Tetrahedron Vol. 48, No. 20, pp. 4171-4182, 1992
Printed in Great Britain

0040-4020/92 $5.00+.00
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A SYNTHETIC PROCEDURE FOR THE PREPARATION OF OLIGONUCLEOTIDES WITHOUT USING AMMONIA AND ITS APPLICATION FOR THE SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING O-4-ALKYL THYMIDINES.

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(*Received in UK* 20 *March* 1992)

Abstract : The preparation of 5'-O-dimethoxytrityl (DMT) and *p*-nitrophenylethyl (NPEOC, NPE) protected nucleosides linked to 4-(2-hydroxyethyl)-3-nitrobenzoic acid derivatives is described. These products attached to controlled-pore glass supports and together with DMT and NPE-protected nucleoside cyanoethyl phosphoramidites allows for the first time the preparation of short oligonucleotides containing the ammonia sensitive mutagenic bases O-4-propyl and O-4-butyl thymidines.

Recent advances in DNA chemistry are making possible to prepare a large variety of chemically modified oligonucleotides with important biological properties. For instance, phosphate-modified oligonucleotides are being studied as antisense inhibitors of gene expression as well as oligonucleotides covalently linked to intercalating and reactive substances[1]. Oligonucleotides containing non- radioactive reporter groups are being used for the detection of bacterial or viral sequences and in DNA sequencing[2]. Finally oligonucleotides containing modified bases are important tools for studying the molecular bases of biochemical process such as carcinogenesis, DNA repair, and protein-DNA interactions[3].

Some of these modified-oligonucleotides are not stable to the standard deprotection protocols that requires a long aqueous ammonia treatment at 55ºC. Two different approaches are being used to circumvent this problem. In a first approach, an aliphatic amino or sulphydryl group is added to the oligonucleotides in a specific site and the ammonia-labile molecule is attached to the oligonucleotide after deprotection using the special reactivity of the amino or sulphydryl group. An example of this approach is the preparation of fluorescently-labelled oligonucleotides[4,5]. A second approach involves the use of base protecting groups different from the standard benzoyl and isobutyryl groups and consequently the use of a different protocol for the deprotection of bases and phosphate groups. In that sense, the more labile amide-type phenoxyacetyl group (Pac)[6,7] and the β-elimination-type groups 9-fluorenyl-methoxycarbonyl (Fmoc)[8,9] and *p*-nitrophenylethyl (NPEOC, NPE)[10] groups have been used for the

preparation of modified oligonucleotides containing ammonia-sensitive functionalities.

When solid-phase methodology is used in this second approach, some nucleophile is still needed to break the standard succinyl linkage between oligonucleotide and the solid support[11]. To resolve that problem we have described in a preliminary communication[12] a new type of linkage between the oligonucleotide and solid support containing a 2-(2-nitrophenyl)- ethoxycarbonyl (NPE-support) group that is labile to both nucleophilic and non-nucleophilic bases. So, in principle, it can be used in conjunction with standard amide-type protecting groups and with β-elimination-type protecting groups. In this communication we would like to describe in detail the preparation of the solid supports and the use of these supports together with NPE-protected cyanoethyl phosphoramidites[13,14] to prepare oligonucleotides using a deprotection protocol that avoids the use of any nucleophile. The methodology described here is extended to the preparation of modified oligonucleotides containing the ammonia-sensitive base analogues O-4-propylthymidine and O-4-butylthymidine.

RESULTS AND DISCUSSION

Preparation of 4-(2-hydroxyethyl)-3-nitrobenzoic acid derivatives.

4-(2-hydroxyethyl)-3-nitrobenzonitrile **2** was prepared as described by Uhlman and Pfleiderer[15] starting from commercially available 2-(4-aminophenyl)ethanol **1**. Acetylation and subsequent nitration of compound **2** gave 4-(2-acetyloxyethyl)-3- nitrobenzonitrile **3** that could be hydrolysed with aqueous HCl or NaOH to give 4-(2-hydroxyethyl)-3-nitrobenzoic acid **4** (see fig. 1). If the removal of the acetyl group of compound **3** is done by transesterification with methanol/HCl (g) , the methyl ester **6** is obtained.

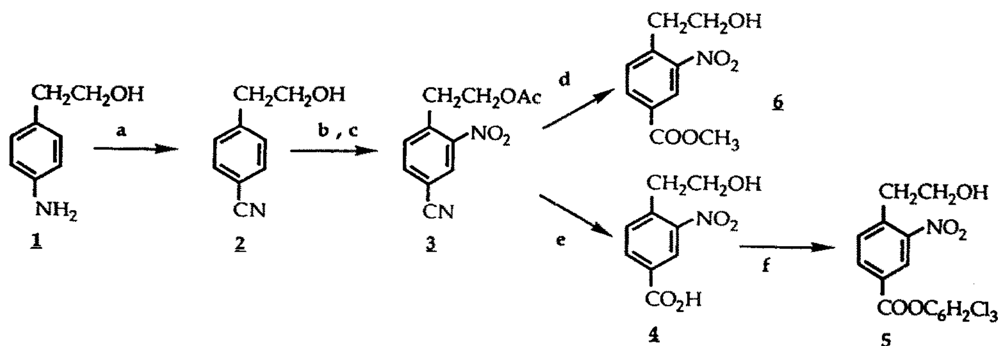


Figure 1 : Preparation of 4-(2-hydroxyethyl)-3-nitrobenzoic acid and methyl and trichlorophenyl esters. a) $NaNO_2$, HCl, CuCN. b) acetyl chloride. c) nitric and sulfuric acids. d) MeOH/ HCl (g). e) EtOH/ HCl (aq) or EtOH/ NaOH aq. f) DCC and 2,4,5-trichlorophenol.

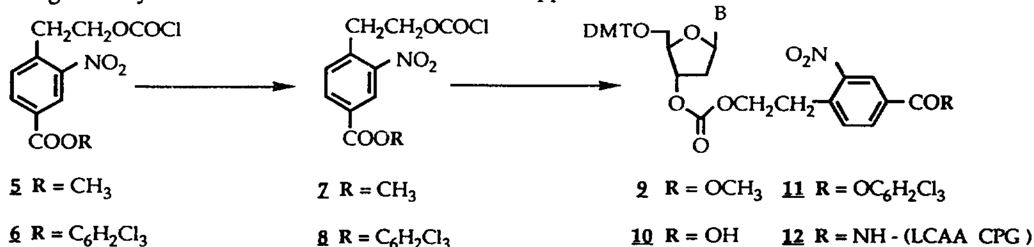
The active ester **5** was prepared by dicyclohexylcarbodiimide-mediated coupling of the carboxylic acid **4** with 2,4,5-trichlorophenol. Reaction of methyl and trichlorophenyl esters **5** and **6** with phosgene affords in quantitative yields the corresponding chloroformates **7** and **8** (see figure 2).

The reaction of the chloroformates **7** and **8** with 5'-O- dimethoxytrityl-thymidine (DMT-T) in pyridine gave the carbonates **9** and **11** in moderate yields (60% **9**, 40% **11**). The carbonate **11** reacted

directly with the solid support containing an amino group (LCAA-CPG). On the other hand, the carbonate **9** was treated with 0.033 M NaOH in dioxane/ water/ acetonitrile (1:1:1) to selectively hydrolyse the methyl ester (using higher concentrations of NaOH leads to severe hydrolysis of the carbonate linkage) and the resulting carboxylic acid **10** was attached to LCAA-CPG support by DCC mediated coupling.

We also tried the reaction of DMT-T with chloroformate **7** in CH_2Cl_2 with a small excess of a base stronger than pyridine. When the reaction was done using 1.2 equivalents of triethylamine the carbonate **9** (B= T) was obtained also in the same 60% yield. The use of 4-(dimethylamino)pyridine (DMAP) gave the desired carbonate in a 90% yield. Because the use of DMAP is not compatible with the trichlorophenyl ester function we recommend the use of the methyl ester chloroformate **7** together with DMAP to prepare the desired nucleoside derivatives although the synthetic procedure is longer.

Using these conditions the carbonate derivatives **9** of DMT-A^{NPEOC} and DMT-C^{NPEOC} were obtained in 50% and 52% yield respectively. After saponification of the methyl ester function, the resulting carboxylic acids **10** were attached to CPG supports as described for the DMT-T derivative.



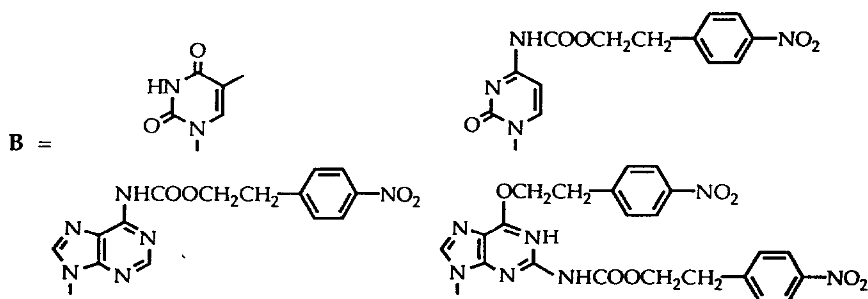


Figure 2 : Preparation of the 4-(2-hydroxyethyl)-3- nitrobenzoic acid derivatives of the 5'-O-DMT, NPE-protected nucleosides.

The new carbonate linkage **12** was stable in all the conditions used during oligonucleotide synthesis, and it could be cleaved in less than 1 hour when it was treated with 0.5 M DBU solution in dioxane or pyridine[12].

Solid-phase synthesis of oligonucleotides with NPE protected phosphoramidites.

The preparation of 2-cyanoethyl phosphoramidites of 5'-O- DMT-NPE protected nucleosides was done following previously described protocols[10,13,14]. The sequences A (5'GACGACTT) and B (5'CAGACGT) were prepared using 1 μmol of the support **12** (B= T) and NPE-protected phosphoramidites on a home-made manual synthesizer. Coupling efficiencies per cycle were about 95%.

After the assembly of the sequences, the supports were treated with a 0.5 M solution of DBU in pyridine for 3 hours at room temperature. HPLC analysis of the oligonucleotide products, after a Sephadex G-10 column, showed an unexpected complex mixture (see figure 3). In the case of the octamer A, the main peak contained the desired oligonucleotide as seen by enzymatic digestion followed by HPLC analysis of the resulting nucleosides. But in the case of the heptamer B any of the three main peaks presented the correct nucleoside composition, being the amounts of 2'-deoxyguanosine and thymidine low and 2'-deoxyadenine high together with the presence of unknown products that did not correspond to any NPE protected nucleosides. We also checked that longer deprotection treatments with DBU did not make any difference on the product composition. So, we conclude that the complexity of the products obtained was not due to incomplete deprotection of NPE groups but on the contrary some side-reaction was produced during the deprotection.

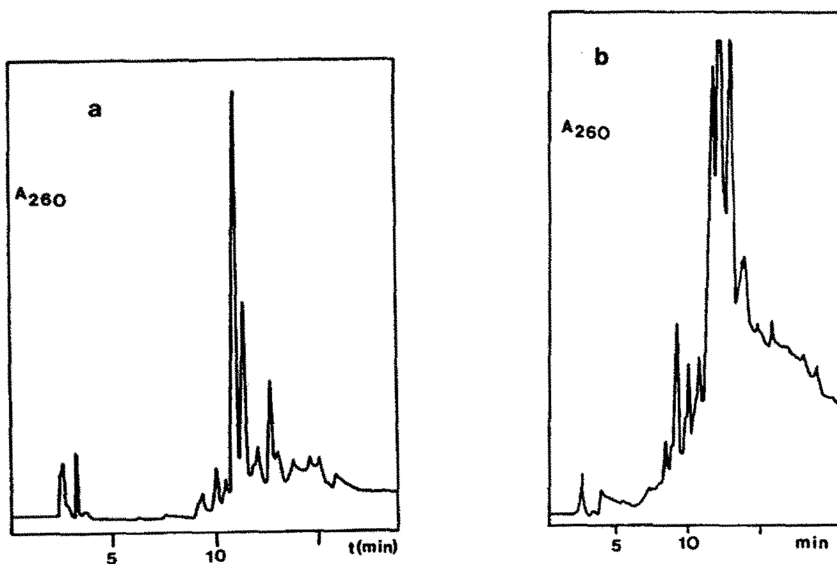


Figure 3 : HPLC profile of oligonucleotides prepared with NPE-protected phosphoramidites and support 12 after the DBU treatment followed by Sephadex G-10 purification. a) octamer A and b) heptamer B.

Our first hypothesis was that the side-reaction was produced by the olefins generated during the deprotection of the *p*-nitrophenylethyl and the 2-cyanoethyl groups. Perhaps, *p*-nitrophenylstyrene and/or acrylonitrile produced during the deprotection could further react with the DNA bases to form the corresponding alkyl derivatives. In order to check that hypothesis we prepare the heptamer B (5'CAGACGT) with standard benzoyl, isobutyryl protected phosphoramidites and aliquots of these oligonucleotides were treated with DBU solutions containing dC^{NPEOC} as a source of *p*-nitrostyrene and bis(2-cyanoethyl)-2-chlorophenyl phosphate as a source of acrylonitrile. The results are shown in figure 4. A severe modification of the oligonucleotide was observed when it was treated with the DBU solution containing bis(2- cyanoethyl)-2-chlorophenyl phosphate while no modification was observed in the DBU solution in the presence of dC^{NPEOC}. The products produced during the treatment of the oligonucleotide with bis(2-cyanoethyl)-2-chlorophenyl phosphate were analyzed by phosphodiesterase-alkaline

phosphatase digestion and it was found no T, low amounts of dG and dA together with some unknown products that had a similar retention time with the unknown products observed on the enzymatic digestion of the oligonucleotide prepared with NPE-protected 2-cyanoethyl phosphoramidites.

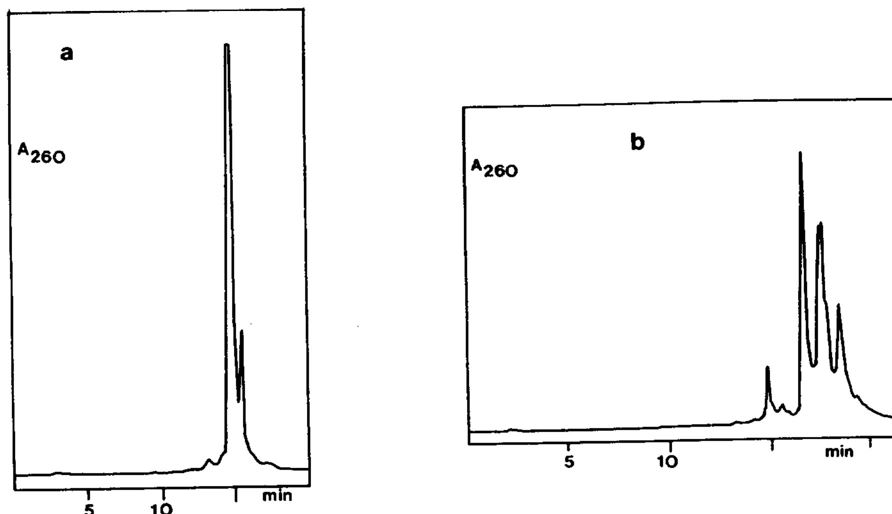


Figure 4 : HPLC profile of the product obtained after a 3 hrs treatment at room temperature of the heptamer 5' CAGACGT 3' with a) 0.5 M DBU solution in pyridine in the presence of dC^{NPEOC} and b) 0.5 M DBU solution in pyridine in the presence of bis (cyanoethyl)-o-chlorophenyl phosphate.

The alkylation of nucleosides in basic media by acrylonitrile has been described and the resulting products have been characterized[16]. As we observed in our experiment, thymidine and dG were the nucleosides modified in much extension by acrylonitrile followed by dA and very little modification has been observed in dC. So, we conclude that the side-reaction observed during the DBU deprotection of the oligonucleotide was due to the alkylation of the DNA bases by the acrylonitrile produced during the β-elimination of the phosphate cyanoethyl esters. It is known, that 2- cyanoethyl phosphates are more labile than NPE-protecting groups and the *o*-nitrophenylethyl carbonate linkage. So, in principle, they can be selectively deprotected with a triethylamine solution allowing the removal of the acrylonitrile before the DBU treatment that deprotects the NPE groups and breaks the oligonucleotide-support bond. In order to check that we prepare oligonucleotide C (5'CATACGT) using the support **12** (B= T) and NPE-protected 2-cyanoethyl phosphoramidites. The support was treated first with a 40% triethylamine solution in pyridine (3 hours) in order to deprotect the 2-cyanoethyl phosphates and second with a 3 hours treatment with 0.5 M DBU solution in pyridine. The HPLC chromatogram of the product obtained, as it can be seen in figure 5, consisted of a major peak that had the expected nucleoside composition indicating that the previously detected side-reaction was prevented. In addition, the 13 mer D (5'TCTCTCTCTCTCT) has been prepared using the two-steps deprotection protocol (data not shown).

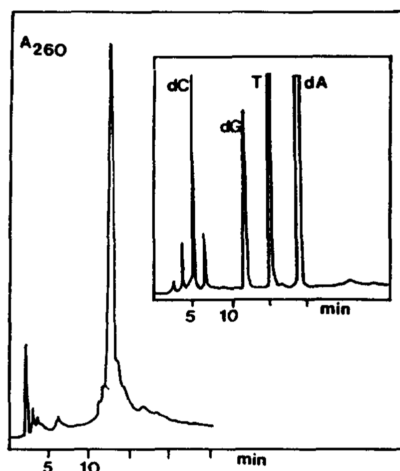


Figure 5 : HPLC profile of oligonucleotide C prepared with NPE protected phosphoramidites and support 12 using the two-steps protocol. The insert shows the HPLC chromatogram of the enzymatic digestion of the purified product.

Synthesis of oligonucleotides containing O-4-alkylthymidines.

O-4-alkylthymidines are one of the products of the reaction of alkylating agents with DNA. Their formation and persistence in DNA have been the object of several works[17]. But, the study of the biological and structural properties of these lesions in DNA is still an unresolved matter due to the difficulty of preparation of synthetic oligonucleotides having the lesion at a predetermined site. The main reason of this difficulty is the great lability of these compounds to ammonia[18].

The methodologies described for the preparation of these modified oligonucleotides are based on the pioneering work from Reese's group in which the ammonia treatment is substituted by a treatment of the protected oligonucleotide with a solution of DBU in methanol[18]. The success of this method is due to the generation, for the cleavage of the base protecting groups, of methoxide ions, the only nucleophile tolerated in the presence of O-methylthymidine. The extension of Reese's method for the preparation of oligonucleotides having 4-O-ethylthymidine needs to change the standard protecting group to the more labile Pac groups[6] or to the NPE protecting groups[10] because of the lower nucleophilicity of ethoxide ions compared to methoxide ions. No other 4-O-alkylthymidine have been efficiently introduced in synthetic oligonucleotides because of the low nucleophilicity of the corresponding alkoxide ion, with the exception of O-4-isopropylthymidine in which the steric hindrance of the isopropyl group slows the exchange of the alkyl group with methoxide ions[19]. For these reasons, we have chosen O-4-propyl and O-4-butylthymidine as ammonia-sensitive molecules to test our methodology.

DMT-protected O-4-propyl and O-4-butylthymidine cyanoethylphosphoramidites were prepared essentially as described previously[10,20]. Oligonucleotides E (5'GCT^{pro}AGC) and F (5'GCT^{bu}AGC) were assembled on an automatic DNA synthesizer using DMT, NPE-protected cyanoethylphosphoramidites and support 12 (B = C^{NPEOC}). After the two-steps protocol described above, the products were purified

by HPLC. As it can be seen in figure 6 both synthesis presented a major product that was isolated in a 13% (hexamer E) and a 9% (hexamer F) overall yield. The presence of the O-4-alkylthymidine and the correct nucleoside composition were confirmed by HPLC analysis of the enzymatic digestion of the products (data not shown).

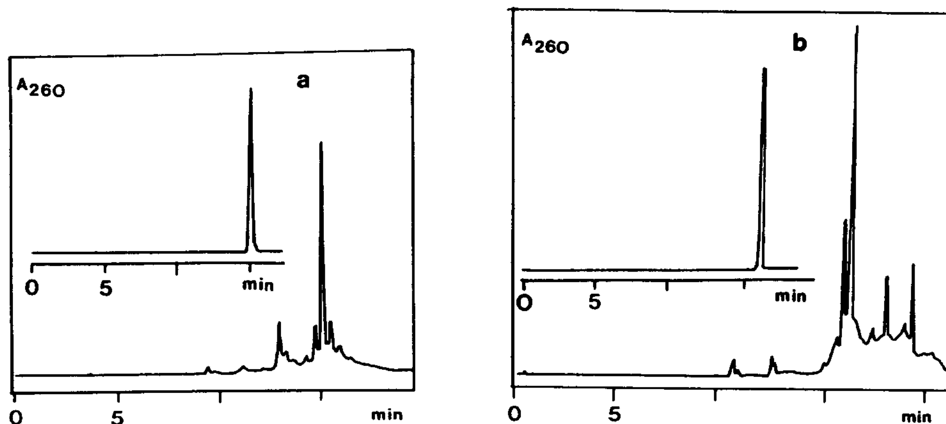


Figure 6 : HPLC profile of oligonucleotides containing 4-O-alkylthymidines. a) hexamer E (5'GCT^{pro}AGC) and b) hexamer F (5'GCT^{bu}AGC). The inserts show the analytical HPLC chromatograms of the purified products.

CONCLUSIONS

We have shown that the new polymeric supports described here can be used for the preparation of modified short oligonucleotides containing ammonia-sensitive molecules without using ammonia for the deprotection at the end of the synthesis. Once the reagents have been made, the methodology presented here is competitive with the standard methodology in terms of time of synthesis and deprotection as well as efficiency of coupling. Unfourtunately, we have detected a severe side-reaction that has been demonstrated to be due exclusively to the cyanoethyl phosphate protecting group. Similar to what has been described for the methyl phosphate protecting group[21], the use of DBU during deprotection produces modified bases, especially G and T derivatives. We have reduced the extent of these side-reactions by eliminating the major part of the cyanoethyl group before the DBU treatment, but we can not recommend this methodology for large oligonucleotides. Improvements of the present work have to come by changing the phosphate protecting groups or by adding a large excess of a molecule that can act as scavenger of acrylonitrile. Thus if this problem can be overcome, this methodology can be used to prepare a large variety of modified oligonucleotides.

EXPERIMENTAL SECTION

Abbreviations: AcOEt: ethyl acetate; CPG^{R}: controlled-pore glass; DBU: 1,8-diazabiciclo[5.4.0]

undec-7-ene; DCC: N,N'-diciclohexylcarbodiimide; DMAP: 4-(dimethylamino)pyridine; DMT: 4,4'-dimethoxytrityl; EtOH: ethanol; HOBt: 1-hydroxybenzotriazole; LCAA-CPG^R: Long chain amino alkyl-controlled-pore glass; MeOH: methanol; NPE: *p*-nitrophenylethyl; NPEOC: *p*-nitrophenylethoxycarbonyl.

DMT-A^{NPEOC}, C^{NPEOC}, $G^{NPE,NPEOC}$, T^{pro} and T^{bu} cyanoethyl phosphoramidites were prepared as described previously[10,13,24,20]. 4-(2-Hydroxyethyl) benzonitrile **2** was prepared as described in ref. 15. 2-Chlorophenyl bis(2-cyanoethyl) phosphate was prepared as described in ref. 22. LCAA- CPG^R was obtained from Pierce and from CPG Inc. Oligonucleotide syntheses were done either in a home-made manual synthesizer or in an automatic Applied-Biosystems DNA synthesizer. Enzymatic digestions of oligonucleotides were performed as described in ref. 10.

4-(2-Acetyloxyethyl)-3-nitrobenzonitrile 3.

4-(2-Hydroxyethyl)benzonitrile (**2**) (5.5 g, 37 mmol) was dissolved in 30 mL of acetic anhydride and 3 mL of acetyl chloride were added (40 mmol). The mixture was stirred for 30 minutes at room temperature and the solution was concentrated to about 15 mL. The resulting solution was added dropwise to a mixture of 18 mL of fuming nitric acid (density 1.52) and 15 mL of concentrated sulfuric acid at -10ºC. The mixture was stirred for additional 2 hours at 0ºC and subsequently poured over ice (200 mL). The precipitated product was collected and washed with water, obtaining 7 gr (80% yield) of the desired compound. ^{1}H-NMR ($CDCl_3$, 60 MHz) : 8.2 (1H, s, Ar); 7.9 (1H, d, J= 8 Hz, Ar); 7.6 (1H, d, J= 8 Hz, Ar); 4.0 (2H, t, J= 6 Hz, $-CH_2$-O-); 3.2 (2H, t, J= 6Hz, $-CH_2$-Ar); 2.0 (3H, s, $-OCOCH_3$).

4-(2-Hydroxyethyl)-3-nitrobenzoic acid 4.

4-(2-Acetyloxyethyl)-3-nitrobenzonitrile (**3**) (7 gr, 30 mmol) was dissolved in 15 mL ethanol and 75 mL of a 10% sodium hydroxide solution were added. After 3 hours at room temperature, a dark solution was obtained with a small amount of some precipitated material. The solution was filtered and washed with 75 mL of dichloromethane. The aqueous phase was acidified with HCl to pH 2 and the product was extracted with ethyl acetate (3 x 80 mL). The organic solutions were pooled, dried (Na_2SO_4) and concentrated to dryness. Purification of the product was done by chromatography on silica gel eluting with hexane-AcOEt (1:1) and pure AcOEt. The desired fractions were pooled and concentrated obtaining 3.8 gr of a yellow solid (60% yield), m.p. : 114-116 ºC.

Elemental analysis calculated for $C_9H_9NO_5$: C: 51.1%, H: 4.3%, N: 6.6%; Found C: 51.3% , H: 4.3%, N: 6.5%. IR (KBr) (cm^{-1}: 3400, 3300-2300, 1690, 1535, 1325. ^{1}H-NMR (CD_3OD, 200 MHz) : 8.17 (1H, d, J= 1.5Hz, Ar); 7.90 (1H, dd, J= 8 and 1.5 Hz, Ar); 7.32 (1H, d, J= 8 Hz, Ar); 4.69 (s, -OH); 3.55 (2H, t, J= 6.5 Hz, $-CH_2OH$); 2.88 (2H, t, J= 6.5 Hz, Ar-CH_2-)

Alternatively, the same product was obtained by acid hydrolysis. 2.0 gr of 4-(2-acetyloxyethyl)-3-nitrobenzonitrile (**3**) (8,5 mmol) were dissolved with 50 mL of 12 N HCl-EtOH (1:1) and heated under reflux for 16 hours. The resulting mixture was concentrated to dryness and the product was purified as described above, obtaining 1.4 gr of the desired product (77% yield).

Methyl 4-(2-hydroxyethyl)-3-nitrobenzoate 6.

4-(2-Acetyloxyethyl)-3-nitrobenzonitrile (**3**) (7 g, 30 mmol) was dissolved in 50 mL of MeOH/ HCl(g). The mixture was heated under reflux for 3 hours and the resulting solution was concentrated to dryness. The product was purified by column chromatography on silica gel eluting with increasing amounts of MeOH (from 0 to 4%) in dichloromethane. Yield : 5.7 gr (85%). IR (KBr) (cm^{-1}): 3600-3200, 1740, 1540, 1365. ^{1}H-NMR ($CDCl_3$, 60 MHz) : 8.4 (1H, d, J= 2 Hz, Ar); 8.1 (1H, dd, J= 8 and 2 Hz, Ar); 7.4 (1H, d, J= 2 Hz, Ar); 4.1 (3H, s, $-OCH_3$); 3.9 (2H, t, J= 6 Hz, $-CH_2$-OH); 3.1 (2H, t, J= 6 Hz, Ar-CH_2-); 1.8 (s, -OH).

2,4,5-Trichlorophenyl 4-(2-hydroxyethyl)-3-nitrobenzoate 5.

A mixture of 2,4,5-trichlorophenol (0.27 gr, 1.35 mmol) and DCC (0.28 gr, 1.35 mmol) dissolved in dichloromethane (10 mL) was added to a solution of 4-(2-hydroxyethyl)-3- nitrobenzoic acid (**4**) (0.32 gr, 1.5 mmol) in AcOEt (1 mL). The mixture was stirred overnight at room temperature and then kept for 2 hr at 4ºC. The N,N'-dicyclohexylurea that was formed was removed by filtration and the filtrate

was washed with pH 9.5 sodium carbonate buffer (3 x 25 mL) and saturated aqueous NaCl solution (25 mL), dried (Na_2SO_4), and concentrated to dryness. The product was recrystallized from toluene/hexane. Yield: 0.44 gr (75%). m.p. 82-84 ºC. ^{1}H- NMR ($CDCl_3$, 200 MHz) : 8.73 (1H, d, J= 1.8 Hz, Ar); 8.35 (1H, dd, J= 8 and 1.8 Hz, Ar); 7.66 (1H, d, J= 8 Hz, Ar); 7.63 (1H, s, Ar'); 7.45 (1H, s, Ar'); 4.02 (2H, t, J= 6.2 Hz, -CH_2-OH); 3.28 (2H, t, J= 6.2 Hz, -CH_2-Ar); 1.94 (s, - OH).

Chloroformate 7.

A solution of methyl 4-(2-hydroxyethyl)-3-nitrobenzoate (5) (0.9 g, 4 mmol) in dry dichloromethane (5 mL) was added dropwise with stirring to a 1.9 M solution of phosgene in toluene (10.5 mL, 20 mmol) cooled with an ice-bath. After stirring at 4ºC for 30 min the ice-bath was removed and the mixture stirred 3 hr at room temperature. Phosgene and dichloromethane were removed using a water aspirator and toluene was eliminated using a rotatory evaporator connected to a high-vacuum pump, obtaining 1 gr (3.5 mmol, 87% yield) of an oil that was used without further purification. IR (KBr) (cm^{-1}): 1790, 1740, 1540, 1365. ^{1}H-NMR ($CDCl_3$, 60 MHz) : 8.6 (1H, s, Ar); 8.2 (1H, d, J= 8 Hz, Ar); 7.5 (1H, d, J = 8 Hz, Ar); 4.5 (2H, t, J = 7 Hz, -CH_2-OH); 4.0 (3H, s, - OCH_3); 3.3 (2H, t, J= 7 Hz, Ar-CH_2-).

Chloroformate 8.

0.39 gr of 2,4,5-trichlorophenyl 4-(2-hydroxyethyl)-3- nitrobenzoate (6) (1 mmol) dissolved in 1 mL of dichloromethane were treated with phosgene (3 mL of a 1.9 M solution in toluene, 5 mmol) essentially as described above. Yield : 0.42 gr (0.9 mmol, 92%). IR (KBr) (cm^{-1}): 1800-1750, 1535, 1355. ^{1}H-NMR ($CDCl_3$, 90 MHz) : 8.82 (1H, d, J= 2.5 Hz, Ar); 8.40 (1H, dd, J= 2,7 and 8.6 Hz, Ar); 7.63 (1H, d, J= 8.6 Hz, Ar); 7.60 (1H, s, Ar'); 7.42 (1H, s, Ar'); 4.65 (2H, t, J= 6.7 Hz); 3.4 (2H, t, J= 6.7 Hz).

4-(2-3'-(5'-O-DMT-N,O-(NPEOC, NPE) protected-2'- deoxyribonucleosidyl) carbonyloxyethyl)-3-nitrobenzoate methyl ester 9.

Using pyridine as solvent and base catalyst.

5'-O-DMT-thymidine (300 mg, 0.55 mmol) was dried by coevaporation with pyridine. The residue was dissolved in pyridine (5 mL) and the solution cooled with an ice-bath and the chloroformate 7 (220 mg, 0.82 mmol) was added. After 4 hrs of magnetic stirring a second portion of the chloroformate 7 was added (100 mg, 0.4 mmol) and the mixture was left overnight at room temperature. The excess of chloroformate was destroyed with 2 mL of methanol and the solvents were evaporated. The residue was purified on a silica gel column eluted with a solution from 0% to 4% of methanol in dichloromethane. Fractions containing the desired product were pooled and evaporated to dryness obtaining 0.25 gr of the desired product (60% yield). TLC (5% MeOH/ CH_2Cl_2) Rf = 0.75. ^{1}H-NMR ($CDCl_3$, 200 MHz) : 8.64 (1H, m, Ar); 8.40 (1H, s, NH); 8.20 (1H, m, Ar); 7.60 (1H, d, H-5); 7.48 (1H, m, Ar); 7.27 (9H, m, Ar DMT); 6.84 (4H, d, J= 9 Hz, Ar DMT); 6.43 (1H, dd, H-1'); 4.46 (3H, m, CH_2 and H-3'); 4.18 (1H, m, H-4'); 3.96 (3H, two s, CH_3 ester); 3.80 (9H, s, CH_3O- DMT); 3.45 (2H, m, H-5'); 3.34 (2H, m, CH_2); 2.450 (2H, m, H-2'); 1.39 (3H, s, 5-CH_3).

Using triethylamine or N,N-dimethylaminopyridine as base catalyst.

5'-O-DMT-N,O-(NPEOC, NPE) protected -2'-deoxynucleoside (0.5 mmol) was dried by coevaporation with toluene. To the residue 5 mL of dry dichloromethane were added and the solution cooled with an ice-bath. 0.070 mL (0.6 mmol) of triethylamine or alternatively 73 mg (0.6 mmol) of DMAP and 144 mg (0.5 mmol) of the chloroformate 7 were added and the reaction was left for 2 hours at 0-4ºC and 16 hours at room temperature. The reaction mixture was diluted with 50 mL of dichloromethane and washed with cooled water (3 x 20 mL) The organic phase was dried and the solvents evaporated. The product was purified on a silica gel column eluted with a 0% to 5% solution of MeOH in dichloromethane.

9 B = Thymine, using triethylamine as base catalyst.

Yield : 60%. Chromatographic and spectral characteristics as described above.

9 B = Thymine, using DMAP as base catalyst.

Yield : 90 %. Chromatographic and spectral characteristics as described above.

9 B = 4-N-NPEOC-cytidine, using DMAP as catalyst.
Yield : 50 %. TLC (2% MeOH/ CH_2Cl_2) Rf = 0.8. ^{1}H-NMR (Cl_3CD, 200 MHz) : 8.6 (1H, m, Ar); 8.2 (3H, m, Ar); 7.5-7.1 (13H, m, Ar and H-5 and H-6); 6.82 (4H, d, Ar DMT); 6.24 (1H, t, H-1'); 4.4 (5H, m, 2 CH_2 and H-3'); 4.3 (1H, m, H-4'); 3.95 (3H, two s, CH_3); 3.78 (6H, s, CH_3O- DMT); 3.5-3.0 (6H, m, 2 CH_2 and H-5'); 2.8-2.4 (2H, m, H-2').
9 B= 6-N-NPEOC-adenine, using DMAP as catalyst.
Yield : 52%. TLC (2% MeOH/ CH_2Cl_2) Rf = 0.7. ^{1}H-NMR (Cl_3CD, 200MHz) : 8.67 (1H, s, H-8); 8.62 (1H, d, Ar); 8.3-8.1 (4H, m, 3 Ar and H-2); 7.6-7.2 (12H, Ar); 6.68 (4H, d, Ar DMT); 6.48 (1H, dd, H-1'); 4.6-4.3 (6H, 2 CH_2 and H-3' and H-4'); 3.95 (3H, s, CH_3 ester); 3.77 (6H, s, OCH_3 DMT); 3.5-3.1 (4H, m, CH_2); 3.16 (2H, t, CH_2); 3.02 (1H, m, H-2'); 2.72 (1H, m, H-2').

4-(2-3'-(5'-O-DMT-N,O-(NPE, NPEOC) protected-2'- deoxynucleosidyl) carbonyloxyethyl-3-nitrobenzoic acid 10.

Compound **9** was dissolved (0.35 mmol) in acetonitrile (20 mL) and 40 mL (2 mmol) of a 0.05 M solution of NaOH in water/dioxane (1:1) were added. After 5 minutes of magnetic stirring at room temperature, the reaction was checked by TLC (10% MeOH /CH_2Cl_2) being always complete at that time. 0.09 mL (2 mmol) of acetic acid were added to neutralize the excess of NaOH and the reaction mixture was concentrated to dryness. The residue was dissolved in dichloromethane (30 mL) and washed with ice-cold 10% aqueous citric acid (2 x 30 mL) and then with water (1 x 20 mL). The organic phase was dried over anhydrous sodium sulphate and evaporated under reduced pressure. The residue was purified by precipitation on hexane or by column chromatography eluted with a 0% to 15% solution of MeOH in dichloromethane. Some small amounts of DMT protected nucleoside coming from partial hydrolysis of the carbonate linkage can be recovered from the column, being the desired product the last DMT positive product.
10 B= T. Yield 60%. TLC (10% MeOH / CH_2Cl_2) Rf = 0.4.
10 B= A^{NPEOC}. Yield 73%. TLC (10% MeOH/ CH_2Cl_2) Rf = 0.5.
10 B= C^{NPEOC}. Yield 62%. TLC (10% MeOH/ CH_2Cl_2) Rf = 0.5.
High-field proton NMR spectra similar to compounds **9** except for the absence of the signals at 3.9 ppm (methyl ester).

4-(2-3'-(5'-O-DMT-thyminidyl) carbonyloxyethyl-3- nitrobenzoate 2,4,5-trichlorophenyl ester 11.

A solution of the chloroformate **8** (1 mmol) in 1 mL of dry dichloromethane was added to a solution of DMT-T (545 mg, 1 mmol) in dry pyridine cooled with an ice-bath. After the addition was complete, the reaction mixture was left 15 min on the ice-bath and overnight at room temperature. 1 mL of MeOH was added to the reaction mixture and the solvents were evaporated. The residue was dissolved in 25 mL of a 5% triethylamine solution in AcOEt, and the solution was washed with a 5% sodium bicarbonate solution (2 x 20 mL) and a saturated NaCl solution (2 x 20 mL). The organic phase was dried and concentrated to dryness and the resulting product was purified on a silica gel column eluted with a 0% to 5% solution of MeOH in dichloromethane. Yield 40%. TLC (5% MeOH / CH_2Cl_2) Rf = 0.70). ^{1}H-NMR ($CDCl_3$, 200 MHz): 8.92 (1H, s, NH-3); 8.79 (1H, d, J= 1.8 Hz, Ar); 8.40 (1H, dd, J= 7 and 1.8 Hz, Ar); 7.63 (1H, d, J= 7 Hz, Ar); 7.62 (1H, s, Ar'); 7.42 (1H, s, Ar'); 7.28 (9H, m, Ar DMT); 6.84 (4H, d, J= 9 Hz, Ar DMT); 6.45 (1H, dd, J= 8.5 and 5.5 Hz, H-1'); 4.72 (1H, m, H-3'); 4.52 (2H, m, -CH_2-O-); 4.19 (1H, m, H-4'); 3,79 (6H, s, CH_3O- DMT); 3.45 (4H, m, -CH_2-Ar, H-5'); 2.45 (1H, m, H-2'); 1.89 (1H, m, H-2'); 1.38 (3H, s, CH_3-5).

Preparation of the DMT-2'-deoxynucleosidyl-NPE supports 12.
Using compound 11.

0.6 gr of Amino-LCAA-CPG (0.15 mmol/g, 0.09 mmol) were reacted with 175 mg (0.18 mmol) of compound **11** (B= T) and 24 mg of HOBt (0.18 mmol) in the minimal amount of DMF. The reaction was kept 3 days with mild agitation at room temperature. The support was transferred to a filtration apparatus and filtered and washed with DMF, dichloromethane, MeOH, DMF, acetonitrile and again with dichloromethane. The unreacted amino groups on the support were acetylated by a 1 hr treatment of the support with 2.5 mL of a 10 % solution of acetic anhydride in pyridine, and, finally, the support

was washed with dichloromethane and dried. The loading of the resulting support was 0.04 mmol of DMT per gram of resin.

Using compound 10.

Compound **10** (0.08 mmol) and HOBt (9 mg, 0.08 mmol) were dissolved in the minimal amount of DMF and the solution was cooled with an ice-bath. To the solution, 12 mg (0.08 mmol) of DCC were added and the mixture was kept for 10 minutes ion the ice-bath and added, afterwards, to 1 gr of amino-LCAA-CPG (0.030 mmol per gram, 0.03 mmol). The reaction was kept at room temperature for 3 days and the resulting support was filtered, washed and acetylated as described above. Loadings were around 0.010 mmol of DMT per gram of resin.

Oligonucleotide synthesis.

Using 5'-O-DMT-NPE-protected nucleosidyl 3'-N,N-diisopropyl-O-2-cyanoethyl phosphoramidites (10, 14) and the support **12**, the following sequences have been synthesized:

A) 5' GACGACTT 3' 8 bases D) 5' TCTCTCTCTCTCT 13 bases

B) 5' CAGACGT 3' 7 bases E) 5' GCT^{pro}AGC 3' 6 bases

C) 5' CATACGT 3' 7 bases F) 5' GCT^{bu}AGC 3' 6 bases

Oligonucleotides A-C were prepared on a 1 μmol scale and using a home-made manual synthesizer and standard phosphite- triester protocols (23). Oligonucleotide D, E and F were prepared on an 392 Applied Biosystems automatic synthesizer using the protocols recommended by the supplier. Coupling efficiencies were around 95% by measuring DMT optical density at 500 nm.

Deprotection and purification.

One-step deprotection.

Aliquotes of oligonucleotidyl-supports corresponding to sequences A and B were treated with approx. 0.5 mL of a 0.5 M solution of DBU in pyridine. After a period of time ranging from 3 to 24 hours, the resin was filtered and washed with pyridine. The filtrates were pooled, neutralized with acetic acid (1.5 equiv. respect to the of DBU) and diluted with 5 mL of 0.1 M triethylammonium acetate pH 7.8. The solution was concentrated to dryness and the residue was dissolved in 0.1 M triethylammonium acetate buffer pH 7.8 and chromatographed on a Sephadex G-10 column (30 x 1 cm) eluted with the same buffer. The fractions corresponding to the first UV-absorbing peak were analyzed by analytical HPLC using a C-18 Nucleosil 120 (5 μm) column (250 x 4 mm) with a 5-20% gradient of acetonitrile in 10 mM triethylammonium acetate over 20 min (see figure 3).

The main product obtained in the case of oligonucleotide A was digested with snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis of the digestion as described in ref. 10. Nucleotide content: dC 2.2 (2); dG 2.6 (2); T 2.0 (2); dA 2.2 (2). No extra peaks were observed.

The three main peaks obtained in the case of oligonucleotide B had the following nucleoside composition. Peak 1 : dC 2.0 (2); dG 1.0 (2); T 0.9 (2); dA 1.8 (2). Peak 2 : dC 1.8 (2); dG 1.1 (2); T 0.9 (2); dA 4.3 (2). Peak 3 : dC 2.0 (2); dG 1.1 (2); T 1.0 (2); dA 2.8 (2). Three different unknown products were observed. One eluting before dA and the other two immediately after dA.

Two-step protocol.

After the synthesis, the resulting oligonucleotidyl- supports (C-F) were separated from the synthesis columns and located on a filtration apparatus where they were treated with a 40% triethylamine solution in pyridine for 3 hours at room temperature (3 washings of 2 mL for 1 hour each). Afterwards, the resins were filtered and washed with pyridine and acetonitrile and finally, treated with a 0.5 M DBU solution in pyridine for 3 hours at room temperature. The resins were filtered and washed with pyridine. The filtrates were pooled, neutralized with acetic acid and diluted with triethylammonium acetate (as described above). After Sephadex G-10 purification, the products were analyzed by analytical HPLC in the same conditions described above. In all cases a main peak was observed (see figures 5 and 6) that had the expected nucleoside composition. The amounts of product obtained after purification were as follows : Oligonucleotide C : 16 optical units at 260 nm, 32% overall (synthesis and purification) yield.

Oligonucleotide D : 9.5 optical units at 260 nm, 17% overall yield. Oligonucleotide E : 4,8 optical units at 260 nm, 13% overall yield. Oligonucleotide F : 3,2 optical units at 260 nm, 9% overall yield.

ACKNOWLEDGEMENTS.

This work was supported by funds from CICYT (PB88-0216 and SAL90-0828) and NATO Collaborative Research Grant 900554. We would like to thank Dr. Dolors Fernández-Forner and Yolanda Palom for their help during the initiation of the work presented here. The encouragement of Drs Manjit K. Dosanjh, Bea Singer and Myron F. Goodman have been very important during this work.

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