Nucleic Acids Research, 1993, Vol. 21, No. 15 3493–3500

The application of the AMB protective group in the solid-phase synthesis of methylphosphonate DNA analogues

W.H.A.Kuijpers, E.Kuyl-Yeheskiely[1], J.H.van Boom[1] and C.A.A.van Boeckel
Organon International BV, PO Box 20, 5340 BH Oss and [1]Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands

Received April 8, 1993; Revised and Accepted June 14, 1993

ABSTRACT

Partially methylphosphonate-modified oligodeoxynucleotides were synthesized on solid-phase by employing the easily removable 2-(acetoxymethyl)benzoyl (AMB) group as base-protecting group. Although a rapid AMB deprotection can be accomplished in methanolic potassium carbonate, the lability of the methylphosphonate linkage towards potassium carbonate/methanol excludes the use of this deprotection reagent. Thus, saturated ammonia solution in methanol was investigated as an alternative reagent for AMB removal. It is demonstrated that the combination of the AMB protective group and ammonia/methanol as deprotection reagent significantly improves the synthesis of methylphosphonate-modified DNA fragments. A mild overnight treatment at room temperature is sufficient for complete removal of the AMB group, whereas deprotection of conventionally protected oligonucleotides requires much longer exposure to basic conditions at elevated temperatures.

INTRODUCTION

The past several years we have been involved in the development of a new approach of cancer radioimmunotherapy.[1] Instead of direct administration of radiolabeled monoclonal antibodies, in our strategy, antibody conjugates with single-stranded oligonucleotides will be targeted to the tumor site. Then, in a second step, the radiolabeled complementary oligonucleotide is administered. The oligonucleotides which mediate in this so-called DNA–DNA pretargeting strategy, should fulfil several criteria. First, they have to be functionalized in such a way that conjugation with monoclonal antibodies or radiolabeling can be accomplished. Moreover, since both oligonucleotides should survive under physiological conditions, precautions should be taken against nuclease-promoted degradation. In this respect the antisense DNA technology offers a wide, still expanding, variety of modified oligonucleotides.[2]

Methylphosphonate-modified DNA fragments are one of the most extensively studied classes of antisense oligonucleotides.[3] In a large number of studies completely and partially methylphosphonate-modified DNA fragments have been reported to exhibit enhanced stability towards nucleases and, moreover, have been shown to function as antisense inhibitors of gene expression.[4,5] Originally, methylphosphonate linkages were introduced by methods based on the phosphotriester approach.[6–8] Later on, modern solid-phase DNA synthesis involving well-known phosphoramidite chemistry has been extended to the preparation of methylphosphonate DNA sequences.[9–11] The hydrolytic lability of the methylphosphonate linkage, however, precludes a straightforward adoption of the aqueous ammonia deprotection procedure used in natural DNA synthesis.[6,12] To overcome hydrolysis of the methylphosphonate diesters more suitable deprotection methods have been developed (*e.g.* hydrazine hydrate followed by ethylenediamine/ethanol[13], or ammonia/methanol[14,15]). Nevertheless, removal of the conventional base-protecting groups (benzoyl for A and C; isobutyryl for G) according to these procedures necessitates extended exposure to the basic deprotection conditions which may have a deleterious effect on the quality of the desired products.

The difficulties encountered in the isolation of well-defined methylphosphonates have recently been stressed in a paper which describes some improvements in the ethylenediamine deprotection procedure.[16] It was shown that the use of the more labile isobutyryl protective group can prevent the transamination at cytosine which has been observed for the conventional benzoyl base-protection.[7,13] Moreover, a short pre-incubation with a mild ammonia solution was recommended to eliminate modifications at guanine arising from the capping procedure.

Here, we present the solid-phase synthesis of methylphosphonate containing oligonucleotides by applying the 2-(acetoxymethyl)benzoyl (AMB) group for nucleobase protection. The AMB protective group was originally developed by us for the synthesis of (partially) phosphate-methylated DNA fragments.[17] In combination with methanolic potassium carbonate as deprotection reagent,[18] the AMB group provides a mild procedure for the preparation of oligomers containing the labile methylphosphotriester linkage. Hence, we anticipated that this strategy should be equally well suitable for the preparation of methylphosphonate-modified oligonucleotides. However, deprotection in potassium carbonate/methanol leads to serious degradation of the methylphosphonate diester. Thus, experiments are presented directed towards the stability of the methylphosphonate linkage under basic deprotection conditions. It is shown in the preparation of a partially methylphosphonate-modified hexadecamer (*i.e.* compound **15**) that the application

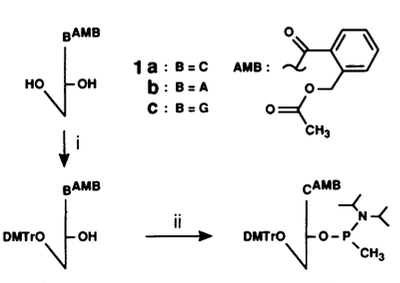
of the AMB protective group in combination with an ammonia/methanol deprotection procedure may significantly improve the synthesis of well-defined methylphosphonate-modified oligonucleotides.

RESULTS AND DISCUSSION

For our investigations on the DNA-DNA pretargeting concept, we have prepared a number of partially methylphosphonate-modified DNA sequences.[1] Those fragments were synthesized by combining methylphosphonamidites[11] of the commercially available base-protected deoxynucleosides with the standard cyanoethyl phosphoramidites in an automated DNA synthesizer. Deprotection of the oligonucleotides was performed in saturated ammonia solution in methanol for 3 days at 50°C to ensure complete removal of the base-protecting groups, in particular the isobutyryl group from the guanine bases.

In order to evaluate the effectiveness of the AMB nucleobase-protecting group, we planned to synthesize the partially methylphosphonate-modified hexadecamer **15**. The introduction of a p-hydroxyphenylethyl moiety at the 5′-end of this oligomer should guarantee efficient radiolabeling with iodine isotopes (^{125}I, ^{131}I).[19] In this way a radiolabeled oligonucleotide becomes available which should hybridize with complementary antibody-linked oligonucleotides in pretargeting experiments.

With the above objectives in mind, we first prepared the methylphosphonamidite of AMB-protected deoxycytidine as a building block for solid-phase DNA synthesis. Thus, DMTr-dC-AMB (**2a**) was readily converted into its methylphosphonamidite **3a** by treatment with bis(*N*,*N*-diisopropylamino)methylphosphine in the presence of a catalytic amount of 2,4,6-collidine.HCl (Scheme 1).[10]



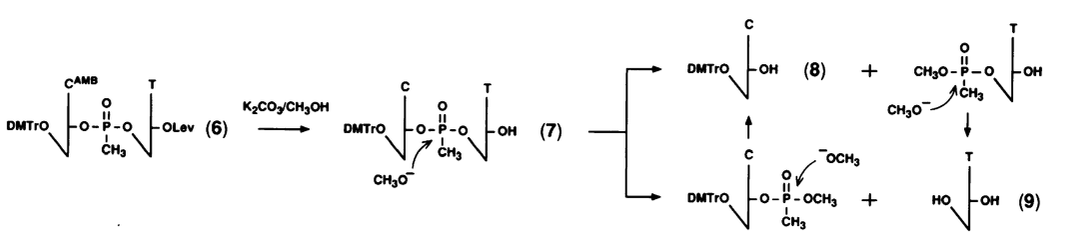
Scheme 1. 2-(Acetoxymethyl)benzoyl (AMB) base-protected deoxynucleosides[17] as starting compounds for the preparation of methylphosponamidites. Reagents: (i) Dimethoxytrityl (DMTr) chloride, pyridine; (ii) Bis(*N*,*N*-diisopropylamino)methylphosphine, collidine.HCl.

The suitability of amidite **3a** for application in automated DNA synthesis[20] was investigated by the assembly of the fully protected partially methylphosphonate-modified hexamer **4** (Figure 1). Cleavage of **4** from the solid support and removal of the cyanoethyl and AMB protective groups were effected by treatment of the solid-support with 0.05 M potassium carbonate in methanol during 4 hours at room temperature.

Analysis of the end product by anion-exchange HPLC (MonoQ) revealed, besides the major component (hexamer **5**) and some material close to the injection peak, the presence of a side-product (Figure 1). At first sight, we reasoned that the side-product might have resulted from incomplete deprotection of the AMB-protected nucleobases. However, upon prolonged treatment of crude hexamer **5** with potassium carbonate/methanol the side-product did not disappear, but instead, new side-products occurred.

The above findings prompted us to examine in more detail the nature of the observed side-reaction. To this end, the dinucleoside methylphosphonate **6** was prepared by coupling phosphonamidite **3a** with 3′-*O*-levulinoyl-thymidine.[21] Dimer **6** was treated with 0.05 M potassium carbonate/methanol at room temperature and the deprotection was monitored by TLC analysis. A rapid removal of the levulinoyl ester was observed reaching completion within 10 minutes. Cleavage of the AMB group was found to be complete after 0.5 − 1 hour, yielding the deprotected dimer **7**. However, at that time the first traces of by-products appeared which accumulated upon further exposure of **7** to methanolic potassium carbonate. After 1 day more than 50% of **7** had been converted into several non-charged products and, after 3 days dimer **7** had been completely degraded. Finally, after 6 days only two products were distinguished, one of which contained a DMTr group. Both compounds were isolated by silica gel chromatography and characterized by NMR spectroscopy.

According to ^{31}P NMR spectroscopy neither one of the two degradation products contained a phosphonate group. The latter observation indicated that chain cleavage had occurred, most likely through attack of methanolate at phosphorus. Such a saponification may lead to the release of one of the two nucleoside constituents, as illustrated in Scheme 2. Repeated methanolate attack should result in the two dephosphorylated nucleoside building blocks. Indeed, ^{1}H NMR spectroscopy, TLC analysis and FAB mass-spectrometry showed that the DMTr-containing compound (**8**) is identical with the product isolated after AMB deprotection of nucleoside **2a**. Likewise, the other degradation product (**9**) turned out to be indiscernible from commercially available thymidine as judged by ^{1}H NMR-, FAB MS-, and TLC analysis. All these results support the degradation mechanism as proposed in Scheme 2.[22,23]



Scheme 2. Mechanism for the degradation of methylphosphonate linkages by the action of potassium carbonate/methanol. AMB: 2-(Acetoxymethyl)benzoyl, DMTr: Dimethoxytrityl, Lev: Levulinoyl.

The lability of the methylphosphonate linkage towards potassium carbonate/methanol is quite remarkable, especially in the light of our former experiments on the synthesis of phosphate-methylated dinucleotides.[18] In this respect it is of interest to note that the phosphate-methylated analogue of dimer **7**, *i.e.* DMTr-dC-P(OCH_3)-T, was found to be considerably more stable in methanolic potassium carbonate than dimer **7**.

Fortunately, for deprotection of the AMB base-protecting group we can resort to other reagents which have been reported not to affect the methylphosphonate linkage. Thus, apart from commonly used ethylenediamine/ethanol[8,13], ammonia/methanol has been applied for the preparation of several partially methylphosphonate-modified oligodeoxynucleotides.[14,15] However, for the deprotection of conventionally protected (benzoyl for A and C, isobutyryl for G) nucleobases long deprotection times (48–72 h) at elevated temperature (50°C) are required. Since cleavage of the AMB protective group in fact is dictated by saponification of the acetyl ester, we expected the AMB group to be much more sensitive to ammonia/methanol than the conventional protective groups. Indeed, treatment of the AMB-protected 5′-*O*-DMTr nucleosides **2a–c** with a saturated solution of ammonia in anhydrous methanol readily resulted in the formation of the deprotected analogues, although the half-lives of deprotection were found to be considerably longer than in the presence of potassium carbonate/methanol (see Table 1). Nevertheless, complete removal of the AMB group can be accomplished within 1 day (6–7 h for A and C; 16 h for G) at room temperature.

Exposure of dimer **6** to ammonia/methanol showed, as judged from TLC analysis, removal of the levulinoyl ester in 2 hours and complete cleavage of the AMB group in 6 hours. The resulting dinucleotide **7** was found to be stable for at least 3 days in ammonia/methanol. Nonetheless, after 6 days slight amounts of side-products had been formed which were, according to TLC analysis, identical to those found in the potassium carbonate/methanol treatment. On the basis of the stability of the dimer model system **7**, it can be concluded that ammonia/methanol is the most convenient deprotecting reagent for AMB base-protected methylphosphonate-modified oligonucleotides.

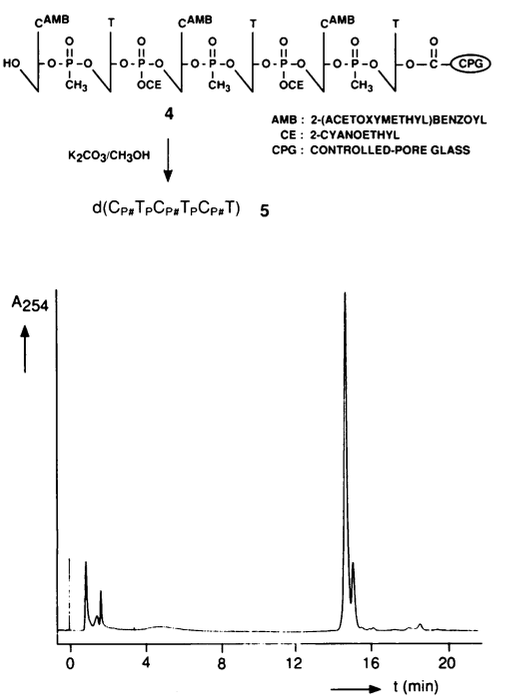


Figure 1. Anion-exchange HPLC (MonoQ) elution profile of crude hexamer **5** obtained after deprotection of immobilized hexamer **4** with 0.05 M potassium carbonate/methanol. P# denotes a methylphosphonate linkage.

In order to further investigate the suitability of ammonia/methanol for AMB deprotection, we aimed at the solid-phase synthesis of the partially methylphosphonate-modified heptamer d(TPCP#TPCP#TPCP#T) (**11**). Thus, the fully protected oligonucleotide **10** was prepared on 10 μmol-scale analogously to the synthesis of hexamer **4**. The additional thymidine nucleotide at the 5′-end was introduced to fully exclude the occurrence of neighboring group participation by the free 5′-hydroxyl group during deprotection. After completion of the solid-phase synthesis, the solid support bound oligonucleotide **10** (6 μmol) was treated with a saturated ammonia solution in dry methanol at room temperature.

As a reference, a portion of the solid support (2 μmol of **10**) was also subjected to methanolic potassium carbonate. HPLC-monitoring of the latter deprotection showed, in full accordance with the observations made for dimer **7** and hexamer **5**, dramatic degradation of the desired heptamer **11** (Figure 2A). Even during the first few hours of the deprotection substantial amounts of side-products had been formed. These results clearly disqualify potassium carbonate/methanol as deprotection reagent for methylphosphonate-containing oligonucleotides.

In contrast, as expected, the deprotection of **10** in ammonia/methanol is not accompanied by cleavage of the methylphosphonate linkages, as was gauged by HPLC analysis (Figure 2B). After a deprotection time of 7 hours, which is sufficient to remove the AMB group from cytidine residues, the mixture was neutralized and further work-up was performed. A small volume of the ammonia/methanol deprotection mixture was kept apart in order to follow the stability of heptamer **11** in the course of time. This revealed no detectable degradation for at least 2 days. After a five days' treatment first traces of side-products were observed (Figure 2B; compare with K_2CO_3/CH_3OH: complete destruction of **11** in 5 days).

The favorable results of the ammonia/methanol treatment of AMB-protected heptamer **10** encouraged us to synthesize the partially methylphosphonate-modified DNA fragment **15** employing this deprotection procedure. To this end, AMB base-protected cyanoethyl phosphoramidites had to be prepared as building blocks for the introduction of phosphodiester linkages. This could be accomplished by phosphitylation of **2a–c** with chloro-(2-cyanoethoxy)-*N*,*N*-diisopropylamino-phosphine in the presence of *N*,*N*-diisopropylethylamine. The cyanoethyl phosphoramidites **12a–c** thus obtained[24] were combined with methylphosphonamidite **3a** in the solid support synthesis (10

Table 1. Deprotection times* of AMB-protected deoxynucleosides at 20°C

Compound	0.05 M K_2CO_3/CH_3OH $t_{1/2}$(min) #	t_∞(min) #	sat. NH_3/CH_3OH $t_{1/2}$(min)	t_∞(h)
DMTr-dC-AMB (**2a**)	4	40	55	7
DMTr-dA-AMB (**2b**)	10	80	75	10
DMTr-dG-AMB (**2c**)	5	45	125	16

*: as determined by TLC experiments ($CHCl_3/CH_3OH$ 9:1 v/v).
#: for charged oligomers longer deprotection times are required (see ref. 17,18).

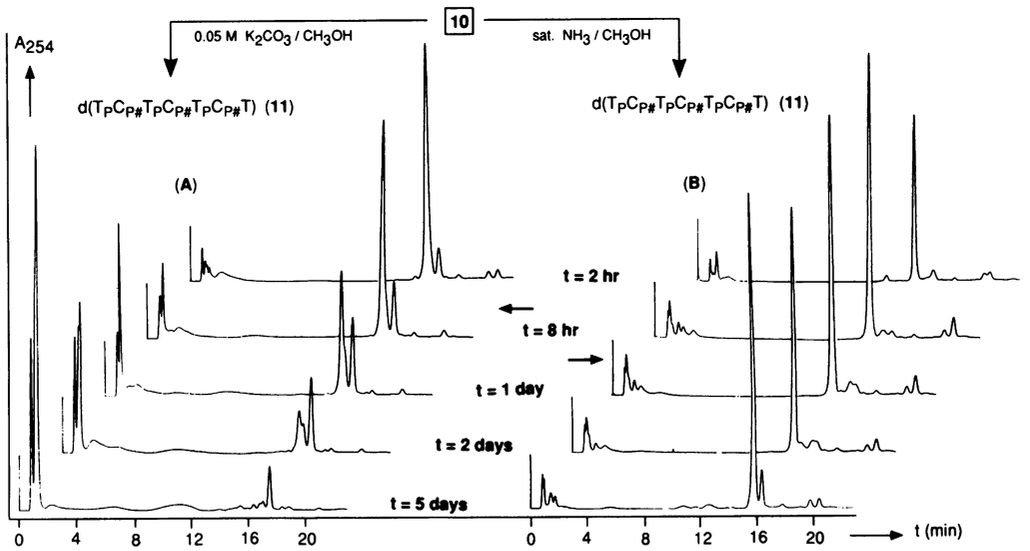
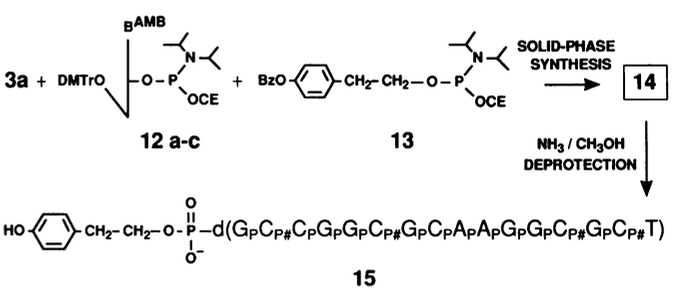


Figure 2. Anion-exchange HPLC (MonoQ) monitoring of the deprotection of **10** (10 μmol-scale) and the stability of the resulting heptamer d(TPCP # TPCP # TPCP # T) (**11**) in (**A**) 0.05 M potassium carbonate/methanol and (**B**) saturated ammonia/methanol. Arrows indicate moment of complete deprotection. P # denotes a methylphosphonate linkage.

μmol) of oligonucleotide **15** (Scheme 3).[25,26] After assembly of the nucleotide building blocks benzoyl-protected phosphoramidite **13** was coupled to the 5'-end of the solid-phase linked oligonucleotide.

The fully-protected oligonucleotide **14** was deprotected in ammonia/methanol for 18 hours at room temperature. In comparison with the deprotection of oligomers **4** and **10** a prolonged (overnight) treatment was performed since **14** contains several AMB-protected guanosine residues. HPLC analysis (MonoQ) of the end product revealed an elution pattern which is comparable with those found for conventional 10 μmol syntheses, *i.e.* a major product (**15**) preceded by some shorter fragments (Figure 3A). The crude oligonucleotide could be readily purified by Sephadex G-50 column chromatography. This afforded pure **15**, the homogeneity of which was established by HPLC anion-exchange chromatography (Figure 3B) and NMR spectroscopy.

The benefit of the AMB group for the synthesis of methylphosphonate containing oligonucleotides was confirmed by an additional experiment in which a small amount of the immobilized oligonucleotide **14** was subjected to the conditions applied for the deprotection of conventionally protected nucleobases, *i.e.* 2–3 days at 50°C. It is evident from Figure 3C that this deprotection protocol renders a much less defined oligonucleotide. Apparently, the prolonged ammonia/methanol treatment at elevated temperature has led to substantial degradation of the methylphosphonate linkages, resulting in various sequences of shorter length. This degradation is clearly reflected in a relatively lower amount of desired product in Figure 3C as compared with Figure 3A.



Scheme 3. Solid-phase synthesis of partially methylphosphonate-modified hexadecamer **15** using AMB base-protected amidites and an ammonia/methanol deprotection procedure. P # denotes a methylphosphonate linkage. AMB: 2-(Acetoxymethyl)benzoyl, Bz: Benzoyl, CE: 2-Cyanoethyl.

CONCLUDING REMARKS

The results described in this paper endorse the value of the AMB base-protecting group for the preparation of labile DNA modifications, here illustrated for the methylphosphonate analogue. Although potassium carbonate/methanol remains the reagent of choice for the deprotection of phosphate-methylated DNA analogues, it is not suitable for preparing methylphosphonate-modified oligonucleotides, due to the lability of the methylphosphonate linkage towards methanolic potassium carbonate. This susceptibility of the methylphosphonate diester to saponification not only calls for mild synthetic procedures for the preparation of methylphosphonated oligonucleotides but might even have implications for the *in vivo* stability of this type of DNA analogues.

As has been demonstrated in the deprotection of a partially methylphosphonate-modified heptamer, the AMB protective group can be fully removed in ammoniacal methanol without any significant degradation of the methylphosphonate linkages. The same deprotection conditions could be successfully applied to deblock an AMB-protected hexadecamer containing four methylphosphonates. On the other hand, it was found that a prolonged ammonia/methanol treatment results in extensive degradation of the methylphosphonate diesters. Future investigations should reveal whether the AMB group is also useful

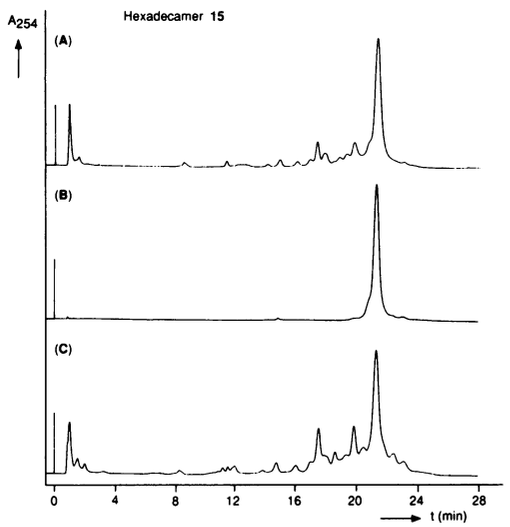


Figure 3. Anion-exchange HPLC (MonoQ) elution profiles of (**A**) crude hexadecamer **15** (10 μmol-scale) after deprotection in saturated ammonia/methanol for 18 hours at room temperature, (**B**) hexadecamer **15** after purification by Sephadex G-50 gelchromatography, and (**C**) deprotection mixture of hexadecamer **15** after prolonged exposure (72 h, 50°C) to saturated ammonia/methanol.

for the synthesis of completely methylphosphonated DNA fragments. In this respect, several other labile base-protecting groups (*e.g.* phenoxyacetyl[27] or other easily removable groups[28]) can be considered as well for improving the preparation of (fully) methylphosphonated oligonucleotides. A further evaluation of base-protecting groups and deprotection procedures should disclose the optimal combination to minimize methylphosphonate degradation.

EXPERIMENTAL

General methods and materials

Methanolic potassium carbonate was prepared as previously described[18]. Methanolic ammonia was prepared by passing ammonia gas over KOH through cooled (ice-water bath) anhydrous methanol (250 mL) until saturation.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM 200 or Bruker WM 360 spectrometer.

Fast atom bombardement (FAB) mass spectra were recorded on a Finnigan MAT 90 mass spectrometer equipped with a WATV Cs ion gun, using either glycerol or m-nitrobenzyl alcohol as the matrix.

High-performance liquid chromatographic (HPLC) analyses were conducted on a Waters 600E (system controller) single-pump gradient system. Ion-exchange chromatography was performed on a Pharmacia Mono Q HR5/5 column at a flow rate of 1.0 mL/min. Oligonucleotides were detected at 254 nm using a Waters model 484 variable wavelength UV detector. Elution conditions **I** (for **5** and **11**) and **II** (for **15**) were applied: (**I**): Buffer A 0.001 M NaH_2PO_4 (pH = 5.5, 20% acetonitrile v/v); Buffer B 0.001 M NaH_2PO_4 and 0.5 M NaCl (pH = 5.5, 20% acetonitrile v/v); gradient: 0–2 min isocratic 100% A, 2–26 min linear 0–80% B; (**II**): Buffer A 0.02 M NaH_2PO_4 (pH = 7.0, 25% acetonitrile v/v); Buffer B 0.02 M NaH_2PO_4 and 2.0 M NaCl (pH = 7.0, 25% acetonitrile v/v); gradient: 0–20 min linear 0–30% B, 20–30 min 30% B.

General procedure for the preparation of AMB-protected nucleosides 1

To a cooled (ice-water bath) suspension of 2′-deoxynucleoside (10 mmol) in dry pyridine (50 mL) was added trimethylsilyl chloride (6.5 mL, 50 mmol). The reaction mixture was stirred for 30 minutes, followed by the addition of 2-(acetoxymethyl)benzoic acid anhydride (6.2 g, 84% w/w, 14 mmol).[17] The solution was stirred for 5 hours at 60°C. Dichloromethane (500 mL) was added. The organic layer was washed with cold saturated aq. $NaHCO_3$ (250 mL) and cold water (250 mL) respectively. Dichloromethane was evaporated under reduced pressure. After cooling of the resulting solution with an ice-water bath, water (20 mL) was added. Hydrolysis of the trimethylsilyl groups was allowed to proceed overnight at 0°C. The reaction mixture was concentrated to dryness and coevaporated with pyridine, toluene and dichloromethane respectively. Further processing of the residue is elaborated for the individual compounds **1a–c**. ^1H NMR data on purified compounds **1** are presented in reference 17.

4-*N*-(2-(acetoxymethyl)benzoyl)-2′-deoxycytidine (1a)

The residue was dissolved in dichloromethane (75 mL) and added dropwise to hexane/diethyl ether (1:1 v/v, 750 mL). The precipitate was collected by filtration and dried *in vacuo* to yield **1a** as a white solid (6.8 g, 76%). R_f ($CHCl_3/CH_3OH$ 85:15 v/v): 0.38. FAB(+): 404.0 $(M+H)^+$. FAB(−): 401.9 $(M-H)^-$.

6-*N*-(2-(acetoxymethyl)benzoyl)-2′-deoxyadenosine (1b)

The residue was applied on a silica gel column and eluted with a mixture of dichloromethane and methanol (9 vol%). This procedure afforded **1b** (3.9 g, 46%) and di-substituted deoxyadenosine (3.0 g, 25%) as white solids. R_f ($CHCl_3/CH_3OH$ 85:15 v/v) (**1b**): 0.37. FAB(+): 428.1 $(M+H)^+$. FAB(−): 426.0 $(M-H)^-$. The di-substituted nucleoside (dA-$(AMB)_2$) can be easily converted into the mono-substituted compound **1b** by treatment with a 1.0 M solution of hydrazine-hydrate in pyridine/acetic acid (3:2 v/v) for 2 hours at 40°C.

2-*N*-(2-(acetoxymethyl)benzoyl)-2′-deoxyguanosine (1c)

The residue was dissolved in dichloromethane/methanol (4:1 v/v) (15 mL) and added dropwise to diethyl ether (250 mL). The precipitate was collected by filtration and dried to afford 3.8 g of a light brown solid. Repetition of the precipitation procedure yielded pure **1c** as a white solid (3.52 g, 85%). R_f ($CHCl_3/CH_3OH$ 85:15 v/v): 0.19. FAB(+): 444.1 $(M+H)^+$. FAB(−): 441.9 $(M-H)^-$.

General procedure for the synthesis of AMB-protected 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleosides 2

AMB-protected deoxynucleoside **1** (5.0 mmol) was dissolved in anhydrous pyridine (25 mL). To the solution was added 4,4′-dimethoxytrityl chloride (2.4 g, 7.0 mmol). The reaction mixture was stirred for 2 hours at room temperature. After the addition of methanol (5 mL), the reaction mixture was concentrated to a small volume and taken up in dichloromethane (100 mL). The solution was washed with saturated aq. $NaHCO_3$ (2×100 mL). The organic layer was dried on $MgSO_4$ and concentrated *in vacuo*. The residue was dissolved in dichloromethane (40 mL) and precipitated from petroleum ether 40–60 (400 mL). The precipitate was collected by filtration and dried. Further purification by silica gel column chromatograpy (eluent: see compounds) yielded pure **2a–c** as white solids.

5'-*O*-(4,4'-dimethoxytrityl)-4-*N*-(2-(acetoxymethyl)benzoyl)-2'-deoxycytidine (DMTr-dC-AMB) (2a)

Yield: 74% (after column chromatograpy using ethyl acetate containing 4 vol% methanol as eluent). R_f ($CHCl_3/CH_3OH$ 9:1 v/v): 0.47. FAB(+): 706.1 $(M+H)^+$. FAB(−): 704.0 $(M-H)^-$. ^1H NMR ($CDCl_3$): δ 8.30 (1H,d,H_6), 7.60 (1H,d,arom. AMB), 7.55−7.15 (13H,m,arom. AMB/DMTr and H_5), 6.86 (4H,d,arom. DMTr), 6.28 (1H,dd,$H_{1'}$), 5.34 (2H,s,CH_2 AMB), 4.52 (1H,m,$H_{3'}$), 4.16 (1H,m,$H_{4'}$), 3.79 (6H,s,OCH_3 DMTr), 3.46 (2H,m,$H_{5'}/H_{5''}$), 2.74 (1H,m,$H_{2'}$), 2.27 (1H,m,$H_{2''}$), 2.10 (3H,s,CH_3 AMB).

5'-*O*-(4,4'-dimethoxytrityl)-6-*N*-(2-(acetoxymethyl)benzoyl)-2'-deoxyadenosine (DMTr-dA-AMB) (2b)

Yield: 85% (after column chromatography using a gradient of methanol (3.5→5 vol%) in dichloromethane, containing 0.5 vol% pyridine). R_f ($CHCl_3/CH_3OH$ 9:1 v/v): 0.43. FAB(+): 730.1 $(M+H)^+$. FAB(−): 728.0 $(M-H)^-$. ^1H NMR ($CDCl_3$): δ 8.75 (1H,s,H_2) 8.13 (1H,s,H_8), 7.77 (1H,d,arom. AMB), 7.55−7.10 (12H,m,arom. AMB/DMTr), 6.80 (4H,d,arom. DMTr), 6.49 (1H,dd,$H_{1'}$), 5.41 (2H,s,CH_2 AMB), 4.72 (1H,m,$H_{3'}$), 4.15 (1H,m,$H_{4'}$), 3.78 (6H,s,OCH_3 DMTr), 3.42 (2H,m,$H_{5'}/H_{5''}$), 2.88 (1H,m,$H_{2'}$), 2.57 (1H,m,$H_{2''}$), 2.08 (3H,s,CH_3 AMB).

5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-(2-(acetoxyethyl)benzoyl)-2'-deoxyguanosine (DMTr-dG-AMB) (2c)

Yield: 85% (after column chromatography using a gradient of methanol (3→8 vol%) in dichloromethane containing 0.5 vol% pyridine). R_f ($CHCl_3/CH_3OH$ 9:1 v/v): 0.36. FAB(+): 746.1 $(M+H)^+$. FAB(−): 743.9 $(M-H)^-$. ^1H NMR ($CDCl_3$): δ 7.79 (1H,s,H_8), 7.50−7.05 (13H,m,arom. AMB/DMTr), 6.73 (4H,d,arom. DMTr), 6.22 (1H,dd,$H_{1'}$), 5.27 (2H,AB,CH_2 AMB), 4.72 (1H,m,$H_{3'}$), 4.09 (1H,m,$H_{4'}$), 3.72 (6H,s,OCH_3 DMTr), 3.33 (2H,m,$H_{5'}/H_{5''}$), 2.81 (1H,m,$H_{2'}$), 2.43 (1H,m,$H_{2''}$), 2.09 (3H,s,CH_3 AMB).

5'-*O*-(4,4'-dimethoxytrityl)-4-*N*-(2-(acetoxymethyl)benzoyl)-2'-deoxycytidine 3'-(*N*,*N*-diisopropyl)-methylphosphonamidite (3a)

To a solution of **2a** (0.81 g, 1.15 mmol), dried overnight *in vacuo*, in dry dichloromethane (5 mL) was added bis(*N*,*N*-diisopropylamino)methylphosphine (0.51 mL, 1.72 mmol)[29] and, subsequently, collidine.HCl (18 mg, 0.11 mmol). The reaction was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate (50 mL) and washed with cold saturated aq. $NaHCO_3$ (2×40 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was redissolved in dry toluene (5.0 mL) and added dropwise to stirred and cooled (−20°C) n-pentane (250 mL). The white precipitate was collected by filtration and further purified by flash chromatography on silica gel. Elution with ethyl acetate/hexane (65:35→80:20 v/v) containing 1 vol% triethylamine, gave, after evaporation of the appropriate fractions, pure amidite **3a** as mixture of diastereoisomers. R_f (ethyl acetate/dichloromethane/triethylamine 9:9:2 v/v/v): 0.66. Yield: 0.82 g (84%). ^{31}P NMR ($CDCl_3$): δ 122.8 and 121.5 ppm. ^1H NMR ($CDCl_3$) of a mixture of diastereoisomers: δ 8.59 (1H,bs,NH), 8.34 and 8.22 (1H,2×d,H_6), 7.65−7.23 (14H,m,arom. AMB/DMTr and H_5), 6.87 and 6.85 (4H,2×d,arom. DMTr), 6.31 and 6.25 (1H,2×dd,$H_{1'}$), 5.35 (2H,s,CH_2 AMB), 4.52 (1H,m,$H_{3'}$), 4.18 (1H,m,$H_{4'}$), 3.81 (6H,s,OCH_3 DMTr), 3.60−3.30 (4H,m,$H_{5'}/H_{5''}$ and 2×CH iPr), 2.72 (1H,m,$H_{2'}$), 2.25 (1H,m,$H_{2''}$), 2.11 (3H,s,CH_3 AMB), 1.23−1.01 (15H,m,P-CH_3 and CH_3 iPr).

Preparation of dimer 6 (DMTr-dC-AMB-P#-T-Lev)

To a solution of 3'-*O*-levulinoylthymidine[21] (0.12 g, 0.35 mmol) in anhydrous acetonitrile (5 mL) were added, respectively, 1*H*-tetrazole (0.14 g, 2.0 mmol) and a solution of phosponamidite **3a** (0.20 g, 0.23 mmol), dried overnight *in vacuo*, in dichloromethane (2.5 mL). After stirring for 45 min at room temperature, *tert*.-butyl hydroperoxide (0.5 mL) was added and the reaction mixture was stirred for another 15 min. The solution was diluted with ethyl acetate (50 mL) and washed successively with saturated aq. $NaHCO_3$ (30 mL), 10% aq. $Na_2S_2O_3$ (30 mL), and saturated aq. NaCl (30 mL). The organic layer was dried ($MgSO_4$), and concentrated under reduced pressure. The crude dimer was purified by silica gel column chromatography using a gradient of methanol (0→10 vol%) in ethyl acetate containing 0.5 vol% triethylamine. This afforded pure **6** (0.13 g, 52% based on **3a**) as a white solid. [A substantial amount of nucleoside **2a** (35% based on **3a**) was isolated as by-product.] R_f (CH_2Cl_2/CH_3OH 85:15 v/v): 0.33.

^{31}P NMR ($CDCl_3$): δ 32.4 and 32.0 ppm. ^1H NMR ($CDCl_3$) of a mixture of diastereoisomers: δ 8.75 (1H,d,H_6(C)), 7.64 (1H,m,arom. AMB), 7.58−7.20 (14H,m,arom. AMB/DMTr, H_6(T) and H_5(C)), 6.87 (4H,d,arom. DMTr), 6.29 (2H,m,2×$H_{1'}$), 5.36 and 5.35 (2H,2×s,CH_2 AMB), 5.30−5.10 (2H,m,2×$H_{3'}$), 4.38−3.92 (4H,m,2×$H_{4'}$ and $H_{5'}/H_{5''}$(T)), 3.81 (6H,s,OCH_3 DMTr), 3.47 (2H,m,$H_{5'}/H_{5''}$(C)), 2.95−2.05 (4H,m,2×$H_{2'}/H_{2''}$), 2.77 (2H,t,CH_2 Lev), 2.57 (2H,t,CH_2 Lev), 2.18 (3H,s,CH_3 Lev), 2.12 (3H,s,CH_3 AMB), 1.92 and 1.89 (3H,2×s,CH_3(T)), 1.56 and 1.47 (3H,2×d,P-CH_3).

Deprotection of dimer 6 and stability of dimer 7 in potassium carbonate/methanol

Dimer **6** (25 mg, 23 μmol), dried overnight *in vacuo*, was dissolved in a freshly prepared 0.05 M solution of anhydrous K_2CO_3 in dry methanol (3.0 mL). The deprotection of **6** and the stability of the resulting dimer **7** were monitored by TLC analysis. R_f (dimer **7**) (CH_2Cl_2/CH_3OH 85:15 v/v): 0.32 and 0.29.

According to TLC analysis, after 6 days at room temperature dimer **7** was completely converted into two degradation products (**8** and **9**). Both degradation products were chromatographed by using, respectively, a gradient of methanol (5→15%) in dichloromethane to obtain pure **8**, and in a second column a gradient of methanol (5→10%) in ethyl acetate to give homogeneous **9**.

R_f (**8**) (CH_2Cl_2/CH_3OH 85:15 v/v): 0.47, R_f (**8**) (ethyl acetate/CH_3OH 9:1 v/v): 0.16. FAB(+) (**8**): 530.0 $(M+H)^+$. FAB(−) (**8**): 528.0 $(M-H)^-$. ^1H NMR ($CDCl_3/CD_3OD$ 1:1 v/v) (**8**): δ 7.94 (1H,d,H_6(C)), 7.46−7.22 (9H,m,arom. DMTr), 6.86 (4H,d,arom. DMTr), 6.25 (1H,m,$H_{1'}$), 5.56 (1H,d,H_5(C)), 4.49 (1H,m,$H_{3'}$), 4.05 (1H,m,$H_{4'}$), 3.81 (6H,s,OCH_3 DMTr), 3.43 (2H,m,$H_{5'}/H_{5''}$), 2.50 (1H,m,$H_{2'}$), 2.20 (1H,m,$H_{2''}$).

R_f (**9**) (CH_2Cl_2/CH_3OH 85:15 v/v): 0.28, R_f (**9**) (ethyl acetate/CH_3OH 9:1 v/v): 0.27. FAB(+) (**9**): 243.0 $(M+H)^+$. FAB(−) (**9**): 241.0 $(M-H)^-$. ^1H NMR ($CDCl_3/CD_3OD$ 1:1 v/v) (**9**): δ 7.80 (1H,s,H_6(T)), 6.30 (1H,m,$H_{1'}$), 4.43 (1H,m,$H_{3'}$), 3.95 (1H,m,$H_{4'}$), 3.80 (2H,m,$H_{5'}/H_{5''}$), 2.31 (1H,m,$H_{2'}$), 2.20 (1H,m,$H_{2''}$), 1.91 (3H,s,CH_3(T)).

Compound **8** was found to be identical with the nucleoside obtained after AMB deprotection of **2a**.

Compound **9** was indistinguishable from thymidine (Sigma) both in NMR- ($CDCl_3/CD_3OD$ 1:1 v/v), FAB MS-, and TLC analysis.

Deprotection of dimer 6 and stability of dimer 7 in saturated ammonia/methanol

Dimer **6** (4 mg, 3.5 μmol), dried overnight *in vacuo*, was dissolved in saturated ammonia solution in dry methanol (0.5 mL). Cleavage of the levulinoyl ester and AMB group were monitored by TLC (CH_2Cl_2/CH_3OH 85:15 v/v). Saponification of the Lev ester was complete in 2 hours affording DMTr-dC-AMB-P#-T (R_f: 0.62 and 0.59). AMB deprotection took 6 hours resulting in dimer **7** (R_f: 0.32 and 0.29). The stability of **7** was further monitored for 10 days.

General procedure for the synthesis of AMB-protected cyanoethyl phosphoramidites 12

AMB-protected 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleoside **2** (1.0 mmol), dried overnight *in vacuo*, was dissolved in dry dichloromethane (5.0 mL). After the addition of *N,N*- diisopropylethylamine (0.7 mL, 3.0 mmol) the solution was cooled with an ice-water bath. Chloro-(2-cyanoethoxy)-*N,N*-diisopropylamino-phosphine (0.33 mL, 1.5 mmol) was added in a dropwise manner. The reaction mixture was allowed to warm to room temperature and stirred for 30 minutes. The reaction was quenched by the addition of methanol (0.25 mL) and the mixture was diluted with ethyl acetate (30 mL). The organic phase was washed with saturated aq. $NaHCO_3$ (3×25 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The residue was dissolved in dry toluene (5.0 mL) and added dropwise to cooled (−20°C) and stirred n-hexane (250 mL). The resulting white precipitate, isolated by filtration, was further purified by silica gel flash column chromatography (eluent: see compounds), yielding pure amidites **12a−c** as white solids.

5′-*O*-(4,4′-dimethoxytrityl)-4-*N*-(2-(acetoxymethyl)benzoyl)-2′-deoxycytidine 3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (12a)

Yield: 80% (after column chromatography using dichloromethane containing 5 vol% pyridine as eluent). R_f ($CHCl_3/CH_3OH$ 95:5 v/v): 0.62 and 0.53. ^{31}P NMR ($CDCl_3$): δ 150.0 and 149.4 ppm.

5′-*O*-(4,4′-dimethoxytrityl)-6-*N*-(2-(acetoxymethyl)benzoyl)-2′-deoxyadenosine 3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (12b)

Yield: 82% (after column chromatography using ethyl acetate containing 0.5 vol% triethylamine as eluent). R_f (ethyl acetate/0.5 vol% triethylamine): 0.53 and 0.43. R_f (ethyl acetate/dichloromethane/triethylamine 9:9:2 v/v/v): 0.61 and 0.54. ^{31}P NMR ($CDCl_3$): δ 149.2 and 149.1 ppm.

5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-(2-(acetoxymethyl)benzoyl)-2′-deoxyguanosine 3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (12c)

Yield: 73% (after column chromatography using a gradient of methanol (0→5%) in ethyl acetate containing 0.5 vol% triethylamine as eluent). R_f (CH_2Cl_2/CH_3OH 95:5 v/v): 0.58. ^{31}P NMR ($CDCl_3$): δ 148.9 and 148.6 ppm.

2-(p-(benzoyloxy)phenyl)ethyl-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (13)

2-(p-Hydroxyphenyl)-ethanol (0.68 g, 5.0 mmol) was added to a 1.0 N NaOH solution in water (5.0 mL; 1 eq.) and additional water was added to give a clear solution. After 5 minutes at room temperature, the solution was evaporated to dryness under reduced pressure. The residue was coevaporated with methanol (twice) and acetonitrile (three times) and, finally, redissolved in acetonitrile/dichloromethane (4:1 v/v; 25 mL). Following the addition of benzoyl chloride (0.65 mL; 5.5 mmol) the solution was stirred for 1 hour at room temperature. The reaction mixture was concentrated to a small volume and diluted with dichloromethane (50 mL). The organic phase was washed with saturated aq. $NaHCO_3$ (2×40 mL), dried ($MgSO_4$) and evaporated to dryness. The resulting solid was purified by column chromatography on silica gel, using a gradient of methanol (0→4 vol%) in dichloromethane as eluent, yielding 2-(p-benzoyloxyphenyl)-ethanol as a white solid (1.10 g, 92%). The latter compound (0.73 g, 3.0 mmol) was phosphitylated as described for phosphoramidites **12**. Compound **13** was purified by flash chromatography (eluent dichloromethane/ethyl acetate 1:1 v/v containing 1 vol% triethylamine) yielding pure amidite as a clear oil (1.18 g, 89%).

^{31}P NMR ($CDCl_3$): δ 147.7 ppm. ^1H NMR ($CDCl_3$): δ 8.20 (2H,m,Bz), 7.68−7.45 (3H,m,Bz), 7.29 (2H,d,Ph), 7.13 (2H,d,Ph), 3.86 (2H,m,CH iPr), 3.74 (2H,m,CH_2), 3.59 (2H,m,CH_2 CE), 2.95 (2H,t,CH_2-Ph), 2.58 (2H,t,CH_2 CE), 1.18 (12H,2×d,CH_3 iPr).

Solid-phase synthesis of immobilized fully protected oligomers 4, 10 and 14

The solid support syntheses of oligonucleotides **4**, **10**, and **14** were performed on a fully automated DNA synthesizer (Applied Biosystems, Model 381A), using the appropriate amidites (**3a**, **12a−c**, **13**) together with commercially available 5′-*O*-(4,4′-dimethoxytrityl)-thymidine 3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (Applied Biosystems). The amidites were dried overnight *in vacuo*, dissolved in anhydrous acetonitrile (ABI, DNA synthesis grade) to give 0.1 M solutions and, finally, filtered through a 0.45 μm filter before use. Controlled pore glass (CPG), loaded with 5′-*O*-(4,4′-dimethoxytrityl)-thymidine (1 or 10 μmol; ABI), was used as solid support. Elongation cycles were performed according to the manufacturer's suggested protocols[20] on 1 μmol (for **4**) or 10 μmol (for **10** and **14**) scale.

During the final synthetic cycle in the assembly of oligomer **14** additional phosphoramidite **13** was passed through the column for 1 min and another 2 min was waited before performance of the oxidation step. No capping was performed in this cycle.

After completion of the solid-phase syntheses, the columns were dried *in vacuo* (oil-pump) for 30 min. Finally, the solid supports were removed from the column under nitrogen and the deprotection procedures were carried out.

Deprotection of oligomers 4 and 10 in potassium carbonate/methanol

To solid support, containing 1 μmol of immobilized fully protected oligonucleotide (**3** or **9**), was added freshly prepared 0.05 M potassium carbonate/methanol (2.5 mL). After a deprotection time of 4 hours at room temperature, the reaction mixture was neutralized by carefully adding acetic acid (5 vol%) to a final pH of 6. The solid support was filtered off and washed

with methanol/water (1:1 v/v; 2×5 mL) and water (5 mL). The filtrate was concentrated to a smaller volume (5 mL) and washed with diethyl ether (5×2 mL). The aqueous layer was further concentrated to a small volume (0.5 mL; pH = 6–7). The stability of heptamer **11** was checked by keeping apart a small amount (0.5 mL) of deprotection mixture before work-up.

Deprotection of oligomers 10 and 14 in ammonia/methanol

Solid support, corresponding with 8 μmol of immobilized oligonucleotide (**10** or **14**) was treated with saturated ammonia solution in dry methanol (25 mL) for 18 hours at room temperature. The mixture was concentrated under reduced pressure and coevaporated once with dry methanol. After the addition of water (15 mL) the solid support was removed by filtration and washed with methanol/water (1:1 v/v) (for **10**) or water (for **14**) (3×5 mL). The aqueous phase was concentrated to a smaller volume (15 mL), washed with diethyl ether (4×15 mL), and concentrated to a small volume (2 mL). For HPLC monitoring of the stability of **11** part of the deprotection mixture of **10** (1.5 mL) was kept apart before work-up.

An additonal amount of immobilized **14** (0.5 μmol) was subjected to saturated ammonia/methanol (2 mL) at 50°C in a sealed flask. After 72 hours the solution was evaporated under reduced pressure, coevaporated with dry methanol and dissolved in water (5 mL).

Purification of oligomers 11 and 15

The crude unprotected oligonucleotides obtained above were purified by Sephadex G-50 chromatography (column 180 cm× 5 cm^2). Elution was performed with 0.05 M triethylammonium acetate (pH = 5.5) at a flow rate of 17 mL/h. The appropriate fractions, as analyzed by MonoQ HPLC, were pooled and lyophilized. The oligonucleotides were brought into the Na^+ form by passing them through a column (1 cm×10 cm) of Dowex 50W X8 cation-exchange resin (100–200 mesh, Na^+ form). The resulting UV-positive fractions were pooled, concentrated to a small volume and lyophilized.

Hexamer **5** : Rt 14.7 min (MonoQ, system I). Not isolated.

Heptamer **11**: Rt 16.8 min (monoQ, system I). ^{31}P NMR (D_2O): δ 36.15 and −0.55 ppm (ratio 1:1). ^1H NMR (D_2O) of a mixture of diastereoisomers: δ 7.84–7.74 (3H,m,3×H_6(C)), 7.60 (1H,s,H_6(T)), 7.50 (1H,s,H_6(T)), 7.48 (2H,s,2×H_6(T)), 6.31–6.19 (7H,m,7×$H_{1'}$), 6.06–5.97 (3H,m,3×H_5(C)), 5.14 (3H,m,3×$H_{3'}$), 4.78 (2H,m,2×$H_{3'}$), 4.55 (1H,m,$H_{3'}$), 4.42–4.26 (10H,m,7×$H_{4'}$ and 3×$H_{5',5''}$), 4.20–4.05 (9H,m,9×$H_{5',5''}$) 3.47 (2H,m,$H_{5'}$/$H_{5''}$(5'-T)), 2.66–2.47 (7H,m,7×$H_{2'}$) 2.43–2.28 (7H,m,7×$H_{2'}$), 1.87 (12H,s,4×CH_3(T)), 1.73 (9H,2×d,P-CH_3; J_{P-H}= 17.5 Hz).

Hexadecamer **15**: Rt 21.4 min (MonoQ, system II). ^{31}P NMR (D_2O): δ 37.07, 36.05, 35.65 and 0.43, −0.77, −0.92, −1.76 ppm (ratio P(CH_3)/P(O^-) 1:3). ^1H NMR (D_2O): δ 6.86 (d,arom. Ph) and 6.62 (d,arom. Ph): p-hydroxyphenyl present; No residual AMB resonances.

ACKNOWLEDGEMENTS

The authors wish to thank Ir J.Huskens for his help with the preparation of the nucleoside building blocks and Mr G.N.Wagenaars (Organon analytical R&D laboratories) for recording the NMR spectra.

REFERENCES

1. Kuijpers,W.H.A., Bos,E.S., Kaspersen,F.M., Veeneman,G.H. and van Boeckel,C.A.A. (1993) *Bioconjugate Chem.*, **4**, 94–102
2. Uhlmann,E. and Peyman,A. (1990) *Chem. Rev.*, **90**, 544–584.
3. Miller,P.S. (1989) In Cohen,J.S. (ed.), Oligonucleotides: Antisense inhibitors of gene expression. Macmillan Press, London, pp 79–95.
4. Miller,P.S. and Ts'o,P.O.P. (1987) *Anti-Cancer Drug Design*, **2**, 117–128.
5. Sarin,P.S., Agrawal,S., Civeira,M.P., Goodchild,J., Ikeuchi,T. and Zamechnik,P.C. (1988) *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 7448–7451.
6. Miller,P.S., Yano,J., Yano,E., Carroll,C., Jayaraman,K. and Ts'o,P.O.P. (1979) *Biochemistry*, **18**, 5134–5143.
7. Miller,P.S., Reddy,M.P., Murakami,A., Blake,K.R., Lin,S. and Agris,C.H. (1986) *Biochemistry*, **25**, 5092–5097.
8. Miller,P.S., Agris,C.H., Murakami,A., Reddy,P.M., Spitz,S.A. and Ts'o,P.O.P. (1983) *Nucleic Acids Res.*, **11**, 6225–6242.
9. Dorman,M.A., Noble,S.A., McBride,L.J. and Caruthers,M.H. (1984) *Tetrahedron*, **40**, 95-102.
10. Jäger,A. and Engels,J. (1984) *Tetrahedron Lett.*, **25**, 1437–1440.
11. Agrawal,S. and Goodchild,J. (1987) *Tetrahedron Lett.*, **28**, 3539–3542.
12. Sinha,N.D., Grossbruchhaus,V. and Koster,H. (1983) *Tetrahedron Lett.*, **24**, 877–880.
13. Miller,P.S., Cushman,C.D. and Levis,J.T. (1991) In Eckstein,F. (ed.), Oligonucleotides and Analogues, A Practical Approach. IRL Press, Oxford, pp 137–154.
14. Marugg,J.E., de Vroom,E., Dreef,C.E., van der Marel,G.A. and van Boom,J.H. (1986) *Nucleic Acids Res.*, **14**, 2171–2185.
15. Roelen,H.C.P.F., van den Elst,H., van der Marel,G.A. and van Boom,J.H. (1992) *Tetrahedron Lett.*, **33**, 2357–2360.
16. Hogrefe,R.I., Vaghefi,M.M., Reynolds,M.A., Young,K.M., and Arnold,L.J.,Jr. (1993) *Nucleic Acids Res.*, **21**, 2031–2038.
17. Kuijpers,W.H.A., Huskens,J. and van Boeckel,C.A.A. (1990) *Tetrahedron Lett.*, **31**, 6729-6732.
18. Kuijpers,W.H.A., Huskens,J., Koole,L.H. and van Boeckel,C.A.A. (1990) *Nucleic Acids Res.*, **18**, 5197–5205.
19. Wilbur,D.S. (1992) *Bioconjugate Chem.*, **3**, 433–470.
20. Applied Biosystems DNA synthesizer Model 381 (Version 1.50 Software Cartridge) User Bulletin Issue No. **56**, April 1990.
21. Hassner,A., Strand,G., Rubinstein,M. and Patchornik,A. (1975) *J. Am. Chem. Soc.*, **97**, 1614–1615.
22. For a methylphosphonothioate diester positioned at the 3'-end of a thymidine nucleoside, degradation has been reported to proceed according to a different mechanism, involving formation of a 2,3'-anhydronucleoside (see ref. 23). However, no products were found which indicate degradation of **7** according to this 'anhydro'-mechanism.
23. Caruthers,M.H., Brill,W. and Dellinger,D.J. (1987) *Phosphorus and Sulfur*, **30**, 549–553.
24. AMB-protected amidites **12a–c** were found to be as stable as the conventional cyanoethyl phosphoramidites, both in acetonitrile solution and on storage as solids.
25. In contrast to our earlier sequences (see ref. 1), this oligonucleotide contains only one methylphosphonate linkage directly at the 3'-end. Recently it has been reported that one 3'-methylphosphonate modification is as effective against exonucleases as two adjacent methylphosphonates (see ref. 26).
26. Quartin,R.S., Brakel,C.L. and Wetmur,J.G. (1989) *Nucleic Acids Res.*, **17**, 7253–7262.
27. Schulhof,J.C., Molko,D. and Téoule,R. (1987) *Nucleic Acids Res.*, **15**, 71–74.
28. For a recent review on base-protective groups see: Beaucage,S.L. and Iyer,R.P. (1992) *Tetrahedron*, **48**, 2223–2311.
29. Maier,L. (1963) *Helv. Chim. Acta*, **46**, 2667–2676.