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Synthesis of the Oligodeoxyribonucleotide, d(CpTpGpGpApTpCpCpApG), and Its Substrate Activity with the Restriction Endonuclease, *Bam*HI

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The synthesis of the DNA fragment, d(CpTpGpGpApTpCpCpApG), using a long-chain alkylamine-controlled pore glass bead polymer support, crystalline (9-phenyl-9-xanthenyl) nucleosides, and the bifunctional phosphorylating reagent, bis(1-benzotriazolyl)-2-chlorophenyl phosphate is described. For high coupling yields a mixed catalyst (1-methylimidazole + diisopropylethylamine) is required. The oligodeoxyribonucleotide shows activity with the DNA restriction endonuclease, *Bam*HI. Kinetic parameters have been determined for the reaction.

INTRODUCTION

The syntheses of DNA fragments on polymer supports involving phosphite triester (*1–3*) or phosphotriester intermediates (*4–7*) are well-developed techniques. However, in both cases significant time is still required to prepare and purify the starting nucleoside-3′-phosphoamidate or nucleoside-3′-phosphodiester intermediates. Recently a report has indicated that 1-hydroxybenzotriazole (HOBT)-activated nucleotides, prepared *in situ* from the corresponding nucleoside derivatives, can be used directly to elongate oligodeoxyribonucleotides bound to a polymer support (*8, 9*). This allows the synthesis of DNA fragments essentially from nucleoside starting materials. Additionally, coupling procedures involving HOBT-activated nucleotides do not result in sulfonation of the primary hydroxy groups or modification of 2′-deoxyguanosine (*10*) or thymidine (*11*) as is common with phosphotriester procedures involving arylsulfonyl activating agents.

In this paper we wish to report that certain 5′-*O*-(9-phenyl-9-xanthenyl)nucleosides, which can generally be obtained rapidly and in high purity as crystalline materials (*12*), can be used efficiently with a HOBT-activated phosphorylating reagent to produce DNA fragments of high quality. As an example, the oligonu-

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cleotide, d(CpTpGpGpApTpCpCpApG), was prepared and examined as a substrate for the DNA restriction endonuclease, *Bam*HI.

EXPERIMENTAL PROCEDURES

Materials. Pyridine (0.01% H_2O), tetrahydrofuran (0.01% H_2O), dioxane (0.01% H_2O), and 1,2-dichloroethane were purchased from Merck (Darmstadt, FRG) and stored over molecular sieves (4 Å). 2'-Deoxynucleosides were from Biomol (Ilvesheim, FRG). Trimethylsilyl chloride and 9-chloro-9-phenylxanthene were products of Fluka AG (Buchs, Switzerland). 1-Methylimidazole and diisopropylethylamine from EGA (Steinheim, FRG) were distilled under reduced pressure and stored over molecular sieves (4 Å). 1-Hydroxybenzotriazole, also from EGA, was dried in high vacuum at 50°C for 72 h. 2-Chlorophenyl phosphorodichloridate was used as obtained from EGA. *Bam*HI was obtained from P. H. Stehelin and CIE AG (Basel, Switzerland) at a concentration of 0.84 mg/ml.

Methods. Melting points (uncorrected) were measured on a Reichert melting point apparatus. ^{1}H NMR spectra were measured at 200 MHz on a Brucker WP005SY spectrometer in the Fourier transform mode. All chemical shifts are given with respect to a tetramethylsilane standard. Preparation of the polymer supports containing the 3'-terminal nucleoside was done essentially as described elsewhere (*9*). Thermal melting of the oligonucleotide helix was obtained using a thermo programmer and spectrophotometer from Gilford Instruments.

2'-Deoxy-N^2-(phenylacetyl)guanosine. To 14.7 g (55 mmol) of 2'-deoxyguanosine (which had been made anhydrous by evaporation three times from dry pyridine) in 250 ml of a 1/1 mixture of pyridine/dichloromethane was added 33 ml (250 mmol) of trimethylsilyl chloride, and the mixture was stirred for 30 min at ambient temperature. After cooling in an ice bath, 8.7 ml (66 mmol) of phenylacetyl chloride was added dropwise and the resulting solution was stirred for 90 min at ambient temperature. The reaction was stopped by the addition of 25 ml of methanol. The solvents were removed by evaporation and the residue was taken up in 200 ml of pyridine to which 50 ml of concentrated aqueous ammonia was added. The reaction mixture was stirred 15 min at ambient temperature after which roughly half of the solvents were removed by rotary evaporation. To the remaining mixture was added 100 ml of dichloromethane. The organic reaction mixture was extracted several times with a large volume of water. The combined aqueous phases were evaporated until crystallization initiated. After cooling for about 12 h at 4°C the crystals were filtered and dried. Further evaporation of the aqueous phase produced a second crop of crystals; total yield, 12.9 g (61%); mp > 300°C. ^{1}H NMR (D_6-DMSO + D_2O): δ = 3.34 (s, HOD), 3.55 (m, 2H), 3.81 (s, 1H), 4.30 (m, 1H), 6.21 (t, 1H), 7.33 (m, 5H), 8.24 (s, 1H). *Anal.* Calcd: C, 56.09; H, 4.96; N, 18.17. Found: C, 55.91; H, 5.11; N, 17.89.

N^4-Anisoyl-2'-deoxycytidine. Prepared by a procedure analogous to that described above for the guanosine derivative but in pyridine. The product was crystallized from the aqueous phase; yield, 17.0 g (86%); mp 93–95°C. ^{1}H NMR (D_6-DMSO + D_2O): δ = 3.61 (s, HOD), 3.85 (s, 2H), 3.91 (d, 1H), 4.26 (m, 1H),

6.15 (t, 1H), 7.05 (d, 2H), 7.36 (d, 1H), 8.03 (d, 2H), 8.39 (d, 1H). The product was in all respects identical to that reported previously (*13*). *Anal.* Calcd: C, 56.50; H, 5.30; N, 11.63. Found: C, 56.47; H, 5.28; N, 11.65.

2'-Deoxy-N^2-phenylacetyl-5'-O-(9-phenyl-9-xanthenyl) guanosine. To 3.85 g (10 mmol) of 2'-deoxy-N^2-(phenylacetyl)guanosine which had been dried by evaporation from dry pyridine was added 150 ml of dry pyridine. The solution was cooled in an ice bath and 3.07 g (10.5 mmol) of 9-chloro-9-phenylxanthene was added. After 5 min at 0°C the reaction mixture was allowed to warm to ambient temperature. After 30 min, TLC analysis (CH_2Cl_2/CH_3OH, 9/1) generally indicated that the reaction was complete at which point the reaction was stopped with 30 ml of methanol for 5 min. The reaction mixture was partitioned between 150 ml of dichloromethane and 250 ml of H_2O. The organic phase was washed with water and dried ($MgSO_4$), and the solvent was removed. The residue was crystallized from dichloromethane; yield, 4.93 g (77%); mp 153–155°C. ^1H NMR (D_6-DMSO + D_2O): δ = 3.36 (s, HOD), 3.81 (s, 2H), 3.97 (m, 1H), 4.42 (m, 1H), 6.21 (t, 1H), 7.03–7.41 (m, 18H), 8.03 (s, 1H). *Anal.* Calcd: C, 69.30; H, 4.90; N, 10.90. Found: C, 69.46; H, 4.94; N, 10.90.

N^4-Anisoyl-2'-deoxy-5'-O-(9-phenyl-9-xanthenyl)cytidine. Prepared from N^4-anisoyl-2'-deoxycytidine by the procedure described above for the guanosine derivative. The product was crystallized from acetonitrile; yield, 5.30 g (68%); mp 220–222°C. ^1H NMR (D_6-DMSO + D_2O): δ = 3.71 (s, HOD), 3.85 (s, 2H), 3.89 (d, 1H), 4.30 (m, 1H), 6.12 (t, 1H), 7.04–7.45 (m, 16H), 8.01 (d, 2H), 8.22 (d, 2H). *Anal.* Calcd: C, 69.99; H, 5.06; N, 6.80. Found: C, 69.96; H, 5.05; N, 6.76.

N^6-Benzoyl-2'-deoxy-5'-O-(9-phenyl-9-xanthenyl)adenosine. Prepared from N^6-benzoyl-2'-deoxyadenosine by the procedure described above for the guanosine derivative but isolated by flash chromatography on silica gel; yield, 4.58 g (75%). ^1H NMR (D_6-DMSO + D_2O): δ = 3.34 (s, HOD), 3.61 (m, 2H), 3.91 (m, 1H) 4.56 (m, 1H), 6.48 (t, 1H), 7.05–7.62 (m, 18H), 8.03 (d, 2H), 8.68 (s, 1H), 8.75 (s, 1H). *Anal.* Calcd: C, 70.70; H, 4.80; N, 11.40. Found: C, 70.89; H, 4.92; N, 11.32.

5'-O-(9-Phenyl-9-xanthenyl)thymidine. Prepared by the procedure described elsewhere (*12*); yield, 4.28 g (86%); mp 139–141°C. ^1H NMR (D_6-DMSO + D_2O: δ = 1.48 (s, 3H), 3.38 (s, HOD), 3.89 (m, 1H), 4.32 (m, 1H), 6.23 (t, 1H), 7.08–7.49 (m, 13H), 7.58 (s, 1H). *Anal.* Calcd: C, 69.90; H, 5.30; N, 5.60. Found: C, 69.82; H, 5.28; N, 5.53.

Bis(1-benzotriazolyl)-2-chlorophenyl phosphate. To 2.87 g (21.25 mmol) of 1-hydroxybenzotriazole suspended in 44 ml of dry tetrahydrofuran or dry dioxane containing 1.8 ml of pyridine (22 mmol) was added dropwise with stirring 2.46 g (10 mmol) of 2-chlorophenyl phosphorodichloridate in 6 ml of tetrahydrofuran or dioxane. After stirring at ambient temperature for 2 h the pyridine hydrochloride was filtered. The solution contained the phosphorylating reagent at a concentration of 0.2 M.

Elongation of a polymer bound oligodeoxyribonucleotide. The following procedure is described for 50 mg of polymer support containing 29 μmol/g (1.45 μmol total) of the nucleoside. The necessary phosphorylated nucleosides are prepared by evaporating the desired quantity of nucleoside from dry pyridine three times. One equivalent of pyridine and one equivalent of phosphorylating reagent is

added. The flask is sealed with a rubber septum and stored in a desiccator. The solution can be used for coupling procedures after 15 min at ambient temperature. Any unused phosphorylated nucleoside is stored dry at −20°C. To 50 μl of mixed catalyst, 1-methylimidazole/dioxane or tetrahydrofuran/diisopropylethylamine (2/2/1, v/v), in a 1-ml vial containing a rubber septum is added, with a dry glass syringe, 150 μl of phosphorylated nucleoside. After a short mixing the solution is removed with a 2-ml gas-tight syringe fitted with a glass frit which contains the polymer support. After a 15-min incubation time the syringe is emptied and washed with dry solvent. The following sequence completes the elongation cycle: (1) 2 × Wash (dioxane or tetrahydrofuran), 2 ml; (2) capping (acetic anhydride/triethylamine/*N*-methylimidazole/dioxane or tetrahydrofuran, 4.5/4.5/1/30 (v/v), 2 ml, 4 min; (3) 3 × wash (dioxane or tetrahydrofuran), 2 ml; (4) 2 × wash (1,2-dichloroethane), 2 ml; (5) 2 × deblock (3% trichloroacetic acid/1,2-dichloroethane, w/v), 1 ml, 1 min; (5) 4 × wash (dioxane or tetrahydrofuran), 2 ml.

Enzyme kinetics. Kinetic parameters were obtained in duplicate from 0.1-ml reaction mixtures containing 20 mM Tris · HCl (pH 8.5), 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 8.4 μg/ml *Bam*HI, and from 7.1 to 106 μM helix concentration of 5′d(CpTpGpGpApTpCpCpApG) at 15 or 30°C. The reactions were initiated by the addition of enzyme and, at reaction times of 0, 5, 10, 15, and 30 min, 15-μl aliquots were removed and immediately frozen in liquid nitrogen. HPLC analysis was done on a 4.6 × 250-mm column of APS-Hypersil using a gradient from 50 mM KH_2PO_4 (pH 6.8) to 0.9 M KH_2PO_4 (pH 6.8) (10% CH_3OH) in 30 min. Alternatively, analyses could be made on an ODS-Hypersil column using 0.1 M triethylammonium acetate (pH 7.0) and a gradient from 0 to 70% acetonitrile in 30 min.

The temperature dependence of the reaction was determined under the same buffer conditions and enzyme concentrations using a helix concentration of the substrate of 17.7 μM at 15, 20, 25, and 30°C. The k_{cat} values for the different experiments were calculated from the equation:

$$\ln k_{cat} = \ln\{v_i(K_m + [S])/[E][S]\} = \Delta G/RT$$

and assuming, based on the kinetic parameters above, that the K_m remained constant at 3.3×10^{-6} M.

RESULTS

Using the bifunctional phosphorylating reagent bis(1-benzotriazolyl)-2-chlorophenyl phosphate (*14*), suitably protected nucleosides can be phosphorylated in the absence of activating agents. The phosphorylation occurs quantitatively at a nucleoside : phosphorylating reagent ratio of 1 : 1 as measured by ^{31}P NMR. Therefore, no excess phosphorylating reagent remains in solution during the coupling procedure which could result in phosphorylation of the 5′-hydroxy group of the polymer-bound oligonucleotide chain and reduce coupling yields. Additionally, no significant 3′-3′ coupling occurs which would reduce the amount of activated phosphorylated nucleoside and decrease coupling yields.

The HOBT-activated phosphorylating reagent was prepared from 2-chloro-

phenylphosphorodichloridate (*14*). It can be used to phosphorylate suitably protected 5′-*O*-[bis(4-methoxyphenyl)(phenyl)methyl]nucleosides, which can be subsequently used to elongate the polymer-bound oligonucleotide (*8, 9*). Howeve, similar 5′-*O*-(9-phenyl-9-xanthenyl)-protected nucleosides can be prepared in crystalline form (*12*), thus avoiding time-consuming purification of the protected nucleoside derivatives by chromatographic techniques. The ease of preparing such derivatives and the high purity obtained led us to examine the possibility of employing such crystalline materials for the synthesis of DNA fragments on polymer supports.

Unfortunately, using a solution of the phosphorylating reagent, bis(1-benzotriazolyl)-2-chlorophenyl phosphate in either dioxane or tetrahydrofuran, we were unable to efficiently phosphorylate either 2′-deoxy-*N*-isobutyryl-5′-*O*-(9-phenyl-9-xanthenyl)guanosine (prepared from 2′-deoxy-*N*-isobutyrylguanosine and 9-chloro-9-phenylxanthene essentially as described for similar derivatives; see Experimental Procedures) or *N*-benzoyl-2′-deoxy-5′-*O*-(9-phenyl-9-xanthenyl)cytidine (*12*) as a result of the poor solubility of these derivatives. Increased solubility of the guanosine and cytidine compounds in dioxane or tetrahydrofuran was achieved by changing the nucleobase blocking groups. The more lipophilic 2′-deoxy-*N*-phenylacetyl-5′-*O*-(9-phenyl-9-xanthenyl)guanosine and *N*-anisoyl-2′-deoxy-5′-*O*-(9-phenyl-9-xanthenyl)cytidine could be phosphorylated in high yield.

N-(4-*tert*-Butylbenzoyl)-2′-deoxy-5′-*O*-(9-phenyl-9-xanthenyl)adenosine has been obtained in crystalline form (*12*). We were, however, unable to prepare the precursor, *N*-(4-*tert*-butylbenzoyl)-2′-deoxyadenosine, in sufficient purity or yield using transient protection of the sugar hydroxy groups as trimethylsilyl ethers (*15*). Therefore we investigated the use of phthaloyl (*16*), pyrazinoyl, isonicotinoyl, isobutyryl, phenylacetyl, or benzoyl as nucleobase blocking groups for 2′-deoxyadenosine. While these could be prepared in acceptable yields we were unable to crystallize the subsequent 5′-*O*-(9-phenyl-9-xanthenyl) compounds. Owing to the high yield which could be obtained for *N*-benzoyl-2′-deoxyadenosine (*15*), we converted this compound to the 9-phenyl-9-xanthenyl derivative. After chromatography using silica gel the product was obtained in analytically pure form (see Experimental Procedures). This adenosine derivative as well as 5′-*O*-(9-phenyl-9-xanthenyl)thymidine could be phosphorylated efficiently in either dioxane or tetrahydrofuran.

Internucleotide coupling of suitable protected nucleoside derivatives occurs by dissolving the nucleoside in a measured amount of phosphorylating reagent. Phosphorylation is complete at ambient temperature within minutes. An aliquot of this solution is then mixed with the catalyst and added to the polymer support containing the oligonucleotide with 5′-hydroxy component, as illustrated in Fig. 1.

This mixed catalyst (1-methylimidazole + diisopropylethylamine) results in higher coupling yields than observed using either component alone, as exemplified with the controlled pore glass bead support (Table 1). The first nucleotide coupling to a polymer-bound nucleoside containing a 5′-hydroxy group catalyzed by 1-methylimidazole in the presence of one equivalent of pyridine from the phosphorylation step occurred after 15 min with a yield of 84%. To assay the effect of diisopropylethylamine alone as a catalyst, the nucleoside starting material was

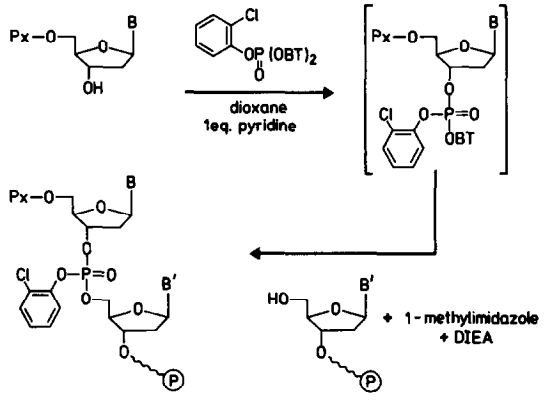

FIG. 1. Elongation of a polymer-bound oligodeoxyribonucleotide using a suitable nucleoside and bis-(1-benzotriazolyl)-2-chlorophenyl phosphate. B or B' = thymine, *N*-benzoyladenine, *N*-anisoylcytosine, or *N*-phenylacetylguanine; DIEA = diisopropylethylamine; P = polymer support; P_x = 9-phenyl-9-xanthenyl.

coevaporated twice from dry toluene after the initial coevaporation from dry pyridine. The phosphorylation took place in the presence of one equivalent of diisopropylethylamine in place of pyridine. A fourfold excess of diisopropylethylamine was added to the phosphorylated nucleoside just prior to coupling on the glass bead support. Under these conditions the yield of the first coupling after 15 min was 35%. The removal of pyridine from the coupling mixture was necessary to assay the effect of diisopropylethylamine. Coupling after 15 min with the addition of diisopropylethylamine in the presence of the one equivalent of pyridine used in the phosphorylation occurred with a 61% yield. The presence or absence of the one equivalent of pyridine resulting from the phosphorylation did not significantly effect the coupling yield using only the 1-methylimidazole catalyst. With no

TABLE 1

EFFECT OF CATALYST ON COUPLING YIELD USING LCAA-CPG POLYMER SUPPORT[a]

Coupling Conditions	Percentage yield
1-Hydroxybenzotriazole-activated nucleotide (1 eq. pyridine) 4-fold excess 1-methylimidazole 4-fold excess diisopropylethylamine	99
1-Hydroxybenzotriazole-activated nucleotide (1 eq. pyridine) 4-fold excess 1-methylimidazole	84
1-Hydroxybenzotriazole-activated nucleotide (1 eq. pyridine) 4-fold excess diisopropylethylamine	61
1-Hydroxybenzotriazole-activated nucleotide (1 eq. diisopropylethylamine) 4-fold excess diisopropylethylamine	35
1-Hydroxybenzotriazole-activated nucleotide (1 eq. pyridine)	0

[a] Coupling yields were determined after 15 min at ambient temperature and based upon the absorbance at 375 nm of the 9-phenyl-9-xanthenyl cation.

catalyst present other than one equivalent of diisopropylethylamine used for the phosphorylation, no significant coupling was observed. Using a mixture of both catalysts after phosphorylation in the presence of one equivalent of pyridine a 99% coupling yield was observed (Table 1).

Previous reports indicate that silica gel (*1, 17*), kieselgur/polyamide (*18, 19*), and controlled pore glass beads containing a long-chain alkylamine (*20, 21*) can be used successfully as supports for a DNA synthesis program. We have therefore examined these three supports for efficiency of nucleotide couplings using HOBT-activated nucleotides. We synthesized the hexamer, d(ApTpCpGpApA), on silica gel, kieselgur/polyamide, and glass bead supports. The hexamer was of interest since it contains all four of the common nucleotides and thus allowed us to confirm that all four nucleotides produce adequate coupling yields. The extent of loading of adenosine through a succinate linkage onto each of the three supports is shown in Table 2.

The coupling yields, based on the 9-phenyl-9-xanthenyl cation absorbance at 375 nm (34 A_{375}/μmol), were generally very high (Table 2). The initial coupling on the silica gel support was observed to be consistently lower than subsequent couplings. Both kieselgur/polyamide and the pore glass beads resulted in high coupling yields at all positions with all four nucleotides.

In an attempt to increase the coupling yield in the first step with the silica gel support the following two modified supports were prepared, whereby the length of the organic spacer between the surface of the support and the 3′-terminal nucleoside was increased:

$-CH_2CH_2CH_2NHCOCH_2CH_2CH_2CH_2CH_2NHCOCH_2CH_2CO-$deoxyribothymidine

$-CH_2CH_2CH_2NHCOCH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2$ $NHCOCH_2CH_2CO$-deoxyribothymidine.

TABLE 2

COUPLING YIELDS IN THE SYNTHESIS OF d(ApTpCpGpApA)[a]

Nucleoside	Silica gel	Kieselgur/ polyamide	Pore glass beads
A	97 μmol/g	81 μmol/g	32 μmol/g
A	61%	95%	99%
G	93%	98%	99%
C	95%	98%	97%
T	98%	97%	99%
A	93%	96%	98%

[a] Coupling mixtures contained a 20-fold excess of 1-hydroxybenzotriazole-activated nucleotide with respect to polymer bound nucleoside. Yields were determined after 15 min at ambient temperature as in Table 1.

Both supports were prepared by coupling the corresponding terminal *N*-(9-fluorenylmethoxycarbonyl)-substituted ω-amino acid (*22*) to 3-aminopropyl–Fractosil 200 using dicyclohexylcarbodiimide. After deprotection of the primary amine using piperidine the nucleoside was coupled to the spacer using a succinate linkage. To both of the modified supports it was possible to load 50–60 μmol nucleoside/g. However, the first coupling to the support with the shorter linker occurred with only a 20% yield. The support with the longer spacer produced an initial coupling with only a 16% yield.

Using the silica gel support, nucleotide couplings were additionally done in solvent mixtures of dioxane/acetonitrile (3/1) and dioxane/dichloromethane (3/1). Adequate coupling yields were obtained in both solvent mixtures but the yields of the first coupling remained low. The yield of the first coupling could, however, be raised to 80–90% if the incubation time was increased to 40 min.

While oligodeoxyribonucleotide synthesis proceeded with high-yield couplings on the kieselgur/polyamide support, the support tended to have unfavorable swelling characteristics, the extent of which appeared solvent dependent. With this support we have additionally used a second washing step with dry dimethylformamide after treatment with trichloroacetic acid, as has been described for polyamide resins (*23*). This is apparently necessary to ensure removal of the acid prior to the next coupling.

In our hands, glass beads containing a long alkylamine spacer (LCAA-CPG) resulted in most efficient oligodeoxyribonucleotide synthesis. While the loading of the glass beads was somewhat lower than either silica gel or kieselgur/polyamide (Table 2), it resulted in high-yield couplings. Additionally, the hydrolysis of the 9-phenylxanthenyl group proceeded to completion generally within 1 min (cytidine required 1.5 min). This allowed for a slightly faster cycle time as well as decreasing the possibility of depurination during the acid treatment.

The oligodeoxyribonucleotide, d(CpTpGpGpApTpCpCpApG), containing the recognition sequence for the DNA restriction endonuclease, *Bam*HI, was synthesized in a glass syringe (*24*) using 50 mg of LCAA-CPG containing 29 μmol/g of 2′-deoxy-*N*-phenylacetyl-5′-*O*-(9-phenyl-9-xanthenyl)guanosine bound through a succinate linkage (*9*). The synthesis sequence as described under Experimental Procedures was employed.

After assembly of the fully protected oligonucleotide, the nucleobases and phosphate groups were deblocked (*8, 9*) and the product containing a 5′-terminal 9-phenyl-9-xanthenyl group was isolated by HPLC (*25, 26*). Subsequently the 9-phenyl-9-xanthenyl group was removed in 80% aqueous acetic acid for 30 min at 0°C. From 1.45 μmol of starting nucleoside component, 30 A_{260} units (0.31 μmol) (*27*) of product was isolated. This represents a 21% overall yield of fully deblocked product with respect to the initial bound nucleoside. The decanucleotide could be completely digested with snake venom phosphodiesterase and bacterial alkaline phosphatase (*25, 26*) to give the correct ratios of 2′-deoxyribonucleosides. Wandering spot analysis confirmed the sequence of the oligonucleotide.

This self-complementary oligonucleotide contains the recognition sequence, d(GpGpApTpCpC), specific for the DNA restriction endonuclease, *Bam*HI. Examination of the thermal stability of the DNA duplex formed by

d(CpTpGpGpApTpCpCpApG) in 20 mM Tris · HCl (pH 8.5) containing 10 mM $MgCl_2$ indicated a T_m value of 45°C ± 1°C. We then monitored enzyme activity in 20 mM Tris · HCl (pH 8.5) containing 10 mM $MgCl_2$ and 1 mM 2-mercaptoethanol in the presence of the oligonucleotide by HPLC (Figs. 2a and b). The substrate molecule was completely converted into d(CpTpG) and d(pGpApTpCpCpApG) as was determined from nucleoside analysis (*25, 26*) after isolation of a small quantity of both product oligodeoxyribonucleotides. Kinetic parameters were determined for the reaction at 15 and 30°C, and the Lineweaver-Burk plots from these measurements are illustrated in Fig. 3a. The apparent K_m at both temperatures was 3.2×10^{-6} M. At 15°C the enzyme exhibited a turnover number of 1.3 min^{-1} and at 30°C 4.7 min^{-1}. The temperature dependence of the cleavage reaction was studied in the range 15 to 30°C as illustrated in Fig. 3b. At the relatively high concentration of substrate used the apparent K_m remained unchanged and the free energy for the enzyme-catalyzed phosphodiester bond hydrolysis could be calculated from the initial velocity measurements at various temperatures (see Experimental Procedures). The activation energy as calculated from this equation for the enzyme-catalyzed phosphodiester bond hydrolysis was 6.6 ± 0.4 kJ/mol.

DISCUSSION

Although the synthesis of DNA fragments by solid-phase procedures is at present a well-established technique, the use of 1-hydroxybenzotriazole-activated nucleotides prepared *in situ* has the advantage that DNA fragments can be synthesized essentially from nucleoside starting materials. That is to say, after purification of the nucleoside starting materials, synthesis procedures can be undertaken without purification of subsequent intermediates. Only after deprotection proce-

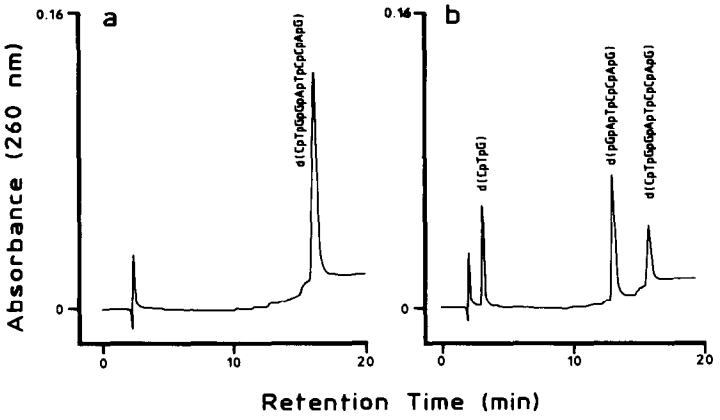

FIG. 2. HPLC analysis of the phosphodiester bond hydrolysis in d(CpTpGpGpApTpCpCpApG) as catalyzed by *Bam*HI after (a) 0 min of incubation at 30°C or (b) 30 min incubation at 30°C. Column, 4.6 × 250-mm APS-Hypersil; buffer A, 0.05 M KH_2PO_4 (pH 6.8); buffer B, 0.9 M KH_2PO_4 (pH 6.8); gradient, 0–100% buffer B in 30 min.

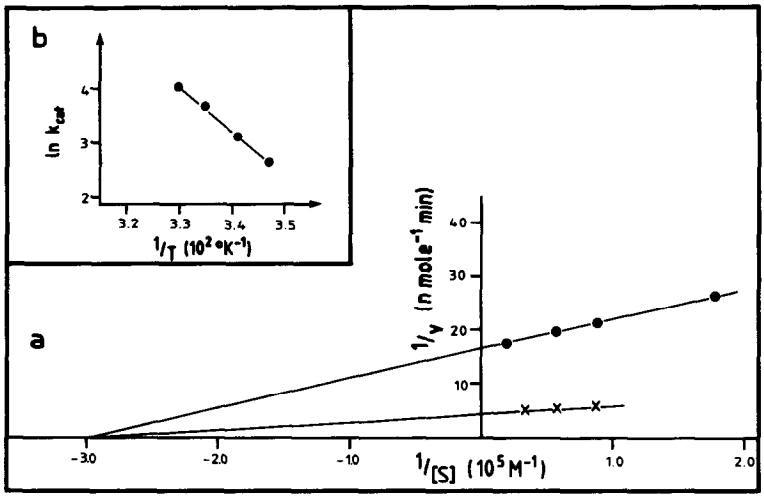

FIG. 3. (a) Lineweaver–Burk plot showing the kinetics of the phosphodiester bond hydrolysis in d(CpTpGpGpApTpCpCpApG) as catalyzed by *Bam*HI at 15°C (●) and 30°C (×). (b) Temperature dependence of enzyme activity. The logarithm of time^{-1} for which the initial velocity was measured is shown as a function of the (absolute temperature)$^{-1}$. The slope was used to determine the activation energy of the reaction.

dures is the final oligonucleotide product purified. The crystalline 5′-*O*-(9-phenyl-9-xanthenyl)nucleoside starting materials can be prepared in high yields and exceptional purity. Although the (phenylxanthenyl)nucleoside derivatives tend to have poorer solubility characteristics in dioxane or tetrahydrofuran than corresponding [bis(4-methoxyphenyl)(phenyl)methyl] derivatives, these problems can be largely overcome by selecting the appropriate nucleobase protecting groups.

High coupling yields were observed with the mixed catalyst consisting of 1-methylimidazole and diisopropylethylamine. The reasons for the success of this mixed catalyst are at present unclear. 1-Methylimidazole is generally considered to act as a nucleophilic catalyst and diisopropylethylamine may be involved in proton transfer processes.

All three polymer supports examined can be used for DNA synthesis, although yields of the first coupling step appear to be in general low when using aminopropylsilyl-modified silica gels. Increasing the distance between the surface of the silica and the first nucleoside using a longer spacer molecule did not improve coupling yields. Yields could, however, be improved to 80–90% for the first coupling by increasing the time to 40 min.

These procedures produce DNA fragments of high quality as shown for the oligonucleotide, d(CpTpGpGpApTpCpCpApG). This synthetic molecule was active with the DNA restriction endonuclease, *Bam*HI. Phosphodiester bond hydrolysis as catalyzed by the enzyme could be monitored by HPLC (Figs. 2a and b). The kinetic parameters obtained are typical for the interaction of an oligonucleotide substrate with a restriction endonuclease (*28, 29*). In comparison with the

pJC 80 DNA substrate (*30*) the K_m for the oligonucleotide is much higher. However, the turnover number of 2.2 min^{-1} in the case of the DNA is comparable with the values of 1.29 min^{-1} at 15°C and 4.70 min^{-1} at 30°C obtained with the oligonucleotide substrate. Additionally, the free energy for the enzyme-catalyzed phosphodiester bond hydrolysis of 6.6 ± 0.4 kJ/mol for the oligonucleotide at 5.6 ± 0.4 kJ/mol for the pJC 80 DNA (*31*) are similar. The kinetic data suggest that the interaction of this oligodeoxyribonucleotide with the endonuclease represents a good model system by which protein–nucleic acid interactions can be studied.

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