151. Improved Synthesis of Oligodeoxyribonucleotides by Solid-Phase Phosphotriester Method Utilizing O^6-[2-(*p*-Nitrophenyl)ethyl]-2'-deoxyguanosine Derivatives[1])

by **André Chollet***, **Edgar Ayala** and **Eric H. Kawashima**

Department of Chemistry, *Biogen S.A.*, P.O. Box, CH-1211 Geneva 4

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Summary

The synthesis of oligodeoxyribonucleotides on a cross-linked polystyrene solid support utilizing stable mono- and dinucleotide phosphotriester building blocks is presented. The use of O^6[2-(*p*-nitrophenyl)ethyl]-2'-deoxyguanosine derivatives yields cleaner DNA fragments by suppressing side reactions. Modifications improving the phosphotriester methodology are presented. The purification methods and analysis of synthetic oligodeoxyribonucleotides are described.

1. Introduction. – Recent advances in molecular biology and developments in recombinant DNA technology have created a strong need for sequence-defined oligodeoxyribonucleotides. The rapid chemical synthesis of DNA fragments is now possible using the phosphotriester [1] and phosphoramidite [2] methods. In addition, the use of solid supports has greatly simplified DNA synthesis, mainly by eliminating purification steps and reducing the synthesis time.

The phosphotriester approach has been widely recognized as a method of choice for the synthesis of well-defined DNA fragments providing molecular biologists with the tools to perform manipulations on DNA of natural sources and allowing them to detect and identify specific sequences [3]. The enzymatic assembly of short synthetic oligodeoxyribonucleotides has resulted in the total synthesis of genes coding for important proteins [4].

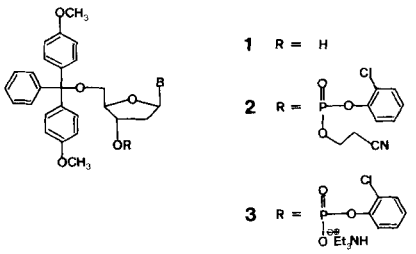
Side reactions of guanine moieties during phosphotriester oligodeoxyribonucleotides synthesis is a serious problem which leads to constant decrease in yield with increasing chain length and guanine content. It has been reported that guanine residues undergo modifications at the O^6-position during phosphorylation reactions and condensation steps [5]. It therefore becomes critical to protect this OH-group of guanine. Recently, several protective groups have been mentioned. *Reese et al.* reported the 6-*O*-(2-nitrophenyl) group [6], *Hata et al.* employed the guanine-glyoxal adduct to simultaneously protect the 6-OH and the 2-NH_2 groups of guanine [7]. Subsequently,

[1]) Part of this work has been presented by one of us *(A.C.)* at the Swiss Chemical Society meeting, October 14, 1983, Bern.

Hata et al. introduced the 6-*O*-diphenylcarbamoyl group [8]. At about the same time, *Jones et al.* [9] and *Pfleiderer et al.* [10] described the use of the 2-(*p*-nitrophenyl)ethyl (npe) group to protect the O^6 function of guanosine derivatives.

We report here the successful use of O^6[2-(*p*-nitrophenyl)ethyl]-protected guanosine derivatives in the efficient and fast solid-phase phosphotriester synthesis of oligodeoxyribonucleotides.

2. Synthesis of Mononucleotides and Dinucleotides Building Blocks. – The 5′- and base-protected nucleosides $xN_d[(MeO)_2Tr]$[2]) **1a–1d** were prepared as described earlier [11]. The *O*-6-(*p*-nitrophenyl)ethyl-guanosine derivative **1e** was prepared from **1c** using a procedure similar to that published by *Pfleiderer et al.* [12]. Acetylation of **1c** (Ac_2O/pyridine; yield 94%) followed by *Mitsunobu* reaction [13] (1.2 equiv. of Ph_3P/1.2 equiv. of diethyl azodicarboxylate/1.2 equiv. of 2-(*p*-nitrophenyl)ethanol/dioxane, 1 h at 20°C) and subsequent acetate hydrolysis (aq. NH_3 in dioxane) afforded **1e** in 77% yield. We have found that the ammonolysis of the transient 3′-acetate is very slow and not reproducible. As an alternative procedure, we have found that the 3′-acetate can be selectively reduced by $NaBH_4$ in *t*-BuOH/MeOH without affecting the N^2-amide group [14].



a B = N^6-benzoyladenine
b B = N^4-benzoylcytosine
c B = N^2-isobutyrylguanine
d B = thymine
e B = N^2-isobutyryl-O^6-[2-(4-nitrophenyl)ethyl]guanine

The npe group can be selectively removed from **1e** by *β*-elimination; using the bicyclic amidine base DBU[2]) in pyridine [10] or tetramethylguanidinium *p*-nitrobenzaldoximate in pyridine H_2O 9:1, **1e** was converted back to **1c** in nearly quantitative yield.

The 5′-protected nucleotides **2** and **3** ($xN_d[(MeO)_2Tr]p(ClPh)(CNEt)$ and $xN_d[(MeO)_2Tr]p(ClPh)(Et_3NH)$) were obtained from **1** by phosphorylation with a phosphorylating agent **4** developed by *van Boom* [15]. The two benzotriazoloxy groups of **4** are sequentially displaced first by $xN_d[(MeO)_2Tr]$ (**1**) and then either by 2-cyanoethanol in presence of *N*-methylimidazole to afford the fully protected nucleotide **2** or by H_2O

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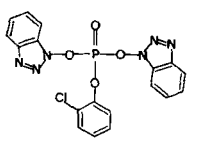
[2]) Abbreviations: N_d = 2′-deoxyribonucleoside, x = base protecting group, $(MeO)_2Tr$ = dimethoxytrityl, ClPh = 2-chlorophenyl, CNEt = 2-cyanoethyl, npe = 2-(*p*-nitrophenyl)ethyl; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, MSNT = 1-(mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole. $(Me_2N)Py$ = 4-(*N*,*N*-dimethylamino)pyridine.

(hydrolytic workup in the presence of $Et_3NH^+HCO_3^-$) leading to the formation of the triethylammonium phosphate **3**. These phosphorylation reactions are easily performed on a 10-mmol scale with yields of 80% after purification by flash chromatography [16] followed by precipitation in petroleum ether. The CNEt group of **2** can be selectively and quantitatively cleaved by Et_3N (β-elimination) to give **3** without deprotection of the O^6-npe group.



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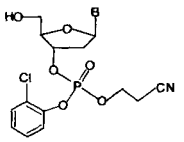

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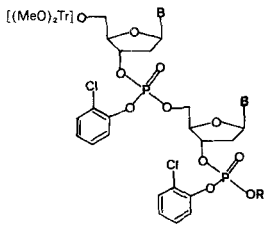

5 R = CH_2CH_2CN

7 R = Et_3NH

The dinucleotides **5** ($xN_d[(MeO)_2Tr]p(ClPh)xN_dp(ClPh)(CNEt)$) have been synthesized by two different methods: a) The first approach, based on *van Boom's* work [17], involves phosphorylation of the nucleoside **1** by **4** and subsequent reaction with the 5′-*O*-unprotected nucleotide **6** (obtained by acidic removal of the 5′-*O*-dimethoxytrityl group from **2**) to give **5**. The O^6-npe protective group is inert under the reaction conditions. b) In the second approach, **5** has been prepared in very good yields by condensation of the charged nucleotide **3** with the 5′-*O*-unprotected nucleotide **6** using a coupling reagent MSNT[2]) in anhydrous pyridine.

The dinucleotides have been purified on short silica gel columns with CH_2Cl_2/MeOH 20:1. HPLC analysis on reverse-phase *C-18* column showed less than 1% of by product. The charged dinucleotides **7** are obtained in quantitative yield from **5** by Et_3N-induced elimination of the CNEt group (followed by column chromatography on silica gel and precipitation in petroleum ether/Et_2O) as a white powder ready to be used in chain elongation reactions.

3. Assembly of Oligodeoxyribonucleotides. – The oligonucleotides are constructed from the 3′-end towards the 5′-end. The elongation is done by successive couplings of mono- or dinucleotides blocks, **3** or **7**, respectively, to a nucleoside covalently bound by its 3′-OH group to the solid support through a succinyl bridge.

As a solid support we used polystyrene cross-linked with 1% divinylbenzene which has been amino-functionalized as described earlier [18]. The typical loading was 0.2 m equiv. of NH_2/g. The nucleoside **1** was first esterified by succinic anhydride as reported [19] and then attached to the polystyrene by condensation with dicyclohexylcarbodiimide. The average loading of the support was 80–120 μmol/g. The remaining NH_2-groups have been deactivated by extensive acetylation (Ac_2O/pyridine $(Me_2N)Py$[2]). Although several supports have been proposed for the solid-phase synthesis of oligodeoxynucleotides [20], we found the polystyrene very suitable for a stop-flow solid-phase method of synthesis.

The reaction vessel was a sintered glass filter equipped with a septum and stopcock [18]. In the typical synthesis conditions, we started with 10–20 mg of resin (1–2 μmol of nucleoside) and performed the cycle reported in *Table 1* for the additon of a mono- or

Table 1. *Standard Synthesis Cycle*[a])

Step	Conditions	Time [min]
1	5% $CHCl_2COOH$ in CH_2Cl_2	1–2
2	CH_2Cl_2 washing	0.5
3	i-PrOH washing	0.5
4	Pyridine washing	0.5
5	Vacuum drying	8
6	0.1M **3** or 7[b]), 0.5M MSNT in pyridine (150–200 µl), 20°, under Ar	20
7	Pyridine washing	0.5
8	0.5 ml of THF/pyridine/Ac_2O 7:2:1 *(v/v)* and 0.5 ml of 0.2M (Me_2N)Py in THF/pyridine 3:2 *(v/v)*	3
9	i-PrOH washing	0.5
10	CH_2Cl_2 washing	0.5

[a]) For 10–20 mg of resin loaded at 0.1 mmol/g.
[b]) 10-Fold excess over 3'-nucleoside.

dinucleotide to the growing chain. Three distinct chemical transformations are performed: 1) Removal of the 5'-*O*-[$(MeO)_2$Tr] group by an acid: we have used 0.2M $CHCl_2COOH$ in CH_2Cl_2 (*Step 1*). 2) Coupling of the incoming nucleotide (*Step 6;* this reaction has to be done under rigorous anhydrous conditions and Ar atmosphere): we have found that the optimum reaction mixture in pyridine was 0.1–0.2M in nucleotide and 0.5M in MSNT. A total volume of 150–200 µl was enough to swell and wet the 20 mg of resin, thus minimizing the amount of nucleotide used per cycle. The reaction time for this step could be reduced to 20 min at room temperature. 3) Blocking of any unreacted 5'-OH material (*Step 8*): this 'capping' step was best done by Ac_2O in THF/2,6-dimethylpyridine in presence of (Me_2N)Py. For the synthesis of short oligonucleotides (up to 20 bases), the capping step can be omitted without appreciable loss in yield or increased difficulties in the final purification.

The time required for one complete cycle was approximately 35 min, thus easily allowing one person to run simultaneously the synthesis of two different heptadecamers in one normal working day.

We have prepared several hundred oligodeoxyribonucleotides ranging form 6 to 41 bases in length (see *Table 2*). The average yield for one coupling cycle, based on spec-

Table 2. *Selected Examples of Synthesized Oligodeoxyribonucleotides*

Length	Sequence	Crude yield[a]) [%]	Isolated yield[b]) [%]
11[c])	d(C-C$\frac{G}{T}$-G-G-G-T-G-T-T-G)	57	23
13	d(G-A-G-C-T-G-G-T-G-G-A-C-G)	70	18
17	d(G-T-G-A-C-G-T-T-A-G-G-C-A-A-T-G-T)	42	9
19	d(A-C-A-G-A-C-C-A-T-G-T-T-T-G-T-G-A-A-C)	26	8
22	d(C-C-C-A-G-C-C-G-C-T-A-A-G-T-T-T-G-T-G-A-A-C)	28	9
27	d(G-T-A-A-T-T-T-C-A-G-C-C-A-T-G-G-T-T-T-G-A-T-T-G-A-T-T)	40	5
41	d(G-G-G-A-A-G-C-T-T-T-A-G-A-C-A-A-A-T-A-C-G-C-T-T-G-C-A-A-C-T-G-C-G-T-T-G-T-T-G-G-T)	10	2

[a]) Determined by measuring UV absorbance (at 260 nm) of the material obtained after NH_3 treatment.
[b]) After final purification by gel electrophoresis, determined by UV absorbance measured at 260 nm.
[c]) These oligonucleotides were successfully used by *Kreil et al.* [3].

trophotometric measurement (at 502 nm) of the $(MeO)_2Tr$ cation released was in the range of 90–99%. The overal yields of oligonucleotides on the solid support were 50–80%.

4. Isolation and Purification of Synthetic Oligodeoxyribonucleotides. – The resin bearing the oligonucleotide was first treated with tetramethylguanidinium pyridine-2-aldoximate [21] in pyridine/H_2O 9:1 at 40°C overnight. This step removes the *O*-chlorophenyl phosphate-protecting group, cleaves the oligonucleotide off the resin, and deprotects all the *O*-6 npe groups on guanosine residues. The solid support was discarded, and the crude oligonucleotide was then heated in 18–20M NH_3 at 50–60°C for at least 6 h to insure complete deprotection of all amino-protecting groups. We have found that a high NH_3 concentration is absolutely necessary in this step. The crude oligonucleotide was then desalted on a size-exclusion *G-50 Sephadex* column at pH 7.5. The final purification was done by polyacrylamide gel electrophoresis in denaturing conditions (7M urea). The overall yield of synthesis, based on UV absorbance at 260 nm, was found to be in the range 10–30%.

In addition, a pre-purification was carried out by *C-18* reverse-phase HPLC (MeCN gradient) on longer oligonucleotides still containing the 5'-*O*-[$(MeO)_2Tr$] group. With oligonucleotides longer than 20 bases, we have found an enrichment although we have not been able to separate traces of material slightly shorter than the wanted oligonucleotide.

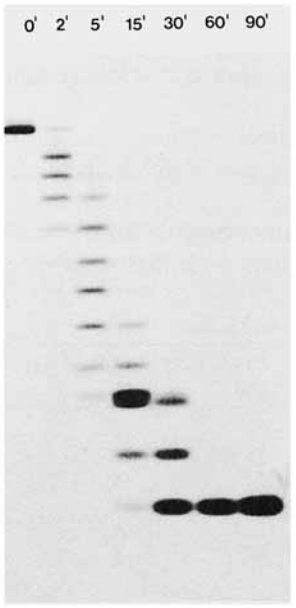

Fig. 1. *Snake venom phosphodiesterase controlled digestion of the tridecamer d(p*G-A-G-C-T-G-G-T-G-G-A-C-G) (37°C)*

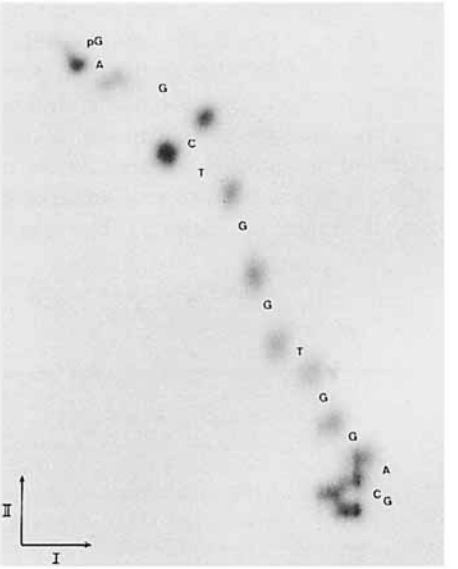

Fig. 2. *DNA sequence analysis of the tridecamer d(p*G-A-G-C-T-G-G-T-G-G-A-C-G).*
I = electrophoresis on cellulose acetate, pH 3.5;
II = homochromatography on DEAE cellulose.

5. Analysis of Synthetic Oligodeoxyribonucleotides. – The purified oligonucleotides were 5′-labeled with [γ-^{32}P]ATP and T4-polynucleotide kinase. Partial enzymatic degradation (3′ to 5′ exonuclease) of the oligonucleotides by snake venom phosphodiesterase was used to analyze the length and purity of labelled oligodeoxyribonucleotides. These digestions proceeded cleanly to completion.

The sequences have been determined either by 2D homochromatography [22] (mobility-shift analysis) or by modified *Maxam-Gilbert* sequencing [23]. The 5′-terminal nucleotide was analyzed by PEI-cellulose TLC in comparison with authentic deoxyribonucleotide standards. All the oligodeoxyribonucleotides prepared show the expected length and the correct sequence, as exemplified in *Fig. 1* and *2* with a G_d-rich fragment.

Conclusion. – The method presented here allows the rapid and efficient synthesis by the phosphotriester solid-phase technique of sequence-defined oligodeoxyribonucleotides. Our results demonstrate that the protection of the O^6- group of guanine with npe is fully compatible with the phosphotriester-synthesis methodology. The synthesis of several G_d-rich fragments has been performed with no discernable side reaction, good yield, and clean purification.

We thank *Jean-Pierre Voegeli* and *Piero Imbruglia* for skillful technical assistance, *Jean-Pierre Saulnier* for recording the NMR spectra, and Dr. *K. Rose* for performing the MS analysis.

Experimental Part

General. The solvents and reagents were purchased from *Fluka AG*, Buchs, and *Merck*, Darmstadt (*puriss.* grade). Pyridine was distilled over CaH_2. Pyridine, *N*-methylimidazole, and 2-cyanoethanol (= 3-hydroxypropiononitrile) were stored over molecular sieves (*Union Carbide*, 4 Å, 8–12 mesh). Commercial grade 1-hydroxybenzotriazole was dried for 48 h in a vacuum oven at 30° prior to use. The protected nucleosides $bzA_d[(MeO)_2Tr]$, $bzC_d[(MeO)_2Tr]$, $ibG_d[(MeO)_2Tr]$, and $T_d[(MeO)_2Tr]$ were prepared according to published procedures [24]. TLC have been performed on precoated silica gel plates (Kieselgel *60F, Merck*) with solvent systems CH_2Cl_2/MeOH 19:1 (A) and CH_2Cl_2/MeOH 9:1 (B) and on reverse-phase plates *($KC_{18}F$, Whatman Inc.)* with solvent system acetone/0.05M $Et_3NH^+HCO_3^-$ (pH 7) 3:1(C). Column chromatographies have been done on silica gel (Kieselgel *60H, Merck*). HPLC analysis have been carried out on a *Waters Assoc.* liquid chromatograph; reverse-phase C_{18} *radial pak* cartridge 100 × 8 mm; 0.05M $Et_3NH^+AcO^-$ (pH 7)/MeCN gradient; pressure 1000 psi, flow rate 2 ml/min. UV: λ_{max} (ε) in nm. 360-MHz-^{1}H-NMR and 145-MHz-^{31}P-NMR spectra have been recorded at the University of Geneva (Dr. *U. Burger*) on a *Bruker WM 360* spectrometer. Mass spectra (MS) have been recorded on a *Cratos MS 50* spectrometer at the Centre Médical Universitaire, Geneva (Dr. *R. Offord*).

5′-O-Dimethoxytrityl-N^2-isobutyryl-O^6-[2-(p-nitrophenyl)ethyl]-2′-deoxyguanosine (**1c**). The protected nucleoside **1c** ($ibG_d[(MeO)_2Tr]$) (12.8 g, 0.02 mol) was evaporated twice with anh. pyridine at 20°/1 Torr, dissolved in 40% Ac_2O in pyridine (100 ml), stirred at 20° for 1 h, and dried to give a gum. The residue was dissolved in CH_2Cl_2 (200 ml) and washed twice with 5% $NaHCO_3$ (100 ml), with H_2O (100 ml), and dried over Na_2SO_4. Evaporation of the solvent and reprecipitation in pentane gave $ib^2G_d(Ac)^{3'}[(MeO)_2Tr]^{5'}$ (12,8 g, 94%) as a white powder; R_f 0.70 (A), 0.48 (C).

To a solution of the crude $ib^2G_d(Ac)^{3'}[(MeO)_2Tr]^{5'}$ (2.8 g, 0.0188 mol) in dioxane (100 ml) was added Ph_3P (5.9 g, 0,0226 mol), 2-(*p*-nitrophenyl)ethanol (3.8 g, 0.0226 mol), and diethyl azodicarboxylate (3.9 g, 0.0226 mol). The mixture was stirred at 20° for 90 min. The solvent was evaporated and the residue dissolved in CH_2Cl_2, washed several times with H_2O and dried over Na_2SO_4. Evaporation of the solvent gave a gum. An aliquot was purified by column chromatography (silica gel), first with CCl_4/CH_2Cl_2 1:1, then with CH_2Cl_2 to elute the $ib^2npe^6G_d(Ac)^{3'}[(MeO)_2Tr]^{5'}$ as a white solid; *Rf* 0.59 (A), 0.89 (B), 0.29 (C). UV(MeOH): 270 (24000). ^{1}H-NMR (360 MHz, $CDCl_3$): 1.20 (*2d*, J = 7, 6H, $(CH_3)_2CH$); 2.12 (*s*, 3H, CH_3CO); 2.60, 2.76 (*m*, 2H,

2H–C(2')); 2.98 (*m*, 1H, $(CH_3)_2CH$); 3.32 (*t*, *J* = 7, 2H, CH_2–C(arom.)); 3.41 (*m*, 2H, 2H–C(5')); 3.77 (*s*, 6H, 2 CH_3O); 4.23 (*m*, 1H, H–C(4')); 4.82 (*t*, *J* = 7, 2H, CH_2O–C(6)); 5.52 (*m*, 1H, H–C(3')); 6.39 (*m*, 1H, H–C(1')); 6.80–8.17 (*m*, 17 H–C(arom.)); 7.99 (*s*, 1H, H–C(8)).

The gum was dissolved in dioxane (200 ml). A 33% NH_3 solution was added until the mixture became cloudy. The mixture was shaken at 20° for 3 days. The solvent was evaporated and the residue dissolved in CH_2Cl_2, washed twice with H_2O, dried over Na_2SO_4, and evaporated. Column flash chromatography on silica gel (130 g, elution with 800 ml of CCl_4/CH_2Cl_2 1:1, 400 ml of CH_2Cl_2, then 1% MeOH in CH_2Cl_2) gave 12.1 g (77%) of **1e** as white solid; R_f 0.29 (A), 0.43 (C). UV (MeOH): 270 (22000). ^{1}H-NMR (360 MHz, $CDCl_3$): 1.20 (*t*, *J* = 7, 6H, $(CH_3)_2CH$); 2.56 (*m*, 1H, H–C(2')); 2.8 (*m*, 2H, H–C(2'), $(CH_3)_2CH$); 3.32 (*t*, *J* = 7, 2H, CH_2–C(arom.)); 3.4 (*m*, 2H, 2H–C(5')); 3.78 (*s*, 6H, $2CH_3O$); 4.15 (*m*, 1H, H–C(4')); 4.73 (*m*, 1H, H–C(3')); 4.82 (*t*, *J* = 7, 2H, CH_2O–C(6)); 6.47 (*m*, 1H, H–C(1')); 6.8–8.2 (*m*, 17 H–C (arom.)); 7.98 (*s*, 1H, H–C(8)). MS (43 eV): 788 (M^+).

Alternatively, the intermediate ib^{2}npe^{6}G$_d$(Ac)$^{3'}$[(MeO)$_2$Tr]$^{5'}$ can be reduced to **1e** as follows: to a solution of ib^{2}npe^{6}G$_d$(Ac)$^{3'}$[(MeO)$_2$Tr]$^{5'}$ (415 mg, 0.5 mmol) in *t*-BuOH (10 ml) was added $NaBH_4$ (190 mg, 5 mmol). The mixture was heated under reflux. MeOH was added after 15 min (0.2 ml) and again after 30 min (0.2 ml). The mixture was further heated under reflux for 15 min. H_2O (50 ml) was added. The mixture was extracted with an equal volume of CH_2Cl_2, dried over Na_2SO_4, and evaporated to leave a solid which was flash-chromatographed as above to yield **1e** (350 mg, 89%), identical in all respects to the material obtained above by ammonolysis.

*5'-O-Dimethoxytrityl-N^2-isobutyryl-O^6-[2-(p-nitrophenyl)ethyl]-2'-deoxyguanosine 3'-(2-Chlorophenyl,2-cyanoethyl)phosphate (***2e***) and 5'-O-Dimethoxytrityl-N^2-isobutyryl-O^6-[2-(p-nitrophenyl)ethyl]-2'-deoxyguanosine 3'-(2-Chlorophenyl, triethylammonium)phosphate* (**3e**). Twice, 1-hydroxybenzotriazole (3.15 g, 0.023 mol) was evaporated with anh. pyridine (5–10 ml). Then, it was dissolved in dry THF (22 ml) and dry pyridine (1.8 ml). To this solution was added 2-chlorophenyl phosphorodichloridate (2.7 g, 0.011 mol) [15]. The mixture was shaken for 90 min. A precipitate formed as reaction proceeded (presumably pyridinium chloride). The solution was filtered under Ar, and the filtrate was added to a flask containing **1e** (7.87 g, 0.010 mol). The mixture was shaken at 20° for 15 min. At that stage, two different workup procedures led to the formation of either **2e** (workup(a)) or **3e** (workup(b)). Workup(a): *N*-Methylimidazole (1.6 ml, 0.02 mol) and 2-cyanoethanol (1.02 ml, 0.015 mol) were added to the solution, and the mixture was further stirred at 20° for 2 h. The solvents were evaporated to yield a gum. A 5% $NaHCO_3$ solution (30 ml) was added. The mixture was extracted with CH_2Cl_2 (50 ml), washed twice with H_2O, dried over Na_2SO_4, and evaporated. Toluene (20 ml) was added, and the mixture was further evaporated. The crude product was flash-chromatographed on silica gel (CCl_4/CH_2Cl_2 1:1, then CH_2Cl_2, then 1.5% MeOH in CH_2Cl_2) to give **2e** as a white solid. The material was dissolved in a minimum volume of pyridine and precipitated into pentane/Et_2O 1:1. Filtration afforded **2e** (8.07 g, 78%) as a white solid; R_f 0.46 (A), 0.30 (C). UV (MeOH): 271 (33400). ^{1}H-NMR (360 MHz, $CDCl_3$): 1.18 (*m*, 6H, $(CH_3)_2CH$); 2.7 (*m*, 4H, CH_2CN, 2 H–C(2')); 3.1 (*m*, 1H, $(CH_3)_2CH$); 3.32 (*t*, *J* = 7, 2H, CH_2-C(arom.)); 3.4 (*m*, 2H, 2H–C(5')); 3.78 (*s*, 6H, $2CH_3O$); 4.38 (*m*, 3H, $P(O)OCH_2$, H–C(4')); 4.83 (*t*, *J* = 7, 2H, CH_2O–C(6)); 5.55 (*m*, 1H; H–C(3')); 6.36 (*m*, 1H, H–C(1')), 6.7–8.2 (*m*, 21 H–C(arom.), H–C(8)). ^{31}P-NMR (145 MHz; $CDCl_3$; ext. ref. H_3PO_4): –7.95.

Workup(b): Pyridine/1M $Et_3NH^+HCO_3^-$ (150 ml) 1:1 was added to the solution. The mixture was stirred at 20° for 10 min, evaporated to a gum, and extracted as in workup(a). The crude material, solubilized in a minimum volume of pyridine, was precipitated by dropwise addition into pentane/Et_2O 1:1 to yield **3e** (9.7 g, 90%) as a white solid; R_f 0.35 (B), 0.70 (C).

The nucleotide salt **3e** has also been obtained from **2e** by selective CNEt group removal: A solution of **2e** (2.07 g, 0.002 mol) in pyridine/Et_3N/H_2O 3:1:1 *(v/v)* was stirred at 20° for 15 min. The solvent was evaporated, H_2O (100 ml) was added, and the mixture was extracted wit CH_2Cl_2 (3 × 100 ml), washed with H_2O (100 ml), dried over Na_2SO_4, and evaporated. The residue was dissolved in a minimum volume of CH_2Cl_2 and precipitated by dropwise addition into pentane (300 ml) to yield, after filtration and drying, **3e** (2.17 g, 100%) as a white solid, identical in all respects to the material obtained above.

N^2-Isobutyryl-O^6-[2-(p-nitrophenyl)ethyl]-2'-deoxyguanosine 3'-(2-Chlorophenyl,2-cyanoethyl)phosphate (**6e**). A solution of **2e** (3.75 g, 0.0036 mol) in 5% $CHCl_2COOH$ in CH_2Cl_2 (60 ml) was stirred at 20° for 15 min. A 5% $NaHCO_3$ solution was added (120 ml), and the mixture was extracted twice with CH_2Cl_2 (100 ml), washed with H_2O (100 ml), and dried over Na_2SO_4. The solution was concentrated to a few ml and added dropwise into pentane to precipitate **6e** (2.42 g, 92%) as a white powder; R_f 0.23 (A), 0.61 (C). UV (MeOH): 270 (26800). ^{1}H-NMR (360 MHz, $CDCl_3$): 1.28 (*d*, *J* = 7, 6H, $(CH_3)_2CH$); 2.6–2.8 (*m*, 2H, $(CH_3)_2CH$, H–C(2')); 2.83 (*t*, *J* = 7, 2H, CH_2CN); 3.21 (*m*, 1H, H–C(2')); 3.32 (*t*, *J* = 7, 2H, CH_2–C(arom.)); 3.8–4.0 (*m*,

2H, 2H–C(5')); 3.35–3.5 (*m*, 3H, H–C(4'), P(O)OCH_2); 4.85 (*t*, *J* = 7, 2H, CH_2O–C(6)); 5.59 (*m*, 1H, H–C(3')); 6.29 (*m*, 1H, H–C(1')); 7.16–8.18 (*m*, 8 H–C(arom.), H–C(8)). ^{31}P-NMR (145 MHz, $CDCl_3$; ext. ref. H_3PO_4): –7.92, –7.95. MS (43 eV): 729 (M^+).

$xN_d[(MeO)_2Tr]p(ClPh)xN_dp(ClPh)(CNEt)$ (**5**) *and $xN_d[(MeO)_2Tr]p(ClPh)xN_dp(ClNEt)(Et_3NH)$* (**7**). *General Procedure.* Method I. Twice, 1-hydroxybenzotriazole (3.15 g, 0.023 mol) was evaporated with dry pyridine. Then, it was solubilized in dry THF (22 ml). Pyridine (1.8 ml, 0.022 mol) and 2-chlorophenyl phosphorodichloridate [24] (2.7 g, 0.011 mol) were added. The mixture was shaken for 90 min at 20°, filtrated into a flask containing $xN_d[(MeO)_2Tr]$ (**1**). The solution was shaken at 20° for 20 min, *N*-methylimidazole (1.6 ml, 0.02 mol) was added followed by a slurry of $xN_dp(ClPh)(CNEt)$ (**6**; 0.01 mol) in dry THF (10 ml). The mixture was stirred at 20° for 2 h, evaporated to a gum, diluted with CH_2Cl_2, washed successively with 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4, and evaporated. The crude material was flash-chromatographed on a silica gel column (CH_2Cl_2, then 1–3% MeOH in CH_2Cl_2) to give 70–80% of **5** as a white foam.

Method II. A mixture of $xN_d[(MeO)_2Tr]p(ClPh)(Et_3NH)$ (**3**; 0.01 mol) and $xN_dp(ClPh)(CNEt)$ (**6**; 0.01 mol) was evaporated twice with dry pyridine. A solution of MSNT[2]) [25] (0.03 mol) in dry pyridine (80 ml) was added. The mixture was stirred at 20° for 30 min. The solution was concentrated to 20 ml, H_2O (200 ml) was added, and the mixture was extracted and evaporated. The residue was flash-chromatographed as in method I yielding 70–90% of **5.**

The triethylammonium phosphates **7** were obtained using the procedure described above for the conversion of **2e** into **3e.**

Isolation and Purification of Oligodeoxyribonucleotides. At the end of the cycle (see *Table 1*) for the addition of the last mono- or dinucleotide, the 5'-$[(MeO)_2Tr]$ group was cleaved off with 5% $CHCl_2COOH$ in CH_2Cl_2 (in some cases, when reverse-phase HPLC analysis was carried out, $[(MeO)_2Tr]$ was not removed). The resin was washed successively with CH_2Cl_2 and i-PrOH, swollen in pyridine, suspended in 0.5M tetramethylguanidinium pyridine-2-aldoximate [21] in pyridine/H_2O 9:1 (2 ml), and heated at 40° for 15 h. The supernatant was removed and the resin discarded after washing with pyridine. The combined solutions were dried, taken up in 0.01M $Et_3NH^+HCO_3^-$ (pH 7.5; 21 ml), extracted 3–4 times with Et_2O (2–3 ml), and dried. Conc. NH_3 (min. 18M; 3 ml) was added, and the mixture was heated at 50–60° for 6–8 h, cooled to r. t., and dried. The residue was dissolved in 0.01M $Et_3NH^+HCO_3^-$ (pH 7.5) and purified by *Sephadex G-50* gel filtration chromatography (column 80 cm × 5 cm^2), eluting with 0.01M $Et_3NH^+HCO_3^-$ at a rate of 2 ml/min. Fractions (5 ml) were monitored for UV absorbance at 254 nm and collected. The oligonucleotide was contained in the excluded volume. Fractions of interest were pooled and lyophilized. The final purification was performed by electrophoresis (600 V, 15 h) on a 40-cm-long 15–20% polyacrylamide slab gel in denaturing conditions (7M urea). See [11] for details. A 90mM *Tris*/90 mM boric acid (pH 8.3)/4mM EDTA buffer (T.B.E.) was used. DNA fragments were visualized by UV shadowing (254 nm) and isolated from the gel by cutting out the gel slice of interest and electroeluting in a dialysis bag in 5 times diluted T.B.E. buffer. Typically, a loading of 10 A_{260} units of crude oligonucleotide in a 20 × 1 mm slot afforded 1.5–3 A_{260} units of pure material.

Analysis of Synthetic Oligodeoxyribonucleotides. The oligonucleotides were labeled at the 5'-OH terminus with T4 polynucleotide kinase *(Boehringer Mannheim GmbH)* and [γ-^{32}P]ATP as described by *Boel et al.* [26]. Partial exonuclease digestion was performed with snake venom phosphodiesterase *(Boehringer Mannheim GmbH)* as described earlier [22]. Sequence analysis was performed either by 2D homochromatography [22] or by a modified *Maxam-Gilbert* method [23]. The 5'-end nucleotide analysis was done by complete snake venom phosphodiesterase digestion of a 5'-labeled aliquot, followed by TLC on polyethyleneimine-coated plates (*Merck*, Darmstadt) (elution with 0.1M Na-phosphate (pH 6.8)/$(NH_4)_2SO_4$/EtOH 100:60:2) and comparison with authentic 5'-deoxyribonucleotides.

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