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Modification of guanine bases by nucleoside phosphoramidite reagents during the solid phase synthesis of oligonucleotides

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ABSTRACT

Nucleoside 3'-phosphoramidite and chlorophosphite reagents have been found to react with the lactam function of guanine. This reaction caused unsatisfactory results when oligodeoxyribonucleotides containing a large number of guanine bases were prepared in an automated solid phase synthesizer. The guanine modification is unstable, and leads to depurination and chain cleavage. This side reaction can be eliminated by protecting the O^6-position. A new O^6-p-nitrophenylethyldeoxyguanosine phosphoramidite derivative, **8**, was used to prepare sequences containing up to 24 guanine bases with greatly improved results. A hexatriacontanucleotide, **d(CGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTCA)**, was also prepared using O^6-protected deoxyguanosine nucleosides.

INTRODUCTION

The chemical synthesis of oligodeoxyribonucleotide sequences has become a common procedure in many laboratories (1, 2). This task can be easily performed on insoluble supports using an automated or semi-automated DNA synthesizer (3-6). The success of these solid phase procedures (7-9) has been due to recent improvements in both the insoluble supports and the coupling reactions.

However, high coupling yields are not sufficient to guarantee successful isolation of the final product. It is also necessary to eliminate any side reactions or modifications of the heterocyclic base residues. For example, substituted side-products on guanine, thymine and uracil bases are known to occur when either arylsulphonyl condensing reagents (11-13) or phosphorylating reagents (14-18) are used. Although some of these modifications can be reversed by extended treatment with aldoximate reagents (11, 19) at the end of the synthesis, the complete elimination of these side-products in long oligonucleotides

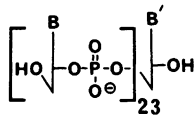
is unproven. To prevent these base modifications, a number of protecting groups have been proposed for use on the O^4- and N^3-positions of thymine (20, 21) and uracil (21-23), and on the O^6-position of guanine (20-22, 24-28).

Alternately, a nucleoside phosphoramidite derivative can be used. Until now, no modification or phosphorylation of the bases has been reported in coupling reactions involving phosphoramidite reagents. Methylation of thymine has been observed, but this requires strongly alkaline conditions (29).

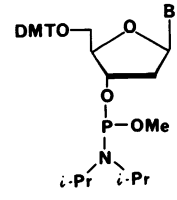
In this report we describe a side reaction involving guanine bases and nucleoside phosphoramidite reagents. This reaction occurs very quickly and causes significant deterioration of sequences which contain a large number of guanine bases. This side reaction can be completely eliminated and we would like to describe how superior results can be obtained by protecting the O^6-position of deoxyguanosine. A preliminary report of some of these results has appeared (30).

RESULTS

A series of six homogeneous, twenty-four unit oligonucleotides **1a-d** were initially prepared as part of a study examining depurination in automated solid phase synthesis. These six



1a, B = B' = Thymine
b, B = B' = Cytosine
c, B = B' = Adenine
d, B = B' = Guanine
e, B = Guanine, B' = Thymine



2a, B = Thymine
b, B = N^4-Benzoylcytosine
c, B = N^6-Benzoyladenine
d, B = N^6-(N-methyl-2-pyrrolidine amidine)-adenine
e, B = N^2-Isobutyrylguanine
f, B = N^2-(N-methyl-2-pyrrolidine amidine)-guanine

tetracosanucleotides were prepared from the deoxyribonucleoside 3'-O(N,N-diisopropylamino)phosphoramidites **2a-f** using an automated DNA/RNA synthesizer (31, 32). The synthesizer program and the coupling yields of these sequences are shown in TABLE 1 and TABLE 2, respectively.

TABLE 1. Automated DNA Synthesis Cycle.

STEP	REAGENT	DURATION	ELAPSED TIME
1	Acetonitrile wash	60 sec	1.0 min
2	3% Trichloroacetic acid/Dichloroethane[a]	90 sec	2.5 min
3	Acetonitrile wash	60 sec	3.5 min
4	Phosphoramidite/Tetrazole addition	12 sec	3.7 min
5	Recycle	288 sec	8.5 min
6	0.1M I_2 H_2O/PYR/THF 1:2:7	30 sec	9.0 min
7	0.25M Ac_2O/DMAP/THF	60 sec	10.0 min

[a] - For sequences longer than 24 units, the detritylation time was increased to 180 seconds after 23 coupling cycles.

After the completion of each synthesis, a portion of the nucleotide containing support was completely deprotected. The crude mixture of products obtained was separated on a 20% polyacrylamide/7M urea electrophoresis gel and then visualized by UV shadowing.

The results (FIGURE 1) for the oligothymidine **1a** and oligodeoxycytidine **1b** sequences were quite good, as expected for these pyrimidine sequences. Only one strongly absorbing and well-defined band was present in each synthesis. Figure 1 also shows the differences in electrophoretic mobility which homooligomers of the same chain length can possess (33).

The oligodeoxyadenosine sequence **1c**, which was prepared from either the N^6-benzoyl (**2c**) or N^6-methylpyrrolidine amidine (**2d**, 34) protected deoxyadenosine derivatives, was also very satisfactory in both instances. No significant depurination was evident

TABLE 2. ^{31}P NMR Chemical Shifts and Average Coupling Efficiencies of Deoxyribonucleoside Diisopropylaminephosphoramidites.

PHOSPHORAMIDITE	^{31}P CHEMICAL SHIFT*	SEQUENCE PREPARED	AVERAGE YIELD**	OVERALL YIELD**
2a	149.7, 149.1	**$(Tp)_{23}T$**	96.8%	47%
2b	150.1, 149.4	**d[$(Cp)_{23}C$]**	97.5%	56%
2c	149.8, 149.6	**d[$(Ap)_{23}A$]**	98.9%	78%
2d	149.4, 149.3	**d[$(Ap)_{23}A$]**	98.8%	75%
2e	149.2, 149.1	**d[$(Gp)_{23}G$]**	93.3%	20%
2f	149.4, 149.3	**d[$(Gp)_{23}T$]**	94.9%	29%

* - ppm (±0.1) downfield from 85% H_3PO_4 in $CDCl_3$
** - determined by trityl colour quantitation

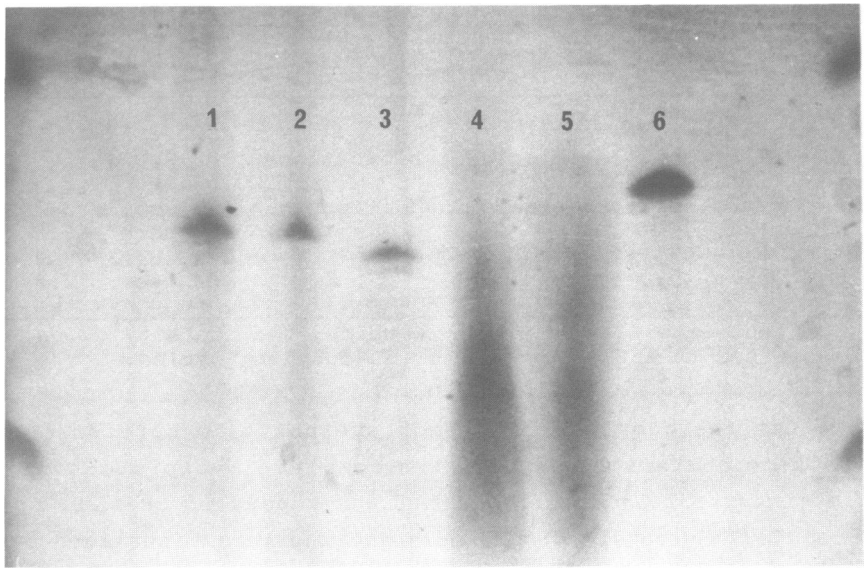


FIGURE 1. 20% Polyacrylamide/7M urea electrophoresis gel of the oligonucleotides **1a-e.** The phosphoramidites used were: Lane 1 - **2c;** Lane 2 - **2d;** Lane 3 - **2b;** Lane 4 - **2f;** Lane 5 - **2e;** Lane 6 - **2a.**

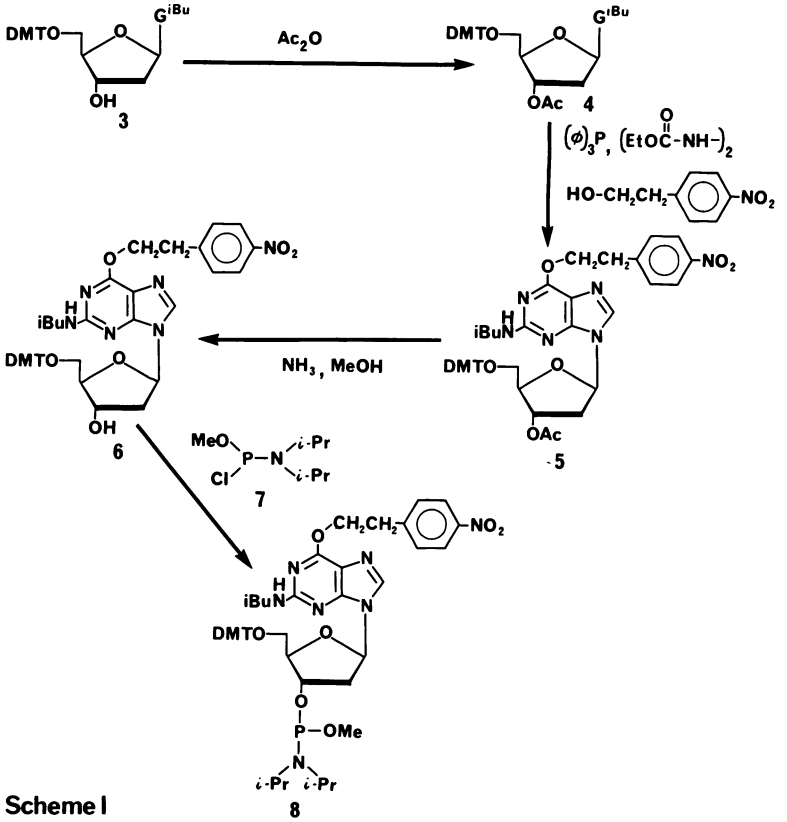
in these preparations since no chain cleavage was observed.

However, the crude reaction mixtures produced when either of the deoxyguanosine phosphoramidites **2e** or **2f** were employed, gave quite different results (FIGURE 1). These reactions did not contain any single well-defined or prominent product. Instead, the material on the electrophoresis gel appeared as a broad smear of faster moving products. This result was indicative of some type of chain degradation and was not due to the slightly lower coupling yields of these syntheses. The very large difference between the guanine containing products and the adenine, cytosine and thymine containing sequences, suggested that some side reaction involving only the guanine base was occurring.

Guanine differs from the other bases by possesing potentially reactive sites at the O^6- and N^1-positions. Indeed, reactions with the O^6-position are well documented, and substitution (11, 15, 16), sulphonation (12, 13), silylation (13), and phosphorylation (13, 14, 17, 18) reactions have been described. Therefore,

we considered the possibility that phosphoramidite reagents might react with guanine to produce phosphite substituted derivatives.

To test this hypothesis, we prepared the O^6-nitrophenylethyl protected deoxyguanosine phosphoramidite **8**, by the route shown in SCHEME I. The introduction of a protecting group on the O^6-position also prevents side reactions at the adjacent N^1-position. The synthesis of nucleoside **6** was performed using the



Scheme I

procedure developed by Pfleiderer et al (27, 28). Derivative **8** was prepared from **6** and **7** (35) in 83% yield.

Phosphoramidite, **8**, was used to synthesize the deoxyguanosine tetracosanucleotide **1d**, using the same synthesis conditions as before. The average coupling yield in this synthesis was 96%. The oligonucleotide containing polymer was deprotected by sequential treatment with thiophenol, ammonium hydroxide, and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The latter step was re-

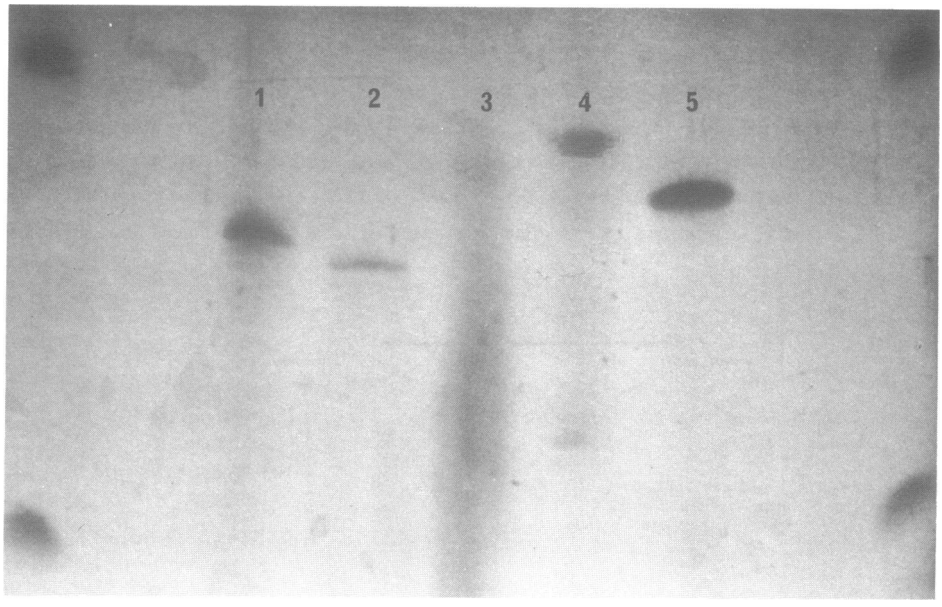


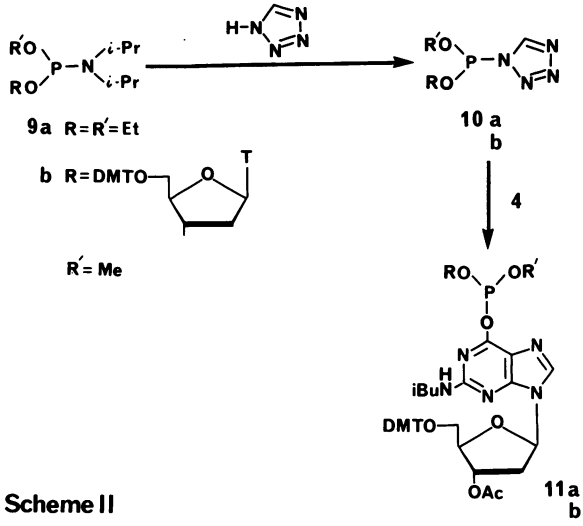
FIGURE 2. 20% Polyacrylamide/7M urea electrophoresis gel showing the oligodeoxyguanosine sequence **1d** prepared from the O^6-protected phosphoramidite reagent **8** (lane 4). Lane 3 contains the synthesis performed using the O^6-unprotected phosphoramidite **2e**. Lanes 1, 2, and 5 contain the tetracosanucleotides prepared from phosphoramidites **2c**, **2b**, and **2a**, respectively.

quired to remove the p-nitrophenylethyl protecting groups from the sequence (27, 28). The crude material was then examined by electrophoresis on polyacrylamide/7M urea gel.

The result, this time, was a great deal better. Only one strongly absorbing and well-defined spot was present when the gel was viewed under UV irradiation (FIGURE 2). The synthetic material was now as good as the oligodeoxyadenosine, oligodeoxycytidine, or oligothymidine sequences. This result confirmed our hypothesis that the lactam function on the guanine base was involved in some side reaction.

We investigated this side reaction using ^{31}P NMR and model nucleoside **4**. In these NMR experiments, either diethyl diisopropylaminophosphoramidite **9a** or 5'-dimethoxytritylthymidine-3'-diisopropylaminomethoxyphosphoramidite **9b** in anhydrous acetonitrile was activated by the addition of tetrazole to produce the

intermediate tetrazolide **10** (SCHEME II). This intermediate was not isolated but reacted immediately with nucleoside **4**. The ^{31}P



Scheme II

NMR spectrum was obtained directly from the reaction mixture (see experimental section) and the course of the reaction was monitored by acquiring a new spectrum every few minutes.

When phosphoramidite **9a** was combined with tetrazole, the signal corresponding to **9a** (146.1 ppm,) was quantitatively converted into a new resonance at 128.9 ppm. This new singlet was due to formation of intermediate **10a**. The activation was very fast and was completed during the acquisition time of the first NMR spectrum. When nucleoside **4** was added to the reaction, the signal at 128.9 ppm was converted into a new resonance at 136.9 ppm. It is reasonable to suggest that this new signal was due to formation of compound **11a**. Approximately 60% of the tetrazolide reacted to give **11a** within the first ten minutes and then no further reaction was observed.

We also obtained the singlet signal at 136.9 ppm by reacting diethyl chlorophosphite with nucleoside **4** in an anhydrous solution of diisopropylethylamine and acetonitrile. In this experiment the chlorophosphite (168.1 ppm) was converted into **11a** in approximately 74% yield within ten minutes. This result indicates that chlorophosphite derivatives as well as activated phosphoramidite derivatives can participate in this side reaction.

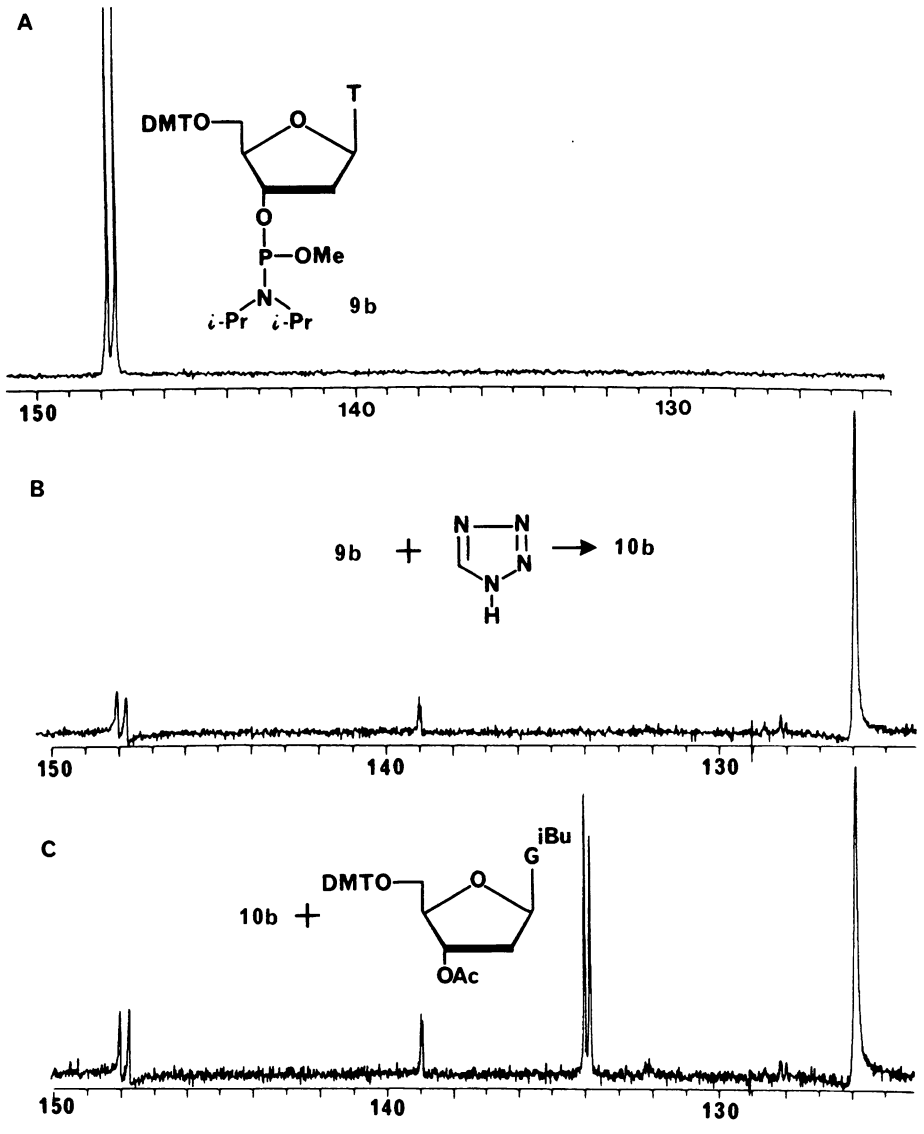


FIGURE 3. 121.5 MHz ^{31}P NMR spectra of (a) **9b**, (b) **9b** and tetrazole, (c) **9b**, tetrazole, and **4** in anhydrous CH_3CN.

The ^{31}P NMR results with the thymidine phosphoramidite **9b** were also similar (FIGURE 3). The two signals corresponding to the diastereoisomers of **9b** (148.Ø, 147.7 ppm) were very quickly converted into the activated derivative **1Ø b** (126 ppm) by the

addition of tetrazole. Only one peak was observed for the tetrazolide derivative **10b** due to rapid exchange of the tetrazole group (36). When nucleoside **4** was added to this mixture, the peak at 126 ppm was partially converted into a doublet at 134.0 and 133.8 ppm (FIGURE 3c). These two signals had chemical shifts which were approximately five ppm upfield of a normal 3'-5' internucleotide phosphite triester linkage (138-140 ppm, 37). This upfield shift of phosphorus was characteristic of an attachment to an aromatic ring system and was consistent with the formation of compound **11b**. The ^{31}P NMR spectrum showed two new peaks because a new chiral center was introduced by the phosphite linkage.

During the two minutes required to obtain the first NMR spectrum, approximately 30% of the tetrazolide was converted into this new material. The reaction appeared to stop after approximately ten minutes, when there was about 50% of the tetrazolide remaining.

The presence of tetrazole was essential for the formation of either **11a** or **11b**. If a solution of either phosphoramidite (**9a** or **9b**) and **4** was prepared with no tetrazole present, only the unreacted starting phosphoramidite was observed in the ^{31}P NMR spectrum, even after standing for several days.

The phosphorus linkage in compounds **11a** and **11b** was quite sensitive towards nucleophilic attack. When an excess of tetrazole was added to the NMR tube containing **11a**, the amount of **11a** decreased, while more of the tetrazolide **10a** was regenerated. Compound **11b** was not affected by excess tetrazole probably because the phosphite linkage was more hindered. However, both **11a** and **11b** were found to be rapidly hydrolyzed by water. The addition of a small amount of water (5-10 ul) into the NMR tube completely converted the phosphorus species into dialkyl phosphonates (9.9 ppm). This lability explained why the modified deoxyguanosine compounds could not be observed on TLC plates.

We also tested the three 3'-acetylated nucleosides **12-14** as well as the fully protected deoxyguanosine nucleoside **5**. These molecules were dissolved in the presence of **10b** and the solutions were checked by ^{31}P NMR after 24 hours. In each case, the only phosphorus containing species was the activated tetrazolide **10b**

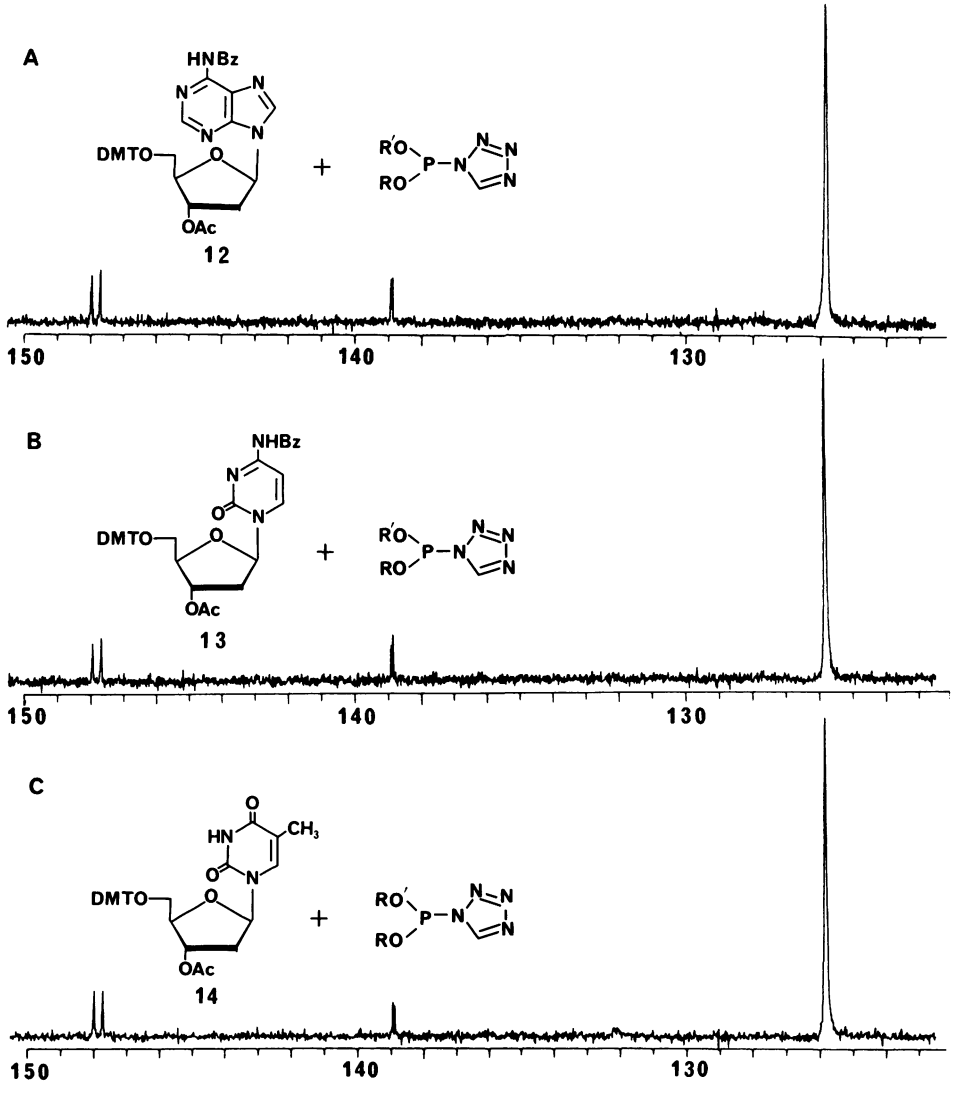


FIGURE 4. 121.5 MHz ^{31}P NMR spectra of (a) **9b**, tetrazole, and **12**, (b) **9b**, tetrazole, and **13**, (c) **9b**, tetrazole, and **14**, in anhydrous CH_3CN.

(126 ppm, FIGURE 4). It is noteworthy that no reaction occurred with the thymidine nucleoside **14**, because the O^4-position of thymine has been known to react with arylsulphonyl condensing reagents (11) and phosphorylating reagents (16).

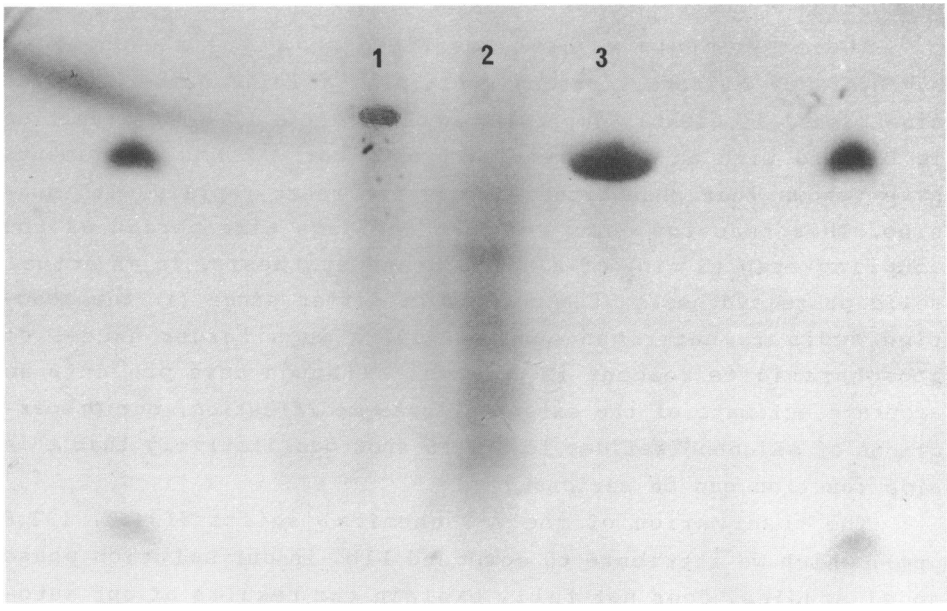


FIGURE 5. 16% Polyacrylamide/7M urea electrophoresis gel containing the hexatriacontanucleotide **d(CGCGGGGTGGAGCAGCCTGGTAGCTCG-TCGGGCTCA)** prepared using O^6-protected guanine (lane 1) and O^6-unprotected guanine (lane 2). A 30 unit long oligothymidylic acid marker is in lane 3.

After having satisfactorily completed the synthesis of the oligodeoxyguanosine tetracosanucleotide using the O^6-protected phosphoramidite **8**, we checked the general utility of this phosphoramidite by preparing another deoxyguanosine rich sequence. This was the hexatriacontanucleotide **d(CGCGGGGTGGAGCAGCC-TGGTAGCTCGTCGGGCTCA)**, which contained sixteen deoxyguanosine nucleosides.

The hexatriacontanucleotide sequence was synthesized twice, first using deoxyguanosine derivative **2e**, and secondly using the O^6-protected deoxyguanosine phosphoramidite **8**. The crude material obtained after completely deprotecting each sequence is shown in FIGURE 5. Although the average coupling yields in each synthesis were similar (96-97%), the final product from the O^6-protected synthesis was significantly better. The product from the first synthesis contains a number of shorter fragments while the second synthesis contained fewer shorter sequences and had a much more intense product band.

DISCUSSION

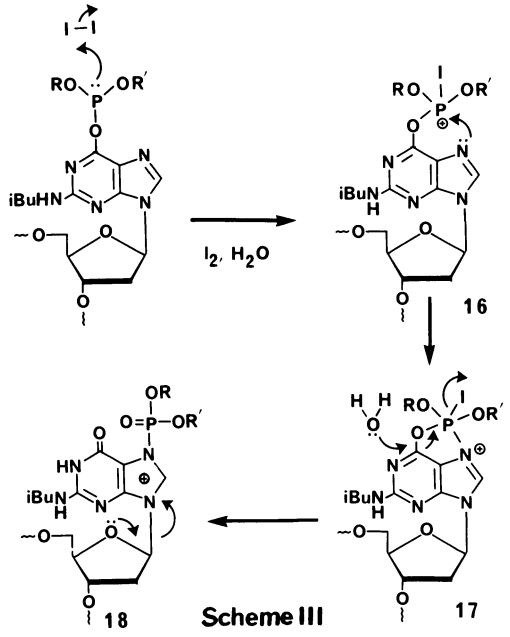
The experiments we have described show that the chemical synthesis of oligonucleotides containing a large number of guanine bases, is greatly improved when guanine's 1,6-amide function is blocked with an O^6-protecting group. Our ^{31}P NMR experiments have shown that phosphite reagents can react rapidly with guanine. This reaction occurs within the same time period as the coupling step (5 min) of a solid phase synthesis. In an actual solid phase synthesis, the conditions differ since (i) the reaction media is heterogeneous and (ii) a much larger excess of phosphoramidite reagent is present. Although this prevents an accurate estimate of the extent of base modification, our preparations of oligonucleotides **1d** and **15** show qualitatively that this side reaction can be serious.

The observation of the ^{31}P chemical shifts (134.Ø, 133.8 ppm), which we attribute to compound **11b**, in our solution phase model studies, does not fully explain the results of our automated solid phase syntheses. Although the model experiments show that phosphitylation of guanine occurs rapidly, we have also found that the phosphite-guanine linkages are easily hydrolyzed by water. This reaction would regenerate the deoxyguanosine nucleoside.

However, the guanine-phosphite linkage may react with iodine before water in the iodine/water oxidation step of the synthesis cycle (SCHEME III). This would lead to intermediate **16** which is susceptible to neighbouring group attack from the N^7-atom. This would allow the phosphorus group to migrate onto the N^7-position instead of being oxidized into an O^6-phosphate linkage. N^7-modified guanosine nucleosides are prone to depurination (38) and apurinic sites might be produced, either immediately, or during the next detritylation step. At the end of the synthesis, the basic conditions (15M NH_4OH, 5Ø°) would cause cleavage at each apurinic site.

Although this mechanism is speculative and we have not been able to estimate the extent to which these side reactions occur, only a very small number of apurinic sites need to be formed during each coupling cycle to seriously affect the synthesis.

The need for an additional protecting group on the O^6-



Scheme III

position of the guanine base is an extra complication which will be required in future solid phase syntheses. Although a number of different types of protecting groups have been described for this position, we decided that the p-nitrophenylethyl protecting group best suited our needs. This group can be easily introduced (27, 28) and is stable to all of the reaction conditions employed.

The removal of the p-nitrophenylethyl protecting group at the end of the synthesis requires an extra step, because this group cannot be quantitatively cleaved with ammonium hydroxide. Instead a treatment with DBU is required. Previous reports, have suggested use of 0.5M DBU for 0.5 to 2 hours (50°) is sufficient for complete deprotection (27, 28). However, we have found that a much longer deprotection step (72 hours, 50°) with a 1M DBU solution is required for quantitative deblocking.

Great care must be taken to ensure complete removal of the p-nitrophenylethyl group because there is no difference in the electrophoretic mobility of the O^6-protected and O^6-unprotected sequences. To confirm complete deprotection, we digest an aliquot of the final material with snake venom phosphodiesterase and alkaline phosphatase and analyze the digest by HPLC (40). The

presence of undegraded material indicates that the deprotection has been incomplete because digestion is blocked by remaining O^6-p-nitrophenylethyl groups.

We have attempted to reduce the time required to remove the p-nitrophenylethyl group, by using tetrabutylammonium fluoride (24) instead of DBU. Preliminary TLC studies showed clean conversion of **6** into **3** within only five minutes. However, an attempted preparation of **1d** using this procedure did not give complete deprotection. This was presumably due to the poor solubility of the oligonucleotide in the THF solvent. Future experiments should investigate other protecting groups for the O^6 -position, such as the cyanoethyl group, which can be more easily removed at the end of the synthesis (24).

CONCLUSIONS

We have found, from ^{31}P NMR observations, that either chlorophosphite reagents or activated phosphoramidite reagents react with guanine bases. This reaction is presumed to occur at the O^6-position of the guanine base and does not occur with either deoxyadenosine, deoxycytidine or thymidine nucleosides. Sequences containing many guanine bases are severely affected by this side reaction and produce poor results. This side reaction can be eliminated by protecting the O^6-position of guanine and sequences prepared with p-nitrophenylethyl protecting groups on the guanine bases produced good results.

This observation has very important significance for any future syntheses of deoxyguanosine containing sequences. The use of deoxyguanosine phosphoramidite reagents which posess the extra protecting group should enable the synthesis and isolation of much longer sequences than is presently possible.

EXPERIMENTAL SECTION

General Materials and Procedures

The protected deoxyribonucleosides: 5'-dimethoxytrityl-thymidine; N^4-benzoyl-5'-dimethoxytrityl-2'-deoxycytidine; N^6-benzoyl-5'-dimethoxytrityl-2'-deoxyadenosine; and 5'-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine were obtained from BIO LOGICALS (Toronto, Ont., Canada). Long chain alkyl amine controlled pore glass (Pierce Chemical Co.) was used as the insoluble support and deoxyribonucleosides were attached to the support via succinate linkages (1). 1-Methyl-2,2-diethoxypyrrolidine was prepared from 1-methyl-2-pyrrolidinone and 5'-dimethoxytrityl-N^6-

(N-methyl-2-pyrrolidine amidine)-2'-deoxyriboadenosine was prepared using the procedure of Caruthers (34).

NMR spectra were obtained on Varian XL-200 and XL-300 spectrometers. ^{31}P NMR chemical shifts are reported in ppm downfield from 85% H_3PO_4 (external standard).

HPLC assays (40) were performed on a Whatman C-8 reversed phase column (0.42 x 25 cm) in a SpectraPhysics SP8000 chromatograph equipped with a UV detector and data system. The eluting solvent was 0.05M KH_2PO_4 containing 2 mM PIC-A (Waters Scientific, Milford, MA).

Preparation of N^2-(N-methyl-2-pyrrolidine amidine)-guanine and 5'-Dimethoxytrityl-N^2-(N-methyl-2-pyrrolidine amidine)-guanine

Deoxyguanosine (5 mmol, 1.38 g) was suspended in 2,2-diethoxy-N-methyl-pyrrolidine (15 mmol, 5 g) and anhydrous pyridine (10 ml) and stirred (3 days, room temperature). The resulting solution was concentrated under vacuum to a thick oil and then coevaporated with anhydrous pyridine (3 x 20 ml). The oil was redissolved in anhydrous pyridine (15 ml) and dimethoxytrityl chloride (5.5 mmol, 1.86 g) was added. After 16 h, TLC (5% MeOH/$CHCl_3$) showed only partial tritylation and more dimethoxytrityl chloride (2.5 mmol, 0.9 g) was added. After another 16 h, the solution was diluted with $CHCl_3$ (100 ml) and neutralized with saturated $NaHCO_3$ solution. The reaction was washed with water and purified on a silica gel column by elution with first $CHCl_3$ and then 3% MeOH/$CHCl_3$. The purified material obtained (2.76 g, 76%) was a mixture of the syn and anti isomers. TLC: 5% MeOH/$CHCl_3$, Rf=0.29 and 0.17. UV (95% EtOH): Max = 234, 302 nm; Min = 222, 256 nm.

Preparation of the Deoxyribonucleoside Phosphoramidites 2a-f

A deoxyribonucleoside (10 mmol) was dissolved in anhydrous $CHCl_3$ (30 ml, distilled from P_2O_5 and stored under Ar) and anhydrous diisopropylethylamine (40 mmol, 7 ml) in a septum sealed bottle. Chloro(N,N-diisopropylamino)methoxyphosphoramidite (11 mmol, 2.2 ml) was added dropwise over 90 seconds and the reaction was stirred at room temperature (1 h). Ethyl acetate (300 ml, prewashed with $NaHCO_3$) was added and the solution washed with saturated NaCl solution (4x 150 ml) and water (1x 50 ml). After drying (anhydrous Na_2SO_4), the solvent was evaporated off to yield the crude material as a foam. The crude was then purified by silica gel chromatography (See TABLE 3 for solvents and physical properties). The purified material was stored in tightly sealed vials at -20°.

General Procedure for Automated Solid Phase Synthesis

An automated synthesizer controlled by an IBM personal computer was used. This machine was originally supplied by BIO LOGICALS but was substantially modified in our laboratory. The modified synthesizer contained a recycle loop (1 ml), a Waters Scientific M-45 high pressure pump, a fraction collector (to collect trityl colours), and a manifold which allowed plastic disposable syringes to be used as the nucleotide resevoirs. Stainless steel columns were used to contain the CPG support (100 mg). Syntheses were performed using the program shown in TABLE 1. Each base addition was performed using 50 umol of nucleoside phosphoramidite reagent. After the required number of coupling cycles, the CPG support was removed from the synthesizer and deprotected manually.

TABLE 3. Physical Properties and Yields of Deoxyribonucleoside 3'-O(N,N-diisopropylamino)phosphoramidite Derivatives.

Compound	F. Wt.	Solvent System (CH_2Cl_2:HEX:TEA)	Rf	UV* (nm) Max	Min	Yield (%)
2a	705.65	45:45:10	0.34	233 268	228 255	97
2b	794.65	50:40:10	0.59	232 257 302	223 248 288	72
2c	818.68	50:40:10	0.56	232 279	223 255	54
2d	795.45	50:40:10	0.50	230 304	221 259	70
2e	800.65	90:00:10	0.32	236 275 280	226 270 278	93
2f	799.91	90:00:10	0.76	234 302	222 256	84
8	939.85	50:40:10	0.64	236 270	233 250	83

* - In 95% ethanol; HEX = hexanes; TEA = triethylamine

Preparation of 3'-Acetyl-5'-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine 4.

Nucleosides **4**, 5, and **6** were prepared using a slight modification of the procedure of Pfleiderer *et al* (27, 28). A solution of **3** (10 mmol, 6.4 g) in anhydrous pyridine (30 ml) and acetic anhydride (20 ml) was stirred at room temperature (2 h). TLC (5% MeOH/$CHCl_3$) showed complete reaction. The solution was evaporated to a gum, coevaporated with toluene (3x 20 ml) and redissolved in $CHCl_3$ (100 ml). The solution was then neutralized with saturated $NaHCO_3$ solution (CAUTION: rapid gas evolution). The $CHCl_3$ solution was washed with water (3x 50 ml), concentrated, and purified by elution on a silica gel column using 1-3% MeOH/$CHCl_3$. The product was obtained as a white foam (12.9 g 94% yield). TLC: 5% MeOH/$CHCl_3$, Rf=0.37; ether/$CHCl_3$/ethanol 71:26:3, Rf = 0.05. UV (95%EtOH): Max = 235, 275, 280 nm; Min = 223, 270 nm.

Preparation of 3'-Acetyl-5'-dimethoxytrityl-N^2-isobutyryl-O^6-p-nitrophenylethyl-2'-deoxyguanosine 5

A solution of **4** (10 mmol, 6.82 g), triphenyl phosphine (12 mmol, 3.15 g) and p-nitrophenylethyl alcohol (12 mmol, 2.01 g) in anhydrous dioxane (150 ml) was stirred at room temperature. Diethylazodicarboxylate (12 mmol, 2.09 g) was then added and the solution quickly became purple coloured. After 30 minutes, the purple colour faded to yellow and the reaction was complete when checked by TLC (5% MeOH/$CHCl_3$). The solution was concentrated to an oil, redissolved in $CHCl_3$ (100 ml) and washed with water (3x 50 ml). The crude material was purified on a silica gel column by elution with ether and then ether:$CHCl_3$ 2:1. The pure product was obtained as a white foam (89% yield). TLC: 5% MeOH/$CHCl_3$, Rf=0.77; ether/$CHCl_3$/ethanol 71:26:3, Rf=0.29; ether, Rf=0.19. UV (95% ethanol): Max = 272 nm; Min = 248 nm.

Preparation of 5'-Dimethoxytrityl-N^2-isobutyryl-O^6-p-nitrophenylethyl-2'-deoxyguanosine 6

A solution of 5 (3.7 mmol, 3.1 g) in 1M NH_4OH/MeOH (150 ml) was stirred at room temperature. The reaction was carefully monitored by TLC (5% MeOH/$CHCl_3$) and after 2.5 h the reaction was concentrated to dryness, redissolved in $CHCl_3$ (75 ml) and washed with water (3x 75 ml). The $CHCl_3$ was evaporated off to yield pure **6** as a white powder (2.9 g, 99% yield). TLC: 10% MeOH/$CHCl_3$, Rf=0.51. UV (95% ethanol): Max = 272, 241 nm; Min = 250 nm.

Deprotection of Oligodeoxyribonucleotides

a, Removal of the methyl and acyl protecting groups.

The controlled pore glass support (30-40 mg) bearing the protected oligonucleotide was treated with a thiophenol:triethylamine:dioxane 1:1:2 solution (1-2 ml) for 1 h at room temperature. The support was then washed with ethanol (3x 5 ml) and the washing were discarded. The support was then sealed into a polypropylene test tube (5 ml) with 15M NH_4OH (2 ml) and the mixture was heated (50°, 4 h). The NH_4OH was decanted off, the support was washed 15M NH_4OH (4x 2 ml) and the combined washings were concentrated to dryness.

b, Removal of the Methyl-pyrrolidine amidine group

After the above deprotection steps, sequences prepared from phosphoramidites **2d** or **2f**, were dissolved in ethylenediamine:phenol 1:3 at 50° and left for 4 h. The phenol solution was then decanted directly onto a Sephadex G-25 column (1x50 cm) and eluted with water. The first UV absorbing peak to elute was collected, combined and evaporated to dryness.

c, Removal of the p-nitrophenylethyl protecting group using DBU

The p-nitrophenylethyl protecting group was always the last protecting group to be removed. The partially deprotected material was dried (Speed-Vac) and then dissolved in 1M DBU/anhydrous pyridine (0.5-1 ml) in a polypropylene test tube. The tube was sealed and then heated at 50° (3 days). The pyridine solution was applied directly onto a Sephadex G-25 column (1x50 cm) and eluted with water. The first band to elute contained the completely deprotected sequence.

Approximately 150 to 200 OD units were obtained from each tetracosanucleotide sequence after cleavage from the polymer support. After performing the ethylenediammine and DBU deprotection steps approximately 75 to 100 OD units remained.

^{31}P NMR Experiments

A Varian XL-300 NMR equipped with a 5 mm broadband probe, tuned to 121.5 MHz was used. All spectra were obtained at 20° C. The spectrometer was referenced and the field homogeneity adjusted by locking onto a CD_3CN sample containing an 85% H_3PO_4 filled glass capillary. Once referenced, spectra could be obtained from solutions of CH_3CN (nondeuterated). Chemical shifts obtained in this manner were precise to $\pm$0.05 ppm and are reported downfield from H_3PO_4.

The phosphoramidite experiments were performed under strictly anhydrous conditions, using freshly distilled CH_3CN (from CaH_2), and oven dried, argon filled NMR tubes and reaction vials. Only 0.5 ml of a reaction solution, which was 0.1M with respect to the phosphoramidite component, was required for these studies. Satisfactory ^{31}P spectra could be obtained after two minutes of acquisition, at this concentration.

The phosphoramidite reactions were monitored by first weigh-

ing the appropriate amounts of nucleoside (1 eq.), phosphoramidite (1 eq.) and tetrazole (1-3 eq.) into a vial. The absence of solvent prevented any reaction from occuring. The reaction was begun by injecting anhydrous acetonitrile into the vial, and immediately transferring an aliquot, via syringe, into an NMR tube. Reactions involving **9a** were begun by adding neat **9a** (1 eq.) into a solution of nucleoside (1 eq.) and diisopropylethylamine (3 eq.) in CH_3CN.

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REFERENCES

1, M.J. Gait, ed., "Oligonucleotide Synthesis - A Practical Approach", IRL Press, Oxford, 1984.
2, R.M. Cook, D. Hudson, E. Mayran, and J. Ott, "Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual", H.G. Gassen and A. Lang ed., pp 111-123, Verlag Chemie, Weinheim, 1982.
3, G.A. Urbina, G.M. Sathe, W.C. Liu, M.F. Gillen, P.D. Duck, R.B. Bender, and K.K. Ogilvie, Science **214**, 270 (1981).
4, M.D. Matteucci and M.H. Caruthers, J. Am. Chem. Soc. **103**, 3185 (1981).
5, F. Chow, T. Kempe, and G. Palm, Nuc. Acids Res. **9**, 2807 (1981).
6, B.D. Warner, M.E. Warner, G.A. Karns, L. Ku, S. Brown-Shimer, and M.S. Urdea, DNA **3**, 401 (1984).
7, G.C. Crockett, Aldrichimica Acta **16**, 47 (1983).
8, S.A. Narang, Tetrahedron **39**, 3 (1983).
9, K. Itakura, J.J. Rossi, and R.B. Wallace, Ann. Rev. Biochem. **53**, 323 (1984).
10, S.P. Adams, K.S. Kavka, E.J. Wykes, S.B. Holder, and G.R. Galluppi, J. Am. Chem. Soc. **105**, 661 (1983).
11, C.B. Reese and A. Ubasawa, Tetrahedron Lett. **21**, 2265 (1980).
12, B.L. Gaffney and R.A. Jones, Tetrahedron Lett. **23**, 2253 (1982).
13, H.P. Daskalov, M. Sekine, and T. Hata, Bull. Chem. Soc. Jap. **54**, 3076 (1981).
14, W.L. Sung, J. Org. Chem. **47**, 3623 (1982).
15, W.L. Sung, and S.A. Narang, Can. J. Chem. **60**, 111 (1982).
16, C.B. Reese and K.H. Richards, Tetrahedron Lett. **26**, 2245 (1985).

17, E. Ohtsuka, A. Yamane, and M. Ikehara, Nuc. Acids Res. **11**, 1325 (1983).
18, T.P. Patel, M.A. Chauncey T.A. Millican, C.C. Bose, and M.A.W. Eaton, Nuc. Acids Res. **12**. 6853 (1984).
19, C.B. Reese and P.A. Skone, J. Chem. Soc. Perkin Trans. I, 1263 (1984).
20, B.S. Schulz and W. Pfleiderer, Tetrahedron Lett. **24**, 3587 (1983).
21, S.S. Jones, C.B. Reese, S. Sibanda, and A. Ubasawa, Tetrahedron Lett. **22**, 4755 (1981).
22, C.J. Welch and J. Chattopadhyaya, Acta Chem. Scand. B **37**, 147 (1983).
23, H. Takaku, S. Ueda, and T. Ito, Tetrahedron Lett. **24**, 5363 (1983).
24, B.L. Gaffney and R.A. Jones, Tetrahedron Lett. **23**, 2257 (1982).
25, M. Sekine, J. Matsuzaki, M. Satoh, and T. Hata, J. Org. Chem. **47**, 571 (1982).
26, T. Kamimura, M. Tsuchiya, K. Koura, M. Sekine, and T. Hata, Tetrahedron Lett. **24**, 2775 (1983).
27, T. Trichtinger, R. Charubala, and W. Pfleiderer, Tetrahedron Lett. **24**, 711 (1983).
28, F. Himmelsbach, B.S. Schulz, T. Trichtinger, R. Charubala, and W. Pfleiderer, Tetrahedron **40**, 59 (1984).
29, X. Gao, B.L. Gaffney, M. Senior, R.R. Riddle, and R.A. Jones, Nuc. Acids Res. **13**, 573 (1985).
30, R.T. Pon, M.J. Damha, and K.K. Ogilvie, Tetrahedron Lett. **26**, 2525 (1985).
31, R.T. Pon and K.K. Ogilvie, Tetrahedron Lett. **25**, 713 (1984).
32, R.T. Pon and K.K. Ogilvie, Nucleosides Nucleotides **3**, 485 (1984).
33, R. Frank and H. Koester, Nuc. Acids Res. **6**, 2069 (1979).
34, L.J. McBride and M.H. Caruthers, Tetrahedron Lett **24**, 2953 (1983).
35, L.J. McBride and M.H. Caruthers, Tetrahedron Lett. **24**, 245 (1983).
36, W.J. Stec and G. Zon, Tetrahedron Lett. **25**, 5279 (1984).
37, S.L. Beaucage and M.H. Caruthers, Tetrahedron Lett. **22**, 1859 (1981).
38, N.K. Kochetkov and E.I. Budovskii, "Organic Chemistry of Nucleic Acids", Plenum Press, London (1971).
39, F. Seela and H. Driller, Nuc. Acids Res. **13**, 911 (1985).
40, J.B. Crowther, J.P. Caronia, and R.A. Hartwick, Anal. Biochem **124**, 65 (1982).