not require the use of O^6-protecting groups. Thus our second approach was an attempt to eliminate chain cleavage by regeneration of the O^6-unmodified bases. If the O^6-phosphitylation could be removed before the iodine/water oxidation step then no O^6-protecting group would be required. Such an approach is possible because the phosphorus atom attached to the O^6-position of guanine is very susceptible to nucleophilic attack and treatment of the O^6-phosphitylated guanosine derivatives with nucleophiles, such as water or acetate ions, will regenerate the O^6-unmodified base (SCHEME 3).

The base regeneration by water was first observed in our early ^{31}P NMR experiments when traces of moisture were present. The water attacked the phosphorus atom of the O^6-phosphite dimer **15a,b** and produced the nucleoside phosphonates **16a,b** as well as regenerating the original deoxyguanosine nucleosides **13a,b**. The nucleoside 3'-O-phosphonates **16a,b** were observed at approximately 9.9 ppm in the ^{31}P NMR spectrum, while the regenerated nucleosides **13a,b** were identified by TLC.

The decomposition of **15a,b** into **13a,b** and **17a,b** by acetate ions can also be monitored by ^{31}P NMR (FIGURE 2). An excellent source of the acetate ions is the acetic anhydride/dimethylaminopyridine solution[22] normally used as the capping reagent. The



addition of a few drops of this solution to a mixture of **13a** and **14a** caused the immediate and complete disappearance of the signals at 134.0, 133.7 and 126 ppm (FIGURE 2-2) and the appearance of two new signals at 131.7 and 131.4 ppm (FIGURE 2-3). Identical results were obtained with the analogous ribose derivatives except that the chemical shifts of the intermediates were slightly shifted. The two new peaks indicated the formation of the acetylated phosphite derivative **17a,b** while the presence of **13a,b** was detected by TLC. Intermediates **17a** and **17b** were prepared by an alternate route which involved adding the capping reagent to a solution of **14a** or **14b** in the absence of any guanine compounds. This confirmed the structure of these intermediates.

The elimination of the guanine modification and the result-

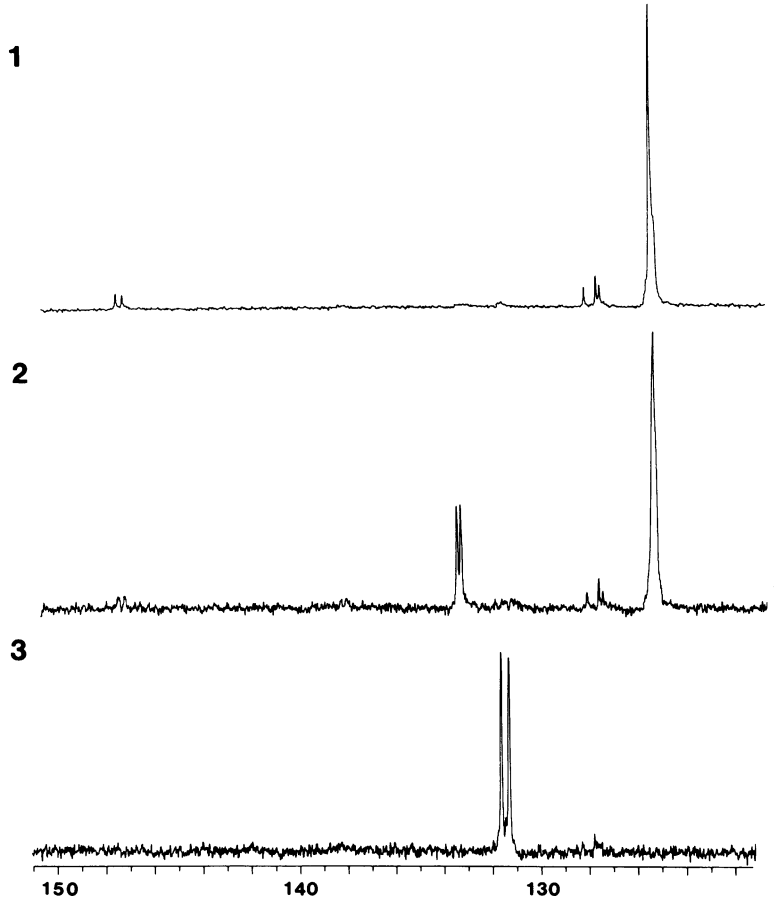


FIGURE 2: 121.5 MHz ^{31}P NMR Spectra of (1) activated thymidine phosphoramidite **14a**, (126 ppm), (2) a mixture of **14a** (126 ppm) and **15a** (134.0, 133.7 ppm), and (3) the spectrum of **17a** (131.7, 131.4 ppm) produced by the addition of acetic anhydride/dimethylaminopyridine solution to the above mixture of **14a** and **15a**. All spectra were obtained from CH_3CN solutions and chemical shifts are reported in ppm downfield from 85% H_3PO_4.

ing chain cleavage in our solid phase syntheses was attempted using both water and acetate (capping reagent) to regenerate the guanine bases. In the first case, a sixty second wash with 25% water/acetonitrile solution was added to the synthesis cycle after the coupling reaction. In the second case, the order of the oxidation and capping steps was reversed. Each modified synthesis cycle was then used to prepare another twenty-four unit long

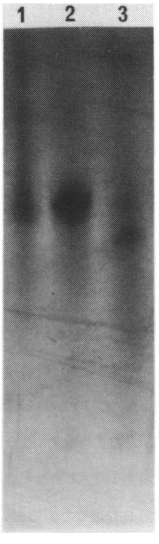


FIGURE 3: Electrophoresis of **d[$(Gp)_{13}$]pG** sequence on a 20% polyacrylamide/7M urea gel. Lane 1, the crude mixture from the synthesis of **$(Gp)_{14}$G** which used acetic anhydride/dimethylaminopyridine solution to eliminate guanine O^6-phosphitylation; Lane 2, the crude mixture from the synthesis of **$(Gp)_{14}$G** which used the O^6-p-nitrophenylethyl protected derivative **12**; Lane 3, the crude mixture from the synhtesis of **d-$(Gp)_{13}$rG**. All samples were loaded with XC and BPB dyes.

oligodeoxyguanylate sequence. The reversed capping/oxidation cycle was also used to prepare a fifteen unit long oligoguanylate sequence.

The synthesis using the 25% water/acetonitrile wash avoided chain cleavage but the coupling yields averaged only 90%. These low yields were presumably due to incomplete removal of moisture from the surface of the insoluble support. However, the syntheses which used the acetic anhydride/dimethylaminopyridine solution to eliminate the O^6-phosphite linkages before the oxidation step produced very high coupling yields, 98-99% in the deoxynucleotide sequence and 97-98% in the ribonucleotide sequence. The crude products from these syntheses (isolated in 55 and 35% respectively) were run on polyacrylamide electrophoresis gels and no fast moving bands indicative of chain cleavage were observed (FIGURE 1-2, 1-3). In the deoxyribonucleotide synthesis the crude product was the same as the product obtained from the synthesis

using the O^6-cyanoethyl protected derivative **8**. In the ribonucleotide case the product was identical to that obtained using the O^6-NPE protection and the gel purified yield of9% was similar. This proved that treatment with acetate ion before oxidation was a viable alternative to the use of O^6-protecting groups.

Another alternative to prevent O^6-modification of guanine bases is the use of N-methylanilinium trifluoroacetate (TAMA)[11] in place of tetrazole as the activating reagent. From a ^{31}P NMR study of the activation of the nucleoside phosphoramidites, and their subsequent reactions with protected guanine residues, we discovered that when a solution of either protected deoxyguanosine derivative **13a** or guanosine derivative **13b** was added to the TAMA activated phosphoramidites no O^6-phosphitylation could be detected in the ^{31}P NMR spectrum. This suggested that base modification and subsequent chain degradation could be eliminated by the use of this activator.

This procedure was tested by using TAMA to synthesize a **d-$(Gp)_{13}$rG** sequence. The coupling cycle used was the same as previously described[10] except that TAMA was used in place of tetrazole and the coupling reaction time was reduced to 30 seconds. The reduction in reaction time was to minimize removal of dimethoxytrityl groups by the acidic TAMA reagent. An average coupling yield of 95% was obtained for the synthesis, (yield following deprotection was 29% and after gel purification 4%) which was slightly lower than the yields achieved using either the O^6-protected phosphoramidites or the capping followed by oxidation cycle. The sequence was analyzed on a 20% polyacrylamide electrophoresis gel (FIGURE 3) and a discrete band with the correct mobility along with a number of fainter, faster bands were observed. However, this result was still much improved from our original results (FIGURE 1-2, lane 1) and confirmed our ^{31}P NMR observations.

In order to ascertain whether the reactions observed in the ^{31}P NMR studies were restricted to methyl protected phosphoramidites, we synthesized the cyanoethyl protected phosphoramidite derivatives of 5'-monomethoxytrityluridine and 5'-dimethoxytritylthymidine and repeated the ^{31}P NMR experiments. The results

from both the capping and the TAMA experiments indicated that no modification of guanine bases occured as in the case of the methyl-protected phosphoramidites. Thus the reactions we have observed occur independently of the type of phosphate protection used on the phosphoramidite reagents.

All of the oligoguanylate and oligodeoxyguanylate sequences were also analyzed by enzymatic degradation using spleen phosphodiesterase and, in the case of the ribonucleotide sequences, ribonuclease T_1. The products of the enzymatic digestions were analyzed by reversed-phase HPLC and gave the correct ratios of nucleotide to nucleoside. Since ribonuclease T_1 is a guanosine specific nuclease this was a very good confirmation of the integrity of the guanine bases obtained.

The identification of the twenty-four unit long oligodeoxyguanylate sequence was complicated by the tendency of the deoxyguanylate strands to form complexes by self-association[20]. These complexes were difficult to denature and even with preheating to 90^{o} and flash cooling, a broad band with very slow electrophoretic mobility appeared as the major product on the electrophoresis gels (FIGURE 1-3). The electrophoretic mobility of the crude mixture of oligodeoxyguanylate sequences was improved by treating the mixture with a formaldehyde solution[23] prior to electrophoresis (FIGURE 1-3). This treatment produced a circular band with electrophoretic mobility similar to a twenty-eight unit long oligothymidylic acid marker.

An alternate method of analyzing the oligodeoxyguanylate sequences which was suitable for preparative purification was liquid chromatography, at pH 13, on NACS-20 resin[24-25]. The crude mixture of sequences produced from the $\mathbf{d(Gp)_{23}G}$ synthesis which used the O^6-cyanoethyl protected derivative **8** produced only one major peak (FIGURE 4-1) when purified on the NACS-20 resin. The product from the $\mathbf{d(Gp)_{23}G}$ synthesis which used acetate ion to regenerate the guanine bases also produced an identical peak when purified on NACS-20 (FIGURE 4-2). Isolation and characterization of this peak indicated that it was the desired $\mathbf{d(Gp)_{23}G}$ sequence. The overall yield of both the $\mathbf{d(Gp)_{23}G}$ sequences following the NACS-20 chromatography was 22%.

The chromatogram of the mixture of crude sequences obtained

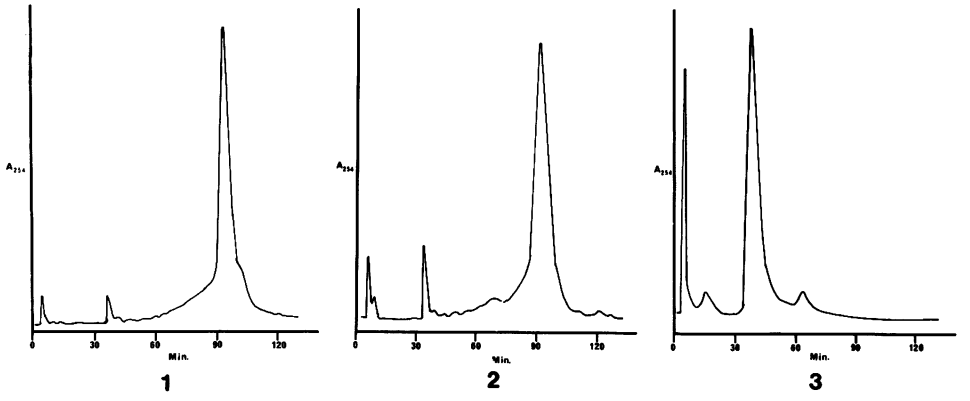


FIGURE 4: Liquid chromatography of oligodeoxyguanylate sequences on NACS-20 resin. (1) The crude **d(Gp)$_{23}$G** sequence prepared using the O^6-cyanoethyl protected derivative 8. (2) The crude **d(Gp)$_{23}$G** sequence prepared using acetic anhydride/dimethylaminopyridine solution to eliminate guanine phosphitylation. (3) The crude mixture of sequences obtained from the attempted synthesis of **d(Gp)$_{23}$G** which did not eliminate guanine phosphitylation. The chromatograms were obtained by elution at 1 ml/min with the following: 0-30 min, 0.1M NaCl, 0.1M NaOH; 30-130 min, gradient between 0.3M NaCl, 0.1M NaOH and 0.6M NaCl, 0.1M NaOH.

when synthesis was performed without eliminating O^6-modification is also shown for comparison (FIGURE 4-3). In this chromatogram a large peak, probably composed of a mixture of shorter fragments, eluted from the NACS-20 column as soon as the salt gradient began and no peak corresponding to the desired **d(Gp)$_{23}$G** product was detected.

A comparison of the results obtained using the different approaches discussed in this manuscript clearly shows the need for eliminating the chain cleavage brought about by phosphitylation of the guanine bases. The use of the first two of the three methods described, that is either O^6-protection or the use of a capping step immediately following the coupling step, to eliminate the O^6-phosphitylation should significantly improve the results of solid phase syntheses, especially when very long sequences are prepared. Although the TAMA reagent can prevent the modification of the guanine bases, its acidity and its hygroscopic nature limit its use as a practical solution to this problem. For most purposes the use of an acetic anhydride/di-

methylaminopyridine solution, to simultaneously cap unreacted sites and regenerate the guanine bases before the oxidation step, is probably the most convenient solution to this problem.

To date the most widely used coupling cycle in phosphite triester synthesis has been oxidation followed by capping[26]. This has clearly been shown to be unsuitable when preparing sequences containing a large number of guanine bases. We have demonstrated that this problem can be eliminated by protecting the O^6-position of guanine nucleosides, by using TAMA as an activating agent, or by using the alternate coupling cycle of capping followed by oxidation. This latter cycle has often been used[27] and this manuscript describes the basis of its success.

EXPERIMENTAL

General Methods and Materials

Oligonucleotide synthesis was performed using the synthesizer and synthesis program previously described[2,10] as well as with an Applied Biosystems 380A DNA synthesizer. The coupling cycle[2] used in the synthesis of the oligoribonucleotide sequences is shown below. The triisopropylsilyl protecting group has been used previously[2,28,29] for the protection of guanosine nucleosides as it allows for a much easier separation of the 2'- and 3'-silylated guanosine isomers than that possible with the more common t-butyldimethylsilyl group. ^{31}P NMR spectra were obtained on a Varian XL-300 spectrometer using the previously described protocols[10].

The deprotection[2] of the oligoribonucleotide sequences were carried out as follows. The methyl phosphate protecing groups were removed using a 30 min. treatment with thiophenoxide ion at room temperature. Cleavage of the oligomer from the solid support and N-debenzoylation were affected simaltaneously with NH_4OH/EtOH : 3/1 at 55°C, 16 hrs.. The triisopropylsilyl groups were removed with 1M TBAF at R.T., 3 hrs. In the case of the NPE protected sequence this step also removed the NPE group and a 6 hr. treatment was used. The fully deprotected sequences were desalted directly after the TBAF treatment by application to a Sephadex G-25F size exclusion column. The final purification was accomplished using preparative (1.5 mm gel thickness) polyacrylamide gel electrophoresis followed by extraction of the gel and desalting.

NACS-20 resin was obtained from Bethesda Research Laboratories (Gaithersburg, MD). A 10mm I.D. x 30mm column containing 1g of NACS-20 resin and run at ambient temperature was used for the purification of the $\mathbf{d(Gp)_{23}G}$ sequences. The flow rate and mobile phase composition are indicated in FIGURE 4 and the products were detected using a 254nm wavelength u.v. detector. N-methylanilinium trifluoroacetate (Aldrich Chemical Co.) was recrystallized from ether and dryed in vacuo prior to use. Enzymes were obtained from Boehringer Mannheim and HPLC analyses of the enzymatic digests were performed on a SpectraPhysics SP8000 liquid chromatograph.

AUTOMATED SYNTHESIS CYCLE FOR RIBONUCLEOTIDE SYNTHESIS

Step	Reagent*	Time (min)
1	5% Trichloroacetic acid/Dichloroethane	3.50
2	Acetonitrile	0.75
3	0.1M Nucleoside Phosphoramidite + 0.5M Tetrazole/Acetonitrile	0.25
4	Recycle	14.75
5 (or 6)	0.25M Ac_2O/DMAP/Collidine/THF	1.50
6 (or 5)	0.1M I_2 THF/Pyridine/H_2O 7/2/1	0.50
7	Acetonitrile	1.25

* Flow Rate = 5 ml min^{-1} Total Cycle Time = 22.50 min

Preparation of O^6-cyanoethyl protected deoxyguanosine nucleosides

1, 5',3'-Di-t-butyldimethylsilyl-2'-deoxyguanosine, **2**.

Deoxyguanosine (50 mmol, 13.35g), imidazole (250 mmol, 17g), and t-butyldimethylsilyl chloride (125 mmol, 18.8g) in anhydrous DMF (400 ml) was stirred at room temperature. After 24 hr., TLC showed a small amount of monosilylated material (5-10%) and more imidazole (50 mmol, 3.4g) and t-butyldimethylsilyl chloride (25 mmol, 3.8g) was added. After stirring (5 hr), the DMF was evaporated off and the residue was suspended in CH_2Cl_2 (150 ml) and washed once with water (75 ml). A thick white precipitate formed and was filtered off. The CH_2Cl_2 filtrate was washed again with water and then concentrated to yield another white precipitate. The combined precipitates were washed with ether and dried to yield 22.99g (46.4 mmol) of pure product (93% yield). UV (95% EtOH): Max. 253 nm, Min. 222 nm; TLC (10% MeOH / $CHCl_3$): Rf = 0.36.

2, 5',3'-Di-t-butyldimethylsilyl-N^2-isobutyryl-2'-deoxyguanosine, **3**.

A solution of **2** (45 mmol, 22.3g) and isobutyryl chloride (56 mmol, 5.9 ml) in anhydrous pyridine (250 ml) was stirred at room temperature. After 24 hr., more isobutyryl chloride (47 mmol, 5 ml) was added and stirring was continued for another 18 hr. The pyridine solution was concentrated to an oil and redissolved in $CHCl_3$ (200 ml). The $CHCl_3$ solution was then added to a rapidly stirred solution of aqueous $NaHCO_3$ (Caution: Rapid gas evolution). The $CHCl_3$ solution was washed with water, concentrated, and applied to a silica gel column. Elution with first $CHCl_3$ and then 5% MeOH/$CHCl_3$ produced the pure product as a yellow foam (21.6g, 75% yield). UV (95% EtOH): Max. 255, 261, 286 nm, Min. 226, 257, 270 nm; TLC (10% MeOH/$CHCl_3$) Rf = 0.55.

3, 5',3'-Di-t-butyldimethylsilyl-N^2-isobutyryl-O^6-(2,4,6-triisopropylbenzenesulphonyl)-2'-deoxyguanosine, **4**.

Compound **3** (30 mmol, 19.2g), triethylamine (120 mmol, 17 ml), and 4-dimethylaminopyridine (1.5 mmol, 183 mg) were dissolved in CH_2Cl_2 (300 ml). 2,4,6-Triisopropylbenzenesulfonyl chloride (60 mmol, 18.2 g) was added. A precipitate of triethylamine hydrochloride quickly formed and the reaction was stirred

at room temperature for three hours. The reaction mixture was applied directly to the top of a silica gel column and the column eluted with 1:1 ether/$CHCl_3$ to yield 28 g of crude product. The orange brown foam recovered was repurified on a second silica gel column to yield 23 g of pure **4** (82% yield) as a light yellow foam. UV (95% EtOH): Max. 232, 258, 280 nm, Min. 254, 270 nm; TLC (1:1 ether/$CHCl_3$) Rf = 0.90.

4, 5',3'-Di-t-butyldimethylsilyl-O^6-(2-cyanoethyl)-N^2-isobutyryl-2'-deoxyguanosine, **5**.

Compound **4** (22.5 mmol, 21.2 g) and 3-hydroxypropionitrile (225 mmol, 15.4 ml) were dissolved in CH_2Cl_2 (22.5 ml) and cooled to 0°. Anhydrous trimethylamine (240 mmol, 22.5 ml) was added followed ten minutes later by DBU (34 mmol, 5.0 ml). The reaction was stirred at 0° for two hours. The trimethylamine was then removed by coevaporation with CH_2Cl_2. The orange residue was washed with saturated NH_4Cl solution (3x) and purified on a silica gel column by elution with first $CHCl_3$ and then 1:1 ether/$CHCl_3$. 11.2 g of pure **5** was obtained (68% yield). UV (95% EtOH): Max. 224, 273 nm, Min. 236 nm; TLC (1:1 ether/$CHCl_3$) Rf = 0.32; TLC (5% MeOH/$CHCl_3$) Rf = 0.44; IR ($CHCl_3$ solution) 2395 cm^{-1}.

5, O^6-(2-cyanoethyl)-N^2-isobutyryl-2'-deoxyguanosine, **6**, and O^6-(2-cyanoethyl)-5'-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine, **7a**.

A solution of anhydrous 2M HF/1M TBAF in pyridine was prepared by adding aqueous HF (2 equiv.) to a THF solution of TBAF. The solution was then evaporated to an oil and coevaporated with anhydrous pyridine (4x) before dilution to the final volume. Compound **5** (3 mmol, 2.18 g) was dissolved in the above solution (6 ml). TLC (20% MeOH/$CHCl_3$) after two hours showed approximately 50% desilylation and TLC after 5.5 hours showed complete desilylation. However, TLC also indicated the loss of the 2-cyanoethyl group from about 30% of the material. The pyridine solution was coevaporated to dryness with xylene and the residue dissolved in $CHCl_3$ (30 ml). The $CHCl_3$ solution was washed with water (3x20 ml). The combined aqueous washes were evaporated to dryness and redissolved in $CHCl_3$. The solution was purified on a silica gel column by elution with a 3-10% methanol/$CHCl_3$ gradient. Elution with 5% MeOH/$CHCl_3$ produced 1.64 g of a mixture of **6** and TBAF. Elution with 10% MeOH/$CHCl_3$ produced 418 mg of N^2 -isobutyryl-2'-deoxyguanosine (1.2 mmol, 40%).

The mixture of **6** and TBAF was coevaporated with anhydrous pyridine (3x10 ml) and dissolved in anhydrous pyridine (10 ml). Dimethoxytrityl chloride (1.5 mmol, 507 mg) was added and the reaction was monitored by TLC. Dimethoxytrityl chloride was added in 0.5 mmol aliquots until 5',3'-ditritylated material began to form. $CHCl_3$ (100 ml) was added to the pyridine and the solution was washed with aqueous $NaHCO_3$ solution. The crude material was purified by silica gel chromatography using a 0-3% MeOH/$CHCl_3$ gradient. 1.09 g of pure **7a** (1.57 mmol, 52%) was obtained along with 0.52 g (0.52 mmol, 17%) of **7b**.

For compound **6**: UV (95% EtOH), Max. 270, Min. 252; TLC (20% MeOH/$CHCl_3$) Rf = 0.43; TLC (10% MeOH/$CHCl_3$) Rf = 0.14. For compound **7a**: UV (95% EtOH), Max. 270, 227 nm, Min. 252 nm; TLC (5% MeOH/$CHCl_3$) Rf = 0.35. For compound **7b**: UV (95% EtOH), Max. 270, 226 nm, Min. 258 nm; TLC (5% MeOH/$CHCl_3$) Rf = 0.63.

6, O^6-(2-cyanoethyl)-5'-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine-3'-(N,N-diisopropylmethoxyphosphoramidite), **8**.

N,N-Diisopropylmethylphosphonamidic chloride (1.2 mmol, 230 ul) was added to a solution of **7a** (1.0 mmol, 693 mg) and diisopropylethylamine (4 mmol, 0.56 ml) in anhydrous $CHCl_3$ (4 ml). Ethyl acetate (25 ml) was added after one hour of stirring. The solution was washed with saturated NaCl solution (3x) and evaporated to a foam. The crude material was purified on a silica gel column using 10% triethylamine/$CHCl_3$. Compound **8** was obtained as a pure white foam and 868 mg (1.0 mmol, 100% yield) was recovered. TLC (10% triethylamine/$CHCl_3$) Rf=0.70. ^{31}P NMR 149.64, 149.59 ppm.

Preparation of N^2-benzoyl-5'-O-monomethoxytrityl-O^6-p-nitrophenylethyl-2'-O-triisopropylsilylguanosine-3'-N,N-diisopropylmethylphosphoramidite **12**.

1, N^2-Benzoyl-5'-O-monomethoxytrityl-O^6-p-nitrophenylethylguanosine **10**.

N^2-Benzoyl-O^6-p-nitrophenylethylguanosine **9** (10.1 g, 18.8 mmol, 1 eq.), prepared according to the procedure of Pfleiderer[12] was dissolved in anhydrous pyridine (100 ml) under argon and monomethoxytrityl chloride (7.55 g, 24.45 mmol, 1.3 eq.) was added. After 16 hours stirring, TLC analysis (10% MeOH/$CHCl_3$) indicated the presence of starting material and additional monomethoxytrityl chloride (7.55 g) was added in 2 aliquots over 8 hrs. After overnight stirring the pyridine was removed by coevaporation with toluene and the resulting oil was dissolved in CH_2Cl_2 (500 ml) and extracted with 5% $NaHCO_3$ solution (2x). The organic layer was dried over $MgSO_4$ and the solvent removed. The crude material was purified by silica gel chromatography using a 0-3% MeOH/CH_2Cl_2 gradient to give 15.75 g of product which was contaminated with ditritylated material. A second purification on a short silica gel column using CH_2Cl_2/Et_2O:1/1 as eluent yielded 12.5 g (82.1%) of pure **10**. U.V. (95% EtOH), Max. 271, 228, Min. 247; TLC (5% MeOH/$CHCl_3$) R_f = 0.40; ^1H NMR (DMSO-d_6) H1' d 5.963, 5.985 ppm; m.p. 126°.

2, N^2-Benzoyl-2'-O-triisopropylsilyl-5'-O-monomethoxytrityl-O^6-p-nitrophenylethylguanosine **11**.

Compound **10** (9.0 g, 11.12 mmol, 1 eq.) was dissolved in anhydrous DMF (50 ml) and imidazole (1.97 g, 28.9 mmol, 2.6 eq.) and triisopropylsilyl chloride (2.4 ml, 14.46 mmol, 1.3 eq.) was added. After overnight stirring, TLC ($Et_2O/CHCl_3$:1/1) still showed the presence of starting material, therefore an additional two aliquots of imidazole (0.65 g) and triisopropylsilyl chloride (0.8 ml) were added over the next 24 hours. The reaction was worked up by removal of the DMF in vacuo and coevaporation (2x) of the residue with toluene. The resulting oil was dissolved in CH_2Cl_2 (250 ml) and extracted with 5% $NaHCO_3$ solution. The organic phase was dried over Na_2SO_4 and the CH_2Cl_2 evaporated to give 11.4 g of crude **11** as a pale yellow foam. The crude product was purified by silica gel chromatography using CH_2Cl_2/hexane/NEt_3 (35/63/2) to give 4.0 g (52%) of **11**. The remainder of the fractions consisted of a mixture of 2'- and 3'- isomers (2.3 g, 30%), 3'-isomer (1.4 g, 18%) and **10** (2.6 g). The yields were based on 6.4 g of **10** as starting material. For **11**: UV (95% EtOH) Max. 271, 228(sh), Min. 247; TLC ($Et_2O/CHCl_3$:1/1) R_f=0.58; ^1H NMR ($CDCl_3$) H1' d 6.037, 6.011 ppm; m.p. 84-87°.

3, N^2-Benzoyl-5'-O-monomethoxytrityl-O^6-p-nitrophenylethyl-2'-O-triisopropylsilylguanosine-3'-N,N-diisopropylmethylphosphoramidite **12**

To a stirred solution of DMAP (97.7 mg, 0.8 mmol, 0.2 eq.), diisopropylethylamine (2.8 ml, 16 mmol, 4 eq.) and N,N-diisopropylmethylphosphonamidic chloride (1.0 ml, 5.2 mmol, 1.3 eq.) in anhydrous THF (15 ml) was added, dropwise by syringe, a solution of **11** (3.86 g, 4 mmol, 1 eq.) in anhydrous THF (10 ml) under an Ar atmosphere. After 8 hr. the reaction was incomplete and therefore additional $NEt(i\text{-}Pr)_2$ (0.9 ml) and phosphitylating reagent (0.3 ml) were added and the reaction stirred overnight. The resulting slurry was filtered, diluted with EtOAc (100 ml), and washed (5x) with saturated brine. The organic phase was dried over Na_2SO_4 and the solvent removed in vacuo to give 4.4 g of crude **12** as a pale orange foam. The crude material was purified by silica gel chromatography, CH_2Cl_2/hexane/NEt_3 (30/68/2) to give 3.37 g (75%) of pure **12** as a white foam. UV (95% EtOH) Max. 271, 228, Min. 247, 223; TLC (Et_2O/Hex:1/1 x 1, $Et_2O/CHCl_3$:1/1 x 1) R_f=0.57, 0.67 (2 diastereomers); m.p. 87-90°; ^1H NMR H1' 5.736, 5.895 $J_{1'-2'}$=7 Hz, P-O-CH_3 2.983, 2.917 and 3.406, 3.340 ppm; ^{31}P NMR 153.875, 149.520 ppm.

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