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SYNTHESIS OF OLIGODEOXYNUCLEOTIDES AND OLIGODEOXYNUCLEOTIDE ANALOGS USING PHOSPHORAMIDITE INTERMEDIATES

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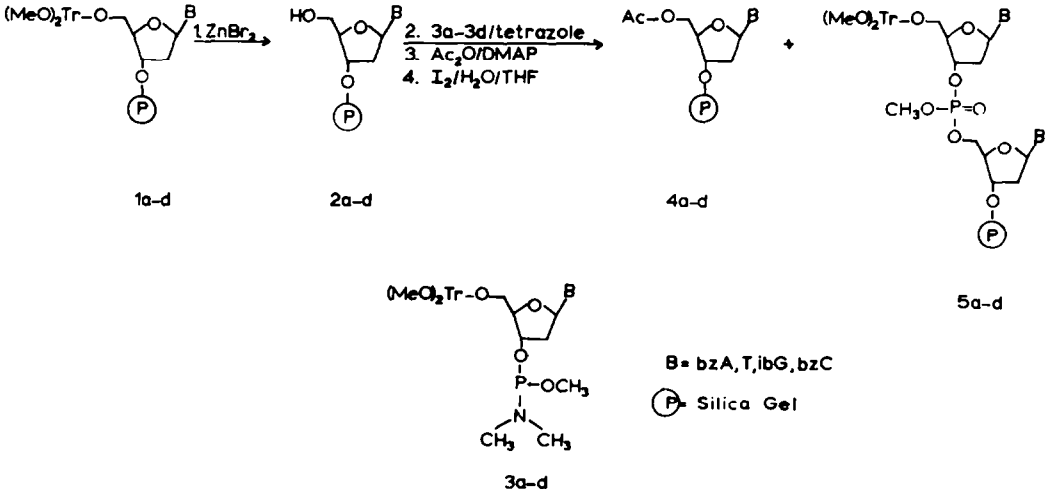
Abstract—The synthesis of two deoxyoligonucleotides, d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) and d(G–T–G–A–G–C–T–A–A–C–T–C–A–C), corresponding to the DNA binding site for cyclic AMP receptor protein is reported. These syntheses have been completed in milligram quantities using a silica gel polymer support methodology and mononucleotide phosphoramidites. Procedures are also reported for synthesizing diastereoisomers of dinucleoside methylphosphonate 3′-phosphoramidites.

A major emphasis of our research has been to develop a methodology that nonchemists can use successfully and routinely for synthesizing oligodeoxynucleotides.[1] The general synthetic strategy (Scheme 1) involves adding mononucleotides sequentially to a nucleoside covalently attached to an insoluble polymer support (**1a–d**). Reagents, starting materials and side products are then removed by filtration. The synthesis cycle begins by removing the dimethoxytrityl group, $(MeO)_2Tr$, from **1a–d** with either a protic acid or $ZnBr_2$.[2] The next step is addition of a mononucleotide to **2a–d**. We routinely use suitably protected deoxynucleotide 3′-phosphoramidites (**3a–d**) which are ideal as intermediates in deoxyoligonucleotide synthesis. These reagents as the dimethylamino, morpholino or diisopropyl derivatives are easy to prepare using standard organic chemical procedures[3,4] and can be stored indefinitely as stable white solids. When mixed with various weak acids such as tetrazole in acetonitrile, phosphoramidites are converted to the corresponding tetrazolides which react very rapidly (5 min or less) with compounds **2a–d** to yield the dinucleotide (90–100%). The final two steps involve first, acylation of unreactive deoxynucleoside or growing oligodeoxynucleotide to form **4a–d** followed by oxidation of the intermediate phosphite to the corresponding phosphate (**5a–d**) with I_2 in water, 2,6-lutidine and tetrahydrofuran. After the addition of all mononucleotides, the oligodeoxynucleotide is chemically freed of protecting groups, hydrolyzed from the support, and purified to homogeneity by polyacrylamide gel electrophoresis. So far, this chemistry has been used to synthesize approx. 2500 base pairs of DNA for various biochemical projects currently in progress.

The development of the phosphoramidite methodology for synthesizing oligodeoxynucleotides has led us to consider several challenging research areas. Currently there is an increasing need for large quantities of sequence specific oligodeoxynucleotides for various biophysical and biochemical studies.[5,6] Moreover clinical and diagnostic applications of sequence defined oligodeoxynucleotides will surely be developed very soon and will require large quantities of these compounds. Because high yields of oligodeoxynucleotides are obtained rapidly using mononucleotide phosphoramidites as starting materials, the procedures outlined in Scheme 1 would appear to



Scheme 1.

be especially attractive for synthesizing milligram quantities of these compounds. This possibility was tested by synthesizing two oligodeoxynucleotides corresponding to the cAMP receptor protein (CRP) binding site of the *lac* operon.[7] The results of these syntheses are reported in this manuscript. Additionally initial results directed toward the incorporation of methylphosphonates into synthetic oligodeoxynucleotides are reported. Methylphosphonates are taken up in intact form by mammalian cells in culture and have been shown to be resistant to nuclease hydrolysis and to exert specific inhibitory effects on cellular DNA and protein synthesis.[8] Clearly of interest would be experiments directed toward testing these effects in specific systems using sequence defined oligodeoxynucleotides containing methylphosphonates. Also of interest would be the use of methylphosphonates for studying protein–DNA recognition problems such as the interaction of *lac* repressor with *lac* operator. A possible route to the synthesis of sequence defined oligodeoxynucleotide methylphosphonates could be through the chemistry outlined in Scheme 1. Dideoxynucleoside methylphosphonates carrying a 3′-phosphoramidite would be substituted for **3a–d**. Otherwise, the chemistry would be similar. Initial experiments outlining procedures for preparing these modified synthons are also reported in this manuscript.

RESULTS AND DISCUSSION

Catabolite activation involves, as a first step, the interaction of CRP with specific DNA located within the control regions of catabolite sensitive operons of *E. coli* (the CRP site) such as *lac*, *gal* and *ara*.[9] In order to probe the CRP–CRP DNA site interaction via various biophysical and biochemical experiments, milligram quantities of the oligodeoxynucleotides (d(G–T–G–A–G–T–T–A–G–C–T–C–A–C), d(G–T–G–A–G–C–T–A–A–C–T–C–A–C)) were synthesized using the method outlined in Scheme 1. However since each condensation was completed using approximately twenty-fold more silica gel (1 g) than for a typical synthesis, the approach varied somewhat from published procedures. Each condensation was completed under N_2 in a silated, 50 ml conical test tube sealed with a serum cap. A wrist action shaker was used to agitate the suspension. After completion of the condensation step, the silica was transferred to a sintered glass funnel and washed thoroughly. The acylation, oxidation and detritylation steps were then completed while the silica gel was in the funnel. Additionally, the condensation reactions were allowed to proceed for 15 rather than the usual 5 min and $ZnBr_2$ rather than a protic acid was used to remove trityl groups. Choosing a Lewis acid for detritylation was considered essential since the DNA was to be used directly for various biochemical and biophysical experiments. We do not know how depurinated oligodeoxynucleotides (which are inevitably formed when protic acids are used in conjunction with current purine protecting groups) will alter various biophysical experiments; but by using $ZnBr_2$, the problem can be avoided.

After synthesis of the oligodeoxynucleotides, the completely deprotected products were isolated and characterized as to sequence and purity. Treatment with triethylammonium thiophenoxide and t-butylamine removed the methyl phosphate protecting group. The next step was hydrolysis of oligodeoxynucleotidic material from silica gel using first concentrated ammonium hydroxide and then 2M triethylammonium bicarbonate. Finally amino protecting groups and the 5′-dimethoxytrityl protecting group were removed by sequential treatment with concentrated ammonium hydroxide at 50° followed by 80% acetic acid. Approximately 90% of the total oligodeoxynucleotidic material was removed from silica gel using concentrated ammonium hydroxide at room temperature. This is usually sufficient for biochemical experiments where the total amount of oligodeoxynucleotide produced (even on a 25 mg scale of silica) far exceeds our requirements. However for various biochemical and biophysical experiments where large amounts of these compounds are needed, recovery of the remaining 10% is desirable. This was accomplished by treatment with 2 M triethylammonium bicarbonate. Based on the initial amount of nucleoside attached to sil ica, the isolated yields of unpurified d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) and d(G–T–G–A–G–C–T–A–A–C–T–C–A–C) were 33% (2500 A_{260} units, 82 mg) and 25% (1700 A_{260} units, 62 mg), respectively. These yields were determined on pooled, crude hydrolysates that had been passed through a G50-40 Sephadex column to remove salt and various hydrolyzed protecting groups. The analysis of this material by polyacrylamide gel electrophoresis is shown in Fig. 1. Sufficient material was loaded on the gel so that the yield could be monitored using UV absorbance. Based on this analysis, approximately 85% of the A_{260} units were assigned to each of the product oligodeoxynucleotides. Thus the yields of d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) and d(G–T–G–A–G–C–T–A–A–C–T–C–A–C) in the silica gel hydrolysates were approximately 69 and 52 mg, respectively, which corresponds to an average of about 90% yield per condensation. Each crude hydrolysate was further analyzed by 5′-end labeling with T4-kinase and [γ-^{32}P]ATP followed by gel electrophoresis. These results are shown in Fig. 2. This technique provided an estimate of the relative molar yield since each oligodeoxynucleotide, including intermediates, received one label. Clearly the desired products were the major oligodeoxynucleotides present in these crude reaction mixtures. Chromatography on Sephadex G50-40 eliminated di-, tri-, tetra-, and pentadeoxynucleotides from the hydrolysate which explains why these compounds were not observed on the gel. The loss of these compounds however does not alter the overall estimate of yields which were based on a comparison of isolated A_{260} units to the amount of nucleoside attached to silica gel.

Homogeneous oligodeoxynucleotides were obtained by gel electrophoresis followed by elution using standard techniques.[10] Further characterization using partial snake venom phosphodiesterase and two-dimension sequence analysis indicated that the oligodeoxynucleotides had been synthesized correctly (data not shown). Recent circular dichroism studies demonstrate that the DNA duplex formed from d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) and d(G–T–G–A–G–C–T–A–A–C–T–C–A–C) exists as right-handed B-form DNA[11] rather than the left

Fig. 1. Polyacrylamide Gel Electrophoresis of Synthetic Oligodeoxynucleotides. Bands one and three (rel. to top of gel) are dye markers xylene cyanol blue and bromphenol blue respectively. Oligodeoxynucleotides and dye markers were visualized by first placing a silica gel plate containing a fluorescent dye behind the gel and then shining UV light on the gel. Lanes 1 and 2 contained the reaction mixtures from the synthesis of d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) while lane 3 contained the reaction mixture from the synthesis of d(G–T–G–A–G–C–T–A–A–C–T–C–A–C). The major UV absorbing band in each lane (excluding dye markers) contained the product.

Fig. 2. Polyacrylamide Gel Electrophoresis of Synthetic Oligodeoxynucleotides After End Labeling with ^{32}P–Phosphate. Lanes 1 and 2 (read left to right) contain reaction mixture hydrolysates from the synthesis of d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) and d(G–T–G–A–G–C–T–A–A–C–T–C–A–C), respectively. The major band in each lane contained the product.

handed form which was predicted from X-ray crystallographic studies with the catabolite activator protein.[12]

These results therefore demonstrate that 50–100 mg of sequence defined oligodeoxynucleotides can be synthesized on polymer supports using mononucleotide phosphoramidite intermediates. The yields per condensation remain high (90%) even when 1 g silica is used per cycle and the molar excess of mononucleotide phosphoramidite is reduced to tenfold. However even lower excesses must be used successfully if these methods are to become feasible on a routine bases for large scale syntheses. This should be possible. Large excesses of mononucleotide phosphoramidites have been used in the past primarily to maintain high yields on small scale reactions where trace contamination with water can be a serious problem (in vessels open to the atmosphere). For large scale syntheses, however, only a very small fraction of the total mononucleotide phosphoramidite is needed to remove trace water contamination. Therefore high yields should be possible with even less excess of the mononucleotide phosphoramidites.

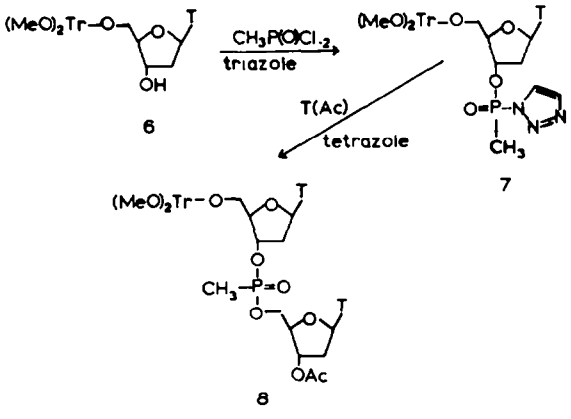
The internucleotide methylphosphonate linkage is chiral. Recent research has shown that dideoxynucleoside methylphosphonate diastereoisomers stack differently and form complexes having different stability with complementary DNA.[13,14] When methylphosphonate dimers are in the stacked conformation, the phosphonate Me group of one diastereoisomer assumes a pseudoaxial position (*S*-form) and the phosphonate Me group of the other isomer assumes a pseudoequatorial position (*R*-form). Based on various biophysical studies (UV, CD and NMR) with the methylphosphonate dimers of d(ApA), stacking for the *S*-form was slightly greater than for the *R*-form. These studies suggest that any oligodeoxynucleotide containing a methylphosphonate at a specific, defined position will form two diastereomeric DNA duplexes with a complementary, unmodified oligodeoxynucleotide.

These duplexes will presumably have different physical–chemical properties. One isomer should conformationally be quite similar to natural DNA whereas the other may have different biophysical properties. Clearly the problem becomes even more complex as additional chiral centers are introduced into an oligodeoxynucleotide. Therefore for any biochemical research involving oligodeoxynucleotides containing methylphosphonates, diastereoisomers should be separately synthesized and studied.

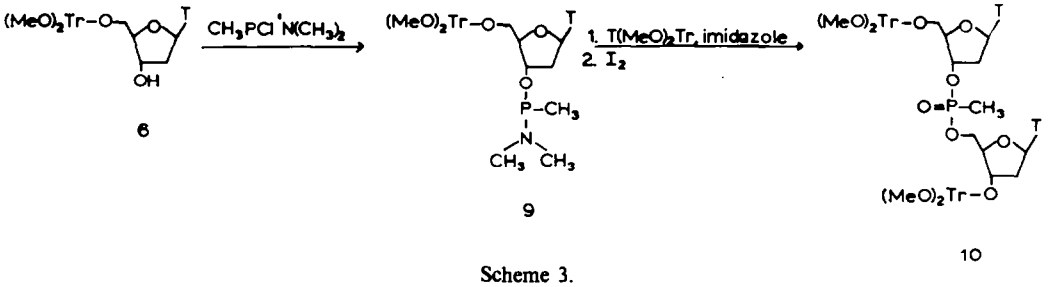
The challenge is to develop procedures for introducing one diastereoisomer of methylphosphonate containing nucleotides either at single or multiple but selected sites within oligodeoxynucleotides. No such procedure currently exists. However considerable success has been achieved in separating, via silica gel column chromatography, the diastereoisomers of dinucleoside methylphosphonates.[16] Our initial strategy therefore was to to first synthesize and separate various sequence defined dideoxynucleoside methylphosphonate diastereoisomers and then to convert these compounds into synthons that were compatible with our overall synthetic strategy as outlined in Scheme 1.

Two procedures have been examined for synthesizing dideoxynucleoside methylphosphonates. The first route (Scheme 2) was analogous with slight modifications to methods described previously.[15–17] Methylphosphonodichloridate[15] was allowed to react with triazole and collidine in pyridine to form the ditriazolide which was used directly in a reaction with **6** to form **7**. Further activation of **7** with tetrazole and condensation with 3′-O-acetylthymidine gave **8** which was a mixture of diastereoisomers. These isomers were separated by silica gel column chromatography.[16] Using the same procedure, separated isomers of $(MeO)_2$TrdbzAp(Me)T(Ac) and $(MeO)_2$TrdbzAp(Me)dbzA(Ac) were also prepared. Alternatively, methylphosphine derivatives can be used for preparing the same compounds (Scheme 3). Methylchloro-N,N-dimethyl aminophosphine, diisopropylethylamine, and 5′-O-dimethoxytrityldeoxythymidine were allowed to react in CH_2Cl_2 for 1 hr at r.t. Following an aqueous extraction procedure, **9** was isolated in approximately 85% yield. The same procedure was used for preparing analogous derivatives of $(MeO)_2$TrdbzC, $(MeO)_2$TrdbzA, and $(MeO)_2$TrdibG. Compound **10** was then prepared by condensation of **9** with 3′-O-dimethoxytritylthymidine (T(MeO_2)Tr) using imidazole in acetonitrile to catalyze the reaction (12 hr, r.t.). After oxidation to the dideoxynucleoside methylphosphonate, analysis by ^{31}P–NMR revealed two diastereoisomers as expected. Based on these NMR studies, the yield of **10** appeared to be in excess of 90%. Of further interest was the activation of **9** using imidazole in acetontrile. Mononucleotide phosphoramidites (**3a–d**) containing OMe rather than Me on phosphorus were not activated by imidazole.[18] Presumably the stronger acid (tetrazole) was required in order to protonate the less basic phosphoramidite. Additionally, activation of **9** with tetrazole was undesirable. Several uncharacterized products were observed in the ^{31}P–NMR spectrum.

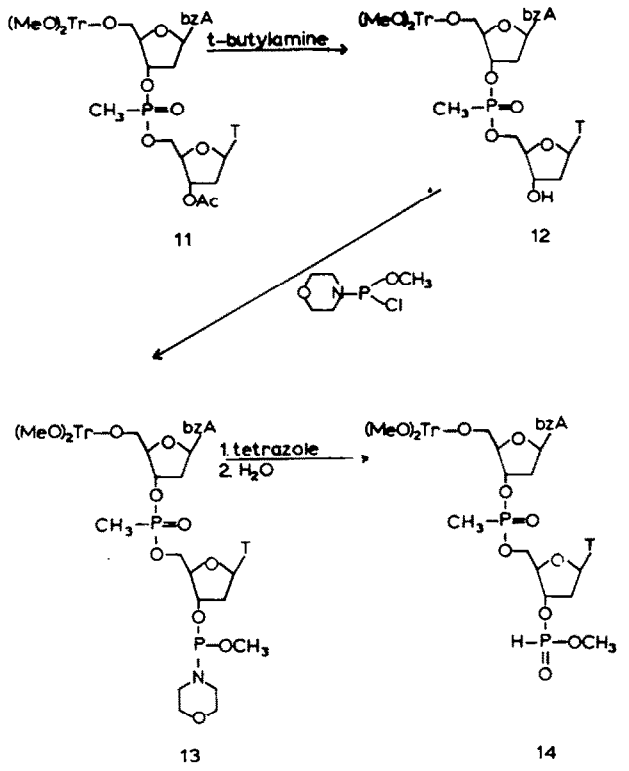
The final step in preparing dideoxynucleoside methylphosphonate synthons was the introduction of the 3′-phosphoramidite. This was accomplished using the chemical reactions outlined in Scheme 4. The first step was removal of the acyl group from the 3′-OH. With compound **8**, this can easily be accomplished by treatment with a precooled mixture (1 : 1) of 30% ammonium hydroxide and pyridine at 4° for 24 hr. No hydrolysis of the internucleotide phosphonate was detected when analyzed by TLC. However when amide protected deoxynucleosides (N-benzoyldeoxyadenosine, N-benzoyldeoxycytidine, and N-isobutyryldeoxyguanosine) were treated with this reagent, considerable loss of the N-benzoyl and N-isobutryl protecting groups was observed. For deacylation of compounds containing these protected bases, a solution of 0.15 M t-butylamine in methanol at 4° for 5 hr was used without loss of amide protecting groups. Thus **11** was converted to **12** completely using the t-butylamine hydrolysis pro-



Scheme 2.



Scheme 3.



Scheme 4.

cedure. An alternative route utilizing 3′-O-levulinyl derivatives of **11** was explored without success. The 3′-O-levulinyl group appeared especially attractive since it can be removed using nonbasic conditions.[19] Unfortunately the two diastereoisomers containing this protecting group could not be separated on silica gel and the approach was abandoned. Synthesis of **13** from **12** involved addition of N-morpholinomethoxychlorophosphine (**4**) to the dideoxynucleoside methylphosphonate in a solution of diisopropylethylamine and CH_2Cl_2. After 15 min at r.t., the reaction was quenched, purified by aqueous extraction and collected by precipitation into hexanes. A similar procedure was used for preparing each of the 3′-N-morpholinomethoxyphosphoramidites of $(MeO)_2$TrdAp(Me)T and $(MeO)_2$TrdTp(Me)T with isolated yields ranging from 68 to 76%. Further activation of these phosphoramidites was investigated by adding first tetrazole in acetonitrile and then water. As has been observed previously for the mononucleotide phosphoramidites,[3,4] **13** was rapidly converted to the phosphonate (compound **14**) suggesting that 3′-phosphoramidites of dideoxynucleoside methylphosphonates can also be used as synthons for oligodeoxynucleotide synthesis. Preliminary results using these synthons and the chemistry outlined in Scheme 1 appear quite promising. Four 21 base paired *lac* operators have been synthesized such that each operator contains a diastereoisomer of d(Tp(CH_3)T) or d(Ap(CH_3)T) at one specific site within the operator. Overall yields for each oligodeoxynucleotide (21 mononucleotides each) were analogous to those reported previously using mononucleotide phosphoramidites. These *lac* operator syntheses will be reported as part of a complete study on the biochemical reactivity of the modified operators with *lac* repressor.

EXPERIMENTAL

5′-O-Dimethoxytrityldeoxythymidine,[20] 5′-O-dimethoxytrityl-N-benzoyldeoxycytidine,[20], 5′-O-dimethoxytrityl-N-benzoyldeoxyadenosine[20] and 5′-dimethoxytrityl-N-isobutyryldeoxyguanosine[21] were synthesized according to published procedures. These compounds were converted to the 3′-N,N-dimethylaminomethoxyphosphines using published procedures.[3,4] 3′-O-Acetyldeoxythymidine,[22] 3′-O-dimethoxytritydeoxythymidine[23] and methylphosphonodichloridate[15] were prepared using published procedures.

B.ps were uncorrected. ^{1}H, ^{13}C and ^{31}P–NMR were recorded on a Bruker WM-250. All ^{31}P–NMR spectra were relative to an external standard of 85% phosphoric acid in acetonitrile. TLC was routinely carried out on Merck analytical silica gel plates (No. 5775) and hplc was completed on a Waters Associates apparatus equipped with a solvent programmer and a Waters Associates C_{18}, reverse-phase, μ-Bondapak analytical column. The organic phase was acetonitrile and the aqueous phase was 0.1 M triethylammonium acetate (pH 7). Silica gel for oligodeoxynucleotide synthesis (Vydac 101TP, 20 μ pore size) was purchased from Separation Group. Acrylamide (Gold Label) was purchased from Bio–Rad. Thiophenol, 4-N,N-dimethylaminopyridine, anhyd. $ZnBr_2$, Ac_2O, Et_3N, t-$BuNH_2$, dicyclohexylcarbodiimide, *p*-nitrophenol, triethoxy-N-propylaminosilane, dimethylformamide, and toluene sulfonic acid were purchased from Aldrich Chemical Co. and used without further purification.

Common solvents such as THF, dioxane, MeCN, nitromethane and MeOH were stored over activated (overnight at 160° in a well ventilated oven) 4 Å molecular sieves and

used without further purification. Dry acetonitrile was obtained by refluxing reagent grade solvent over P_2O_5 for 6 to 8 hr and distilling the constant boiling fraction. This fraction was then refluxed over CaH_2 for 6–8 hr and distilled. The constant boiling fraction was stored over activated molecular sieves. Anhyd. pyridine was obtained by refluxing reagent grade solvent over toluene-*p*-sulfonyl chloride for 6–8 hr and distilling the constant boiling fraction. This fraction was then refluxed over CaH_2 for 6–8 hr and distilled. The constant boiling fraction was stored over activated molecular sieves. Reagent grade toluene was dried by distillation from CaH_2. CH_2Cl_2 was distilled from P_2O_5. 1-H-Tetrazole (Aldrich) was sublimed at 110–115° at 0.05 mm Hg prior to use.

Preparation of 5′ - O - *dimethoxytrityl* - N - *benzoyldeoxycytidine covalently joined to silica gel* (**1**). 5′ - O - Dimethoxytrityl - N - benzoyldeoxycytidine 3′ - *p* - nitrophenylsuccinate was prepared using a procedure modified from published results.[24–26] 5′ - O - Dimethoxytrityl - N - benzoyldeoxycytidine (3.17 g, 5.0 mmol), N,N-dimethylaminopyridine (0.60 g, 5.0 mmol) and succinic anhydride (0.40 g, 4.0 mmol) were dissolved in anhyd. pyridine (10 ml). The reaction was monitored by TLC (Me_3CN/H_2O, 9 : 1 v/v) and terminated after 12 hr at 20°. The products were first concentrated to a viscous oil under reduced pressure, reconcentrated twice (5 ml each) with toluene, dissolved in CH_2Cl_2 (60 ml) and fractionated by aqueous extraction at 0° with 10% citric acid (w/v, 30 ml). The organic phase was first extracted twice with water (30 ml each) and then pyridine (0.6 ml) was added. After drying over Na_2SO_4 and concentrating under reduced pressure to 20 ml, the organic phase was added dropwise to a stirred soln of hexane: ether (1 : 1, 1 l.). The white ppt was collected by centrifugation. Analysis by TLC indicated succinylation was approx. 80%. The 5′ - O - dimethoxytrityl - N - benzoyldeoxycytidine as a crude product (0.73 g, approx. 1 mmol), *p*-nitrophenol (0.14 g, 1 mmol), and dicyclohexylcarbodiimide (0.22 g, 1.1 mmol) were dissolved in dioxane (5 ml) and pyridine (0.2 ml). After 2 hr at 20°, conversion of the nucleoside 3′-succinate to the *p*-nitrophenyl ester was complete when analyzed by TLC (benzene–dioxane, 3 : 1 v/v). After centrifugation to remove dicyclohexylurea, the supernatant liquid containing the crude product was used directly.

Functionalization of silica gel was completed using modifications of a published procedure.[26] Silica gel (Vydac 101TP, 20 μ pore size, 4.0 g) was exposed to 15% relative humidity for 24 hr by placement in a closed container with a soln of sat. LiCl. The silica was then transferred to dry toluene (100 ml) containing 3-aminopropyltriethoxysilane (3.3 g, 0.3 M) and the suspension maintained at reflux for 18 hr. The derivatized silica was isolated by centrifugation, washed successively (twice each) with toluene, MeOH and MeOH–water (1 : 1 v/v). The silica was next shaken with MeOH–water (1 : v/v, 50 ml) for 12 hr washed successively (twice each) with MeOH and ether, and dried under reduced pressure. The dried silica was suspended in 50 ml anhyd. pyridine and treated with trimethyl silylchloride (2.5 g, 2.4 M) at 20° for 12 hr. After isolation by centrifugation, the silica was washed 4 times with MeOH, twice with ether and dried under reduced pressure.

Compound **1** was prepared by mixing aminopropyl derivatized silica suspended in DMF (5 ml) and Et_3N (1 ml) with a soln containing dioxane (5 ml), pyridine (0.2 ml) and 5′ -O dimethoxytrityl - N - benzoyldeoxycytidine 3′ - *p* - nitrophenylsuccinate. A bright yellow color indicating the release of *p*-nitrophenol developed immediately. After 2 hr at 20°, Ac_2O (0.7 ml) in pyridine (5 ml) was added and the mixture was shaken for 20 min at 20°. A negative ninhydrin test on an aliquot of silica indicated that all aminopropyl groups had been derivatized. The silica was isolated by centrifugation, washed successively with DMF, 95% EtOH, dioxane, and diethyl ether, and then dried under reduced pressure. Analysis for the amount of 5′ - O - dimethoxytrityl - N - benzoyldeoxycytidine attached to the silica was done spectrophotometrically. An accurately weighed sample of silica gel was treated with 5 ml of 0.1 M *p*-toluenesulfonic acid in acetonitrile and the absorbance measured at 498 nm. The extinction coefficient of an acid soln of dimethoxytritanol (70 cm^2 μ $mole^{-1}$) was used to calculate a loading of 45 μ mole nucleoside/g silica.

Preparation of d(G–T–G–A–G–T–T–A–G–C–T–C–A–C). Compound **1** (1 g, 45 μ mol) was placed on a sintered glass funnel and converted to 2 using MeOH–nitromethane (5 : 95 v/v, 30 ml) containing 0.1 M $ZnBr_2$ (5 min). This soln was removed with the aid of a water aspirator and a side-arm suction flask. After a wash with MeOH–nitromethane (5 : 95 v/v, 30 ml), the detritylation step was repeated two additional times in order to completely remove the dimethoxytrityl protecting group. The silica was then washed with MeOH (30 ml), THF (2 × 30 ml), anhyd. MeCN (2 × 10 ml) and transferred to a 50 ml silated conical centrifuge tube using anhyd. MeCN. The silica was concentrated by centrifugation, freed of MeCN by decantation, and dried *in vacuo* for 1 hr. The test tube containing silica was sealed with a rubber serum cap and purged with dry N_2. 5′ - O - Dimethoxytrityl - N - benzoyldeoxyadenosine 3′ - N,N - dimethylamino, ethoxyphosphine (0.43 g, 570 μ mol) and 1-H-tetrazole (0.14 g, 2 mmol) in MeCN (10 ml) were added to 2 and the mixture shaken at 20° for 15 min. The mixture was then transferred to a sintered glass funnel where the remaining steps of the synthesis cycle were completed by sequentially washing the silica on the funnel with various reagents. These reagents according to the order of addition were as follows: (1) wash with THF–water–lutidine (2 : 2 : 1 v/v, 2 × 30 ml); (2) oxidation with 0.2 M I_2 in THF–water–lutidine (2 : 2 : 1 v/v, 20 ml, 5 min); (3) wash with THF–water–lutidine (2 : 2 : 1 v/v, 3 × 20 ml); (4) wash with MeCN (3 × 30 ml); (5) wash with THF (30 ml); (6) acylation with Ac_2O–lutidine–THF (1 : 1 : 10 v/v, 24 ml) containing 5.4% (w/v) 4-N,N-dimethylaminopyridine (5 min); (7) wash with MeOH (2 × 3 ml); (8) wash with nitromethane (2 × 30 ml). This cycle was then repeated twelve additional times using the appropriately protected deoxynucleotide 3′-phosphoramidites in order to synthesize the tetradecadeoxynucleotide.

Completely deprotected d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) was isolated by the following procedure. Silica gel containing the completely protected tetradecadeoxynucleotide was transferred to a 50 ml conical test tube and treated with thiophenol–Et_3N–dioxane (1 : 2 : 2 v/v, 5 ml) at r.t. for 90 min. After washing with MeOH (6 × 30 ml) and diethyl ether (4 × 30 ml), the silica was treated with t-$BuNH_2$–MeOH (1 : 1, 5 ml) for 24 hr at 50°. Based on hplc analysis, no nucleotidic material was removed from the support by these procedures. Deoxyoligonucleotides were then hydrolyzed from the support using concentrated ammonium hydroxide (5 ml) at 50° for 15 hr. The liquid phase containing the product was removed by decantation. Further treatment with 2 M triethylammonium bicarbonate (5 ml) at r.t. overnight hydrolyzed the remaining product from silica gel. The assay involved treating an aliquot of washed silica with strong mineral acid. The lack of an orange color indicated that no trityl groups and, therefore, no oligodeoxynucleotidic material remained on the silica gel. The combined ammonium hydroxide and 2 M triethylammonium bicarbonate extracts were concentrated under reduced pressure to dryness, reconcentrated with EtOH–water (1 : 1, 3 × 5 ml), and treated with 80% AcOH (5 ml) at r.t. to remove the dimethoxytrityl group. After concentration *in vacuo*, the crude product was dissolved in 10 mM triethylammonium bicarbonate and freed of hydrolyzed protecting groups by chromatography on a G50-40 Sephadex column equilibrated with the same solvent. The pooled peak of oligodeoxynucleotides contained 2500 A_{260} units corresponding to 82 mg of d(G–T–G–A–G–T–T–A–G–C–T–C–A–C) using a molar

extinction coefficient of 1.5×10^5. Based on polyacrylamide gel electrophoresis of sufficient material such that purity could be estimated by UV absorbance, the sample appeared approx. 85% pure (Fig. 1). The major UV absorbing compound visualized on the gel shown in Fig. 1 was eluted from the gel and characterized by two dimension sequence analysis. The results confirmed the sequence as d(G–T–G–A–G–T–T–A–G–C–T–C–A–C).

The same procedure was used to synthesize d(G–T–G–A–G–C–T–A–A–C–T–C–A–C). A comparable yield (1700 A_{260} units, 62 mg) of the product (85% homogeneous) was obtained after hydrolysis from silica gel. Purification of an aliquot by polyacrylamide gel electrophoresis followed by two dimension sequence analysis confirmed the identity of the product.

$(MeO)_2TrdTp(Me)T(Ac)$ (**8**). Methylphosphonodichloridate (320 mg, 2.4 mmol) was added to a solution of triazole (660 mg, 9.6 mmol) and collidine (2.5 ml, 2.32 g, 19.2 mmol) in dry pyridine (5 ml) and the resulting suspension stirred at 0° for 10 min under argon. A soln of 5′ - O - dimethoxytritylthymidine (1.1 g, 2 mmol) in dry pyridine (8 ml) was added and the mixture stirred at ambient temp. for 30 min. Based on TLC analysis on silica gel using $CHCl_3$–EtOH (9 : 1), the reaction appeared complete at this time. Tetrazole (1.34 g, 19.2 mmol) and 3′-O-acetylthymidine (512 mg, 1.8 mmol) were then added and the soln stirred at ambient temp for 4 hr. The mixture was then cooled in dry ice–EtOH and quenched by addition of 1 M $NaHCO_3$ (40 ml). After addition of $CHCl_3$ (200 ml), the organic phase was separated, washed with water (2×100 ml) and 80% sat. NaCl (2×100 ml), dried over Na_2SO_4, and concentrated to a viscous oil under reduced pressure. Analysis by TLC of the products in $CHCl_3$–EtOH (9 : 1) revealed the presence of two major components running at R_f 0.56 and 0.52. Chromatography on silica gel using $CHCl_3$–EtOH (1–5%) in the presence of pyridine (1%) as the eluant partially separated the diastereoisomers. The faster and slower moving diastereoisomers on TLC were designated isomer I and isomer II, respectively. Pooled fractions were concentrated under reduced pressure, reconcentrated with toluene, and lyophilized from dioxane to yield isomer I (450 mg), isomers I + II (397 mg) and isomer II (160 mg). The overall yield was 63%. Isomer I, ^{13}C–NMR ($CDCl_3$): 170.35, 163.55, 163.47, 158.86, 150.44, 150.37, 144.03, 135.11, 135.01, 130.09, 128.17, 127.99, 127.24, 113.37, 111.60, 87.31, 85.18, 84.75, 84.64, 84.36, 82.93, 82.82, 73.87, 66.02, 65.15, 65.04, 63.19, 61.69, 55.25, 39.33, 37.18, 20.80, 12.36, 11.71, 12.88, 10.58 (P–CH_3, $J_{P\,C} = 14.49$ Hz). Isomer I, ^{31}P–NMR ($CDCl_3$): −31.663. Isomer I, R_f ($CHCl_3$–EtOH, 9 : 1) = 0.56. Isomer II, ^{31}P–NMR ($CDCl_3$): −32.22. Isomer II, R_f (chloroform–ethanol, 9 : 1) = 0.52.

$(MeO)_2TrdbzAp(Me)T(Ac)$ (**11**). Methylphosphonodichloridate (638 mg, 4.8 mmol) was added to a soln of triazole (1.32 g, 19.2 mmol) and collidine (5.1 ml, 4.65 g, 38.4 mmol) in dry pyridine (10 ml) and the resulting suspension stirred at 0° for 10 min under argon. A soln of 5′ - O - dimethoxytrityl - N - benzoyldeoxyadenosine (2.63 g, 4 mmol) in dry pyridine (14 ml) was added and the mixture stirred at ambient temp. for 30 min. Tetrazole (2.69 g, 38.2 mmol) and 3′-O-acetylthymidine (1.02 g, 3.6 mmol) were added and the soln stirred at ambient temp. for 4 hr. The mixture was then cooled in dry ice–EtOH and quenched by addition of 1 M $NaHCO_3$ (80 ml). After addition of $CHCl_3$ (200 ml), the organic phase was separated, washed with water (2×100 ml) and 80% sat. NaCl (2×100 ml), dried over Na_2SO_4, and concentrated to a viscous oil under reduced pressure. Chromatography on silica gel using $CHCl_3$–EtOH (1.5%) in the presence of pyridine (1%) as the eluant partially separated the diastereoisomers. The faster and slower moving diastereoisomers on TLC ($CHCl_3$–EtOH, 9 : 1; R_f 0.54 and 0.52) were designated isomer I and isomer II, respectively. Pooled fractions were concentrated under reduced pressure, reconcentrated with toluene and lyophilized from dioxane to yield isomer I (910 mg), isomers I + II (610 mg), and isomer II (890 mg). The overall yield was 67%. Isomer I, ^{31}P–NMR ($CDCl_3$): −31.51. Isomer II, ^{31}P–NMR ($CDCl_3$): −32.15.

$(MeO)_2TrdTp(Me)T$, *isomer I.* $(MeO)_2TrdTp(Me)T(Ac)$ (350 mg, 0.39 mmol) was dissolved in a precooled soln of aqueous 30% NH_4OH–pyridine (1 : 1, 100 ml) and stirred at 4° for 24 hr. The product was concentrated under reduced pressure, reconcentrated with toluene, lyophilized from dioxane, and isolated as a white foam (300 mg, 91%). Chromatography on TLC in $CHCl_3$–EtOH (9 : 1) gave a single compound (R_f 0.36). Isomer I, ^{31}P–NMR (CD_3CN): −31.21.

$(MeO)_2TrdTp(Me)T$, *isomer II.* $(MeO)_2TrdTp(Me)T(Ac)$ (150 mg, 0.17 mmol) was dissolved in a precooled soln of aqueous 30% NH_4OH–pyridine (1 : 1, 50 ml) and stirred at 4° for 24 hr. The product was concentrated under reduced pressure, reconcentrated with toluene, lyophilized from dioxane, and isolated as a white foam (135 mg, 93%). Chromatography on TLC in $CHCl_3$–EtOH (9 : 1) gave a single compound (R_f 0.36).

$(MeO)_2TrdbzAp(Me)T$, *isomer I* (**12**). $(MeO)_2TrdbzAp(Me)T(Ac)$ (350 mg, 0.35 mmol was dissolved in a precooled 0.15 M soln of t-$BuNH_2$ in MeOH (46 ml) and stirred at 4° for 5 hr. After concentration to a gum under reduced pressure, products were fractionated on a silica gel column using a soln of $CHCl_3$–EtOH (98 : 2) with 1% pyridine as the eluting solvent. Fractions containing the product were pooled, concentrated under reduced pressure, reconcentrated with toluene, and lyophilized from dioxane. The isolated yield was 256 mg (76%). Chromatography on TLC in $CHCl_3$–EtOH (9 : 1) gave a single compound (R_f 0.36). Isomer I, ^{31}P–NMR (CD_3CN): −30.95.

$(MeO)_2TrdbzAp(Me)T$, *isomer II* (**12**). $(MeO)_2TrdbzAp(Me)T(Ac)$ (500 mg, 0.49 mmol) was dissolved in a precooled 0.15 M soln of t-$BuNH_2$ in MeOH (66 ml) and stirred at 4° for 5 hr. After concentration to a gum under reduced pressure, products were fractionated on a silica gel column using a soln of $CHCl_3$–EtOH (98 : 2) with 1% pyridine as the eluting solvent. Fractions containing the product were pooled, concentrated under reduced pressure, reconcentrated with toluene and lyophilized from dioxane. The isolated yield as a white foam was 300 mg (63%). Isomer II, ^{31}P–NMR (CD_3CN): −31.25.

Preparation of **13**. $(MeO)_2TrdbzAp(Me)T$ (210 mg, 0.22 mmol, isomer I) was dissolved in diisopropylethylamine (0.3 ml) and CH_2Cl_2 (5 ml) and cooled to 0°. After addition of N-morpholinomethoxychlorophosphine (0.061 ml, 0.48 mmol), the soln was stirred at ambient temp. for 15 min. The mixture was quenched with a sat. $NaHCO_3$ aq (25 ml). After addition of EtOAc (25 ml), the organic phase was separated, washed with 80% sat. NaCl (5×25 ml), dried over Na_2SO_4 and concentrated to a viscous oil. After addition of CH_2Cl_2 (3 ml) to the oil, the product was precipitated into hexanes, collected by filtration, and dried under reduced pressure. The product was collected as a white amorphous powder (180 mg, 74%). $(MeO)_2TrdbzAp(Me)T$ (210 mg, 0.22 mmol, isomer II) was used to prepare **13** (Isomer II, 172 mg, 71%) by the same procedure. Analysis by TLC in $CHCl_3$–hexane–morpholine (1 : 1 : 0.1) revealed homogeneous compounds with R_f 0.4.

$(MeO)_2TrdTp(Me_3)T$ (150 mg, 0.157 mmol, isomer I) and $(MeO)_2TrdTp(Me)T$ (135 mg, 0.142 mmol, isomer II) were converted to the 3′-morpholinomethoxyphosphines using the same procedure as outlined for preparing **13**. Yields were 131 mg (76%) and 106 mg (68%), respectively. Analysis by TLC in $CHCl_3$–hexane–morpholine (1 : 1 : 0.1) revealed single compounds (R_f 0.4) were present for each isomer.

The reactivity of each isomer was tested. Compound **13** (5 mg, each isomer) was dissolved in a 0.45 M soln of tetrazole in MeCN (0.1 ml). After addition of water (25 μl), analysis by TLC indicated that the phosphoramidite had been converted to base line material.

N,N-Dimethylaminomethylchlorophosphine. N,N-Di-

methylaminotrimethylsilane (17.3 ml, 111 mmol) was added to methyldichlorophosphine (14.2 g, 121 mmol) under argon at 0° during 1 hr with stirring. The distillation under reduced pressure gave a colorless liquid (b.p. 58–60°/30 torr, 12 g, 87%). ^{31}P–NMR ($CDCl_3$): −151.

Preparation of **9**. $(MeO)_2$TrdT (0.54 g, 1 mmol) was added to a soln containing diisopropylethylamine (0.4 ml, 2 mmol) and CH_2Cl_2 (1.5 ml). N,N-Dimethylaminomethylchlorophosphine (0.13 ml, 1.1 mmol) was added to this soln and the mixture was stirred for 1 hr at ambient temp. The mixture was diluted with CH_2Cl_2 (25 ml) and extracted with sat. $NaHCO_3$ aq. The organic phase was dried over Na_2SO_4, concentrated under reduced pressure, and lyophilized from benzene (15 ml) to yield a white powder (584 mg, 95%). Based on ^{31}P–NMR, the purity of the product was at least 90%. ^{31}P–NMR ($CDCl_3$): −140.3, −139.8.

Preparation of **10**. The synthesis of **10** involved ^{31}P–NMR studies. A procedure for isolating 10 has not been completed although no complications are expected. 5′ - O - Dimethoxytrityldeoxythymidine - 3′ - N,N - dimethylaminomethylphosphine (170 mg, 0.27 mmol) was added to 0.4 M imidazole in D_3-acetonitrile (2.5 ml). 3′ - O - Dimethoxytrityldeoxythymidine (130 mg, 0.24 mmol) was added to this solution and the mixture was stirred at ambient temp. for 12 hr. The mixture was quenched and oxidized using 0.2 M I_2 in THF–H_2O–lutidine (2 : 2 : 1, 1 ml). ^{31}P–NMR (CD_3CN): −32.5, −33.0.

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