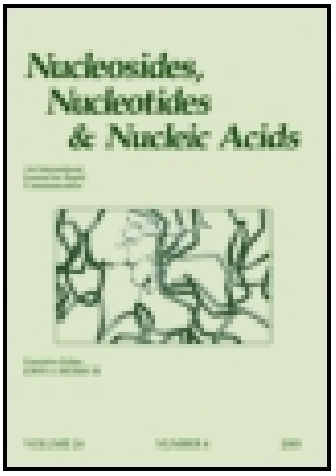

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Use of NPE-Protecting Groups for the Preparation of Oligonucleotides Without Using Nucleophiles During the Final Deprotection

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USE OF NPE-PROTECTING GROUPS FOR THE PREPARATION OF OLIGONUCLEOTIDES WITHOUT USING NUCLEOPHILES DURING THE FINAL DEPROTECTION.

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Abstract: The preparation of O-(4-nitrophenyl)ethyl phosphoramidites and H-phosphonate derivatives of Npe protected nucleosides is described together with the use of these products to prepare oligodeoxynucleotides without using nucleophiles during the final deprotection.

There is a growing interest on modified oligonucleotides because of their important biological properties. This interest has led to the development of methodology in order to fulfil the demand of modified oligonucleotides[1,2]. The standard (Bzl, ibu) protecting groups have been succesful to solve a large part of the synthetic problems encountered during the preparation of these modified oligonucleotides[2] except for some special cases including sensitive base analogues, digoxigenin, alkylating agents and so on . In these cases the sensitive molecule is introduced, if possible, after oligonucleotide deprotection[1,2].

The use of more ammonia labile protecting groups such as phenoxyacetyl (Pac)[3], *tert*-butylphenoxyacetyl (Tac, Expedite)[4], formamidine (FOD)[5], isopropoxyacetyl[6,7], and 2-(acetoxymethyl)benzoyl (Amb)[8] groups has been showed to solve some of the problems related to the sensitivity of some modified oligonucleotides to ammonia but, there is still a need for alternative methods to prepare modified oligonucleotides containing ammonia-sensitive functions.

Among the protecting groups described in nucleoside chemistry that are cleaved in mild (non-nucleophilic) conditions three types have been tested in oligonucleotide synthesis : the allyl type that are removed using Palladium compounds in very mild

conditions[9], the 2-(*tert*-butyldiphenylsilyloxymethyl)benzoyl (SiOMB)[10] that are removed with fluoride and the ß-elimination type (Fmoc[11], Npeoc[12]) that are removed with bases preferentially strong non-nucleophilic bases in aprotic solvents. We have focussed on the (*p*-nitrophenyl)ethyl (Npe and Npeoc) protecting groups developped by the group of Dr. Pfleiderer[12-15]. In a previous communication we have described the preparation of short oligonucleotides using non-nucleophilic deprotection conditions[16]. Unfortunately, base alkylation due to the cyanoethyl phosphate protecting group was detected as side-reaction preventing the use of this method for oligonucleotides longer than 10 bases. In the present comunication we describe the search for better phosphate protecting goups and the use of scavengers in order to reduce base alkylation during the deprotection. The optimized protocol described here allows the preparation of longer and cleaner oligonucleotides using deprotection conditions compatible with ammonia-sensitive products.

RESULTS AND DISCUSSION

Preparation of Npeoc, Npe-protected nucleoside Npe phosphoramidites and H-phosphonates. During our previous work[16] it was clear that the acrylonitrile generated in the deprotection of 2-cyanoethyl phosphates was the main cause of base modification during the DBU deprotection. On the other hand *p*-nitrostyrene arising from Npe and Npeoc groups did not cause base alkylation. So, the first obvious modification was to replace the 2-cyanoethyl group for (*p*-nitrophenyl)ethyl as phosphate protecting group. The preparation of (*p*-nitrophenyl)ethyl phosphoramidites has been described using either the corresponding N,N,N',N'-tetraalkyl-[(*p*-nitrophenyl)ethyl] phosphoro-diamidite[17] (**1**, FIG. 1) or the appropiate chloro-N,N-dialkyl-O-[(*p*-nitrophenyl)ethyl] phosphoramidite[14,18-20]. Compounds **2**a-d (FIG. 1) were synthesized from the corresponding 5'-DMT-protected nucleoside, the appropiate phosphorodiamidite[17] (**1**) and tetrazole as activating agent. The resulting deoxynucleoside 3'-O-(*p*-nitrophenyl)ethyl phosphoramidites were purified on silica gel in moderate yields (55-67%).

An alternative solution was to use H-phosphonate chemistry because no phosphate protection was needed. So, deoxynucleoside 3'-H-phosphonates (**3**a-d, FIG. 1) were synthesized from the corresponding 5'-DMT-protected nucleoside and tris (1,2,4-triazolyl) phosphite[21]. The resulting products were purified in good yields (68-80%).

Solid-phase synthesis of oligonucleotides using compounds 2a-d and 3a-d. Heptamers A and B (see TABLE 1) were prepared using Npe phosphoramidites (2a-d) and H-phosphonates (**3**a-d). The first Npeoc-protected nucleoside was linked to solid

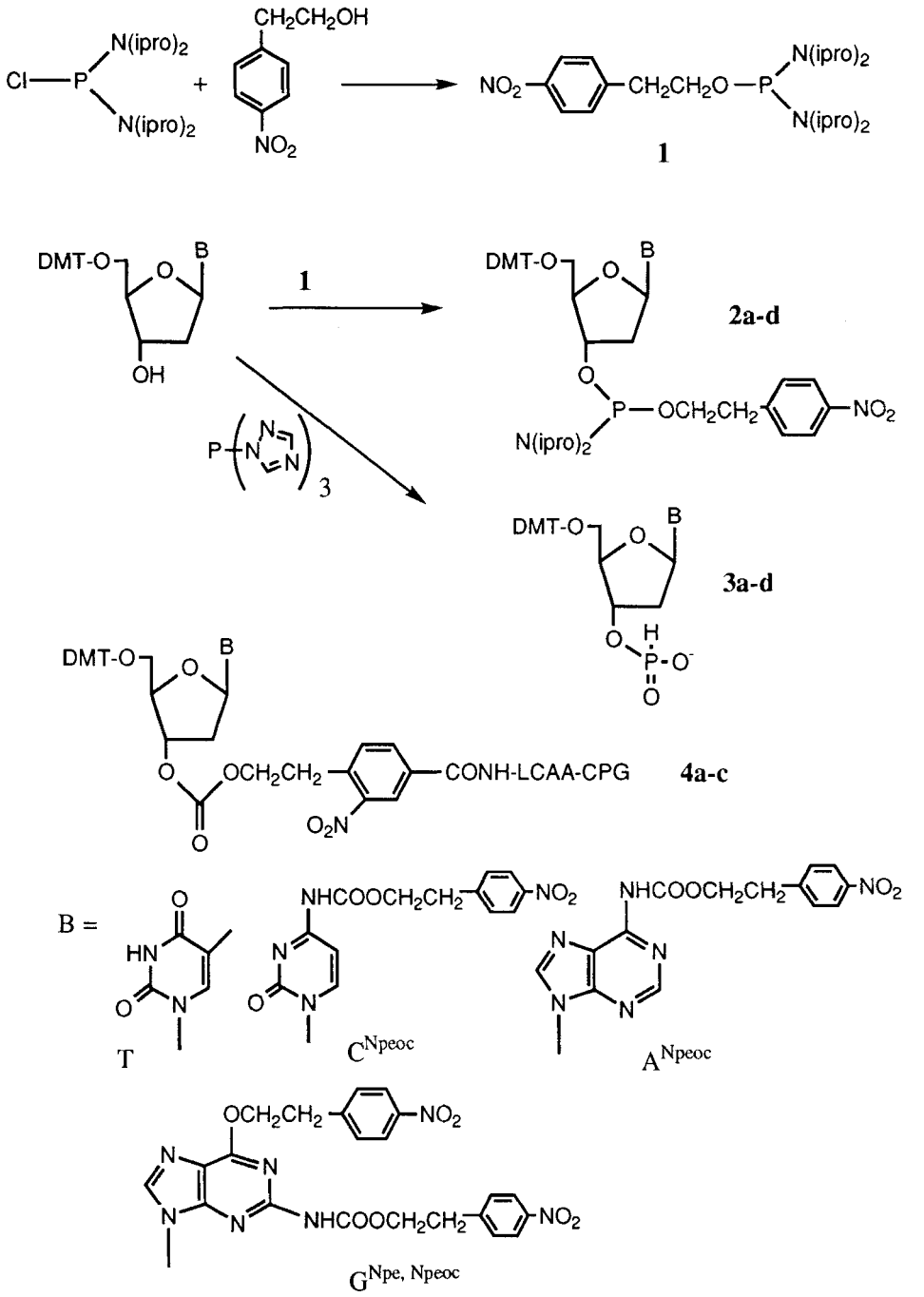

Figure 1 : Preparation of Npe, Npeoc-protected derivatives used in this work.

TABLE 1 : Oligonucleotide sequences prepared throughout this work.

SEQUENCE	Coupling units	Deprotection with Thy
A) 5' TTTCCTC 3' 7mer	H-phosphonate, Npe-amidites	No
B) 5' CAGACGT 3' 7mer	H-phosphonate, Npe-amidites	No
C) 5' CATGACGT 3' 8mer	Npe-amidites	Yes and No
D) 5' TAGCGAGTAC 3' 10mer	Npe-amidites	Yes and No
E) 5' CTGTCGAGCCGCGTAC 3' 16mer	Npe-amidites	Yes and No

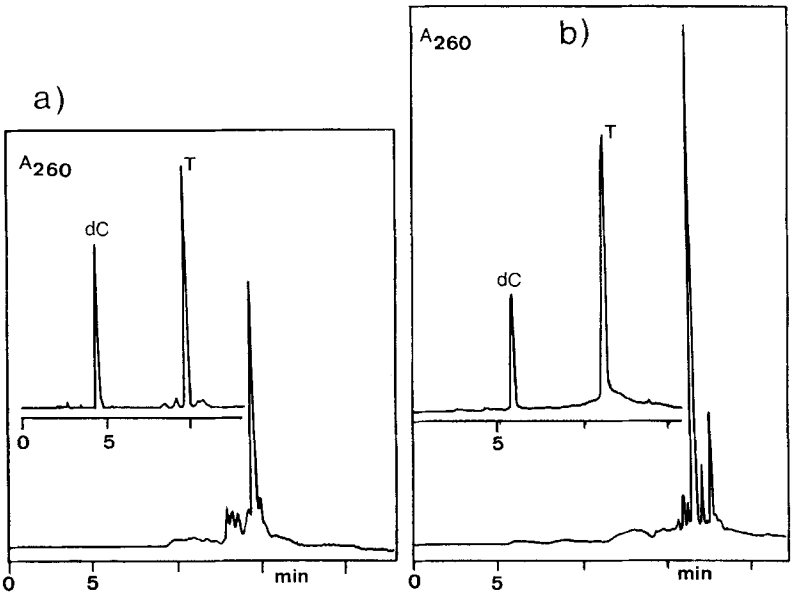

FIGURE 2. HPLC profile obtained after synthesis and DBU deprotection of heptamer A. a) using DMT, Npeoc-protected nucleoside H-phosphonates and b) using DMT,Npeoc-protected nucleoside Npe-phosphoramidites. The insert shows the enzymatic digestion of the main products.

support (CPG) through a base-labile (*o*-nitrophenyl)ethyl carbonate bond[16] (**4a-c**, FIG. 1). After the assembly of the sequences, the last DMT group was removed and solid supports were treated overnight with 0.5 M DBU/ *anhydrous* pyridine at room temperature. The resulting products were analyzed by analytical HPLC (FIG. 2).

As seen in FIGURE 2, a major peak was obtained using either H-phosphonates or Npe-phosphoramidites. The main products had the correct nucleoside composition as

shown by HPLC after snake venom phosphodiesterase and alkaline phosphatase digestion. Small differences were observed on the nature of the side-products. Syntheses that were made with H-phosphonates contained some peaks eluting before the main product (FIG. 2a). These products were shorter sequences and they are present due to a lower coupling efficiency observed during the manual assembly of the sequences. Syntheses that were made with Npe-phosphoramidites contained some peaks eluting after the main product (FIG. 2b). These peaks were similar to the peaks observed when 2-cyanoethyl groups were used for phosphate protection[16] and they were assigned to 4-nitrostyrene alkylation side-products. The amount of these side-products was higher when Npe phosphoramidites were used compared with the use of H-phosphonates but in both cases they were very low compared with the amount obtained when 2-cyanoethyl phosphoramidites were used[16]. Although some alkylation products in the DBU deprotection were observed, the replacement of the 2-cyanoethyl group by H-phosphonate or p-nitrophenylethyl groups has clearly reduced the formation of these side products.

In addition to change phosphate protection, we studied the addition of scavengers to the deprotection solution. Thiols have been used to avoid alkylation reaction during DBU deprotection[21] but they slow down the deprotection reaction and the thiolates are nucleophilic so they can react with ammonia-sensitive products. We thought that a nucleic acid base would be most convenient as scanvenger. We selected thymine among other bases because of its better solubility in the deprotection solutions and because previous results show us that is the most sensitive base to alkylation[16].

Sequences C (8 bases), D (16 bases) and E (10 bases) (see TABLE 1) were prepared on 1 μmol scale using Npe-phoshoramidites. The DMT group of the last phosphoramidite was left on during deprotection of sequence E to facilitate HPLC purification. Half of the solid supports were treated overnight with 0.5 M DBU / *anhydrous* pyridine and the other half with the same solution containing 5 mg of thymine (0.04 mmol). The resulting solutions were neutralized, concentrated and desalted on Sephadex G-10. Analysis of the products by analytical HPLC showed that addition of thymine during the deprotection had two beneficial effects. First, as shown in FIGURES 3 and 4, the amount of side products eluting after the main product was reduced. And second, the total amount of the desired product obtained after purification increased significantly (overall yields were 10-15% with thymine and 2-5% without thymine).

The increase on the yield could not be explained only in terms of reducing the formation of side products but also of a more efficient cleavage. Aliquots of DMT-nucleoside and DMT-dinucleotide-Npe-solid supports were treated at different times with

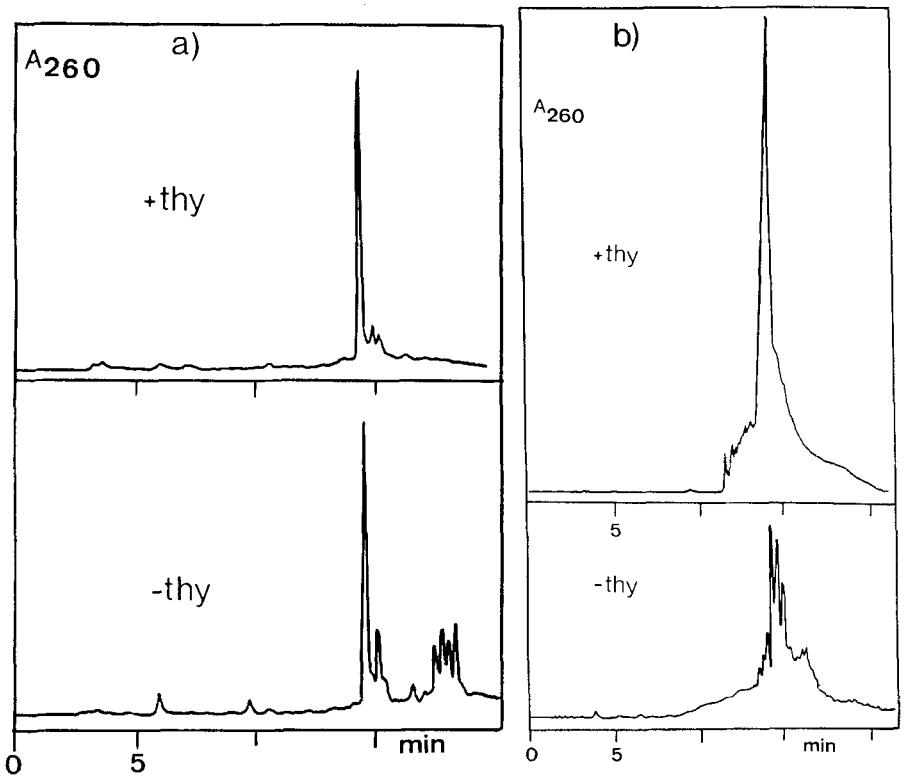

FIGURE 3. HPLC profile obtained after synthesis and DBU deprotection of a) octamer C and b) hexadecamer E using DMT, Npeoc, Npe-protected nucleoside Npe-phosphoramidites. The upper part shows the chromatogram obtained when thymine is added as scavenger during DBU deprotection. The lower part shows the chromatogram obtained without thymine.

0.5M DBU / *anhydrous* pyridine with and without thymine and cleavage was determined by quantitation of DMT groups in the filtrates compared with the amount present on the support (data not shown). Some differences were observed on the DMT-dinucleotide-Npe-supports, especially on DMT-TT-Npe-support where a 50% reduction on the cleavage yield was observed without thymine. These data suggest that the polymeric 2-nitrostyrene derivative formed during the cleavage can react to a certain extent with the released oligonucleotide and the presence of free thymine on the deprotection solution prevents this reaction.

In conclusion we have shown that the use of either H-phosphonate or Npe-phosphoramidite derivatives are useful to reduce base alkylation, a side-reaction previously

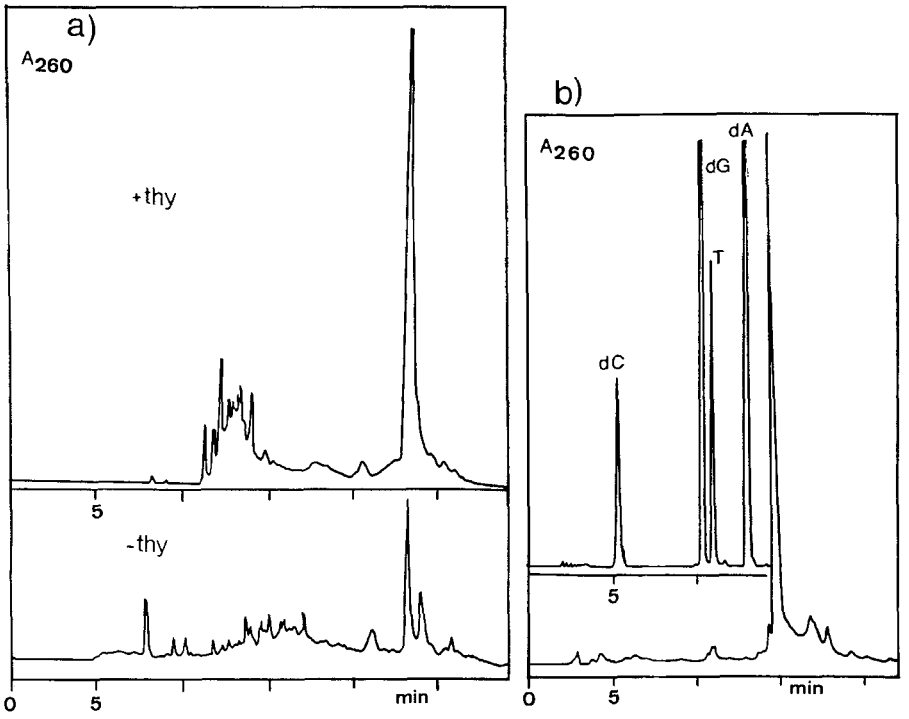

FIGURE 4. HPLC purification of decamer D after synthesis (DMT on) using DMT, Npe, Npeoc-protected nucleoside Npe-phosphoramidites. a) HPLC profile of DMT-decamer obtained when thymine is present during DBU deprotection (upper part) and when thymine is not present (lower part). b) HPLC profile of decamer coming from previous HPLC (deprotection with thymine) after removal of the DMT group. The insert shows the enzymatic digestion of the main product.

described during DBU deprotection when Npeoc, Npe-protected 2-cyanoethyl phosphoramidites were used[16]. Furthermore, the addition of thymine to the deprotection solution cleary improves the quality and quantity of desired oligonucleotides. The results presented here provide an alternative methods to prepare oligonucleotides containing ammonia-sensitive molecules.

EXPERIMENTAL SECTION

Abbreviations used : A_{260}: absorbance at 260 nm, AcOEt : ethyl acetate, CPG: controlled-pore glass, DBU : 1,8-diazabicyclo[5.4.0]undec-7-ene, DCM : dichloromethane, DMT :

dimethoxytrityl, LCAA : long chain amino alkyl, Npe : (*p*-nitrophenyl)ethyl and (*o*-nitrophenyl)ethyl linkage, Npeoc : (*p*-nitrophenyl)ethyloxycarbonyl. DMT-dA^{Npeoc} [12], DMT-dC^{Npeoc} [12], DMT-dG^{Npe,Npeoc} [15], DMT-dA^{Npeoc}-Npe-CPG (**4a**) [16], DMT-dC^{Npeoc}-Npe-CPG (**4b**) [16], DMT-T-Npe-CPG (**4c**) [16] and DMT-T 3'-O-H-phosphonate (**3d**) [22] were prepared following published procedures.

N,N,N',N'-Tetraisopropyl[(p-nitrophenyl)ethyl] phosphorodiamidite (1).

To a dry ether solution of bis(diisopropylamino)chloro phosphine (2.66 g, 10 mmol) cooled with ice bath, *p*-nitrophenethyl alcohol (10 mmol, 1.67 g) and dry triethylamine (1.3 ml, 10 mmol) were added using a syringe under argon atmosphere. The reaction mixture was gradually warmed to room temperature and stirred for 16 hr. Petroleum ether was subsequently added and the precipitated salt was removed by filtration. Filtrates were concentrated to dryness yielding an oil that was dried under high vacuum. Yield 95% . ^{31}P-NMR: 123.1 ppm.

5'-O-DMT-N,O-(Npeoc, Npe) protected-2'-deoxyribonucleoside 3'-N,N-diisopropyl-O-(*p*-nitrophenyl)ethyl phosphoramidite (2a-d).

The appropiate 5'-O-DMT-N,O-(Npeoc, Npe) protected 2'-deoxynucleoside (0.66 mmol) was dried by coevaporation with dry acetonitrile. The residue was dissolved in 10 ml of dry acetonitrile and treated with phosphorodiamidite **1** (0.33 g, 0.83 mmol) and tetrazol (0.032 g, 0.46 mmol). After 30 min, TLC analysis showed the formation of the desired product. Triethylamine (0.5 ml) were added and the solution was concentrated to dryness. The residue was dissolved 5% triethylamine in DCM and the solution was washed with a saturated aqueous solution of NaCl (3 x 30 ml). The organic layer was dried over *anhydrous* Na_2SO_4 and evaporated to dryness. The residue was applied to a silica gel column that was eluted with a solution of DCM-AcOEt-Et_3N (45:45:10, v/v). The appropiate fractions were concentrated to dryness.

Phosphoramidite	Yield	^{31}P-NMR	TLC*	
	(%)	(Cl_3CD)	nucleoside	phosphoramidite
DMT-T	67	148.1; 147.6	0.25	0.71
DMT-C^{Npeoc}	55	148.5; 148.1	0.37	0.87
DMT-A^{Npeoc}	58	148.2; 147.9	0.42	0.81
DMT-G$^{Npeoc, Npe}$	60	148.1; 148.0	0.44	0.83

* DCM / AcOEt / Et_3N 45:45:10

5'-O-DMT-N,O-(Npeoc, Npe) protected-2'-deoxyribonucleoside 3'-O-H-phosphonate (3a-d).

To a stirred solution of PCl_3 (0.44 ml, 5 mmol) and N-methylmorpholine (5.5 ml, 50 mmol) in 50 ml of *anhydrous* DCM, 1,2,4-triazole (1.1 g, 16.6 mmol) was added. After 30 min of magnetic stirring at room temperature, the reaction mixture was cooled to 0 °C and the corresponding 5'-O-DMT-N,O-Npeoc, Npe-protected nucleoside (1 mmol, dried by coevaporation with *anhydrous* acetonitrile) in 15 ml of *anhydrous* DCM was added dropwise over 10 min. The reaction mixture was stirred for 15 min and was poured into 80 ml of 1 M aqueous triethylammonium bicarbonate (pH 8). The phases were separated with a separatory funnel and the aqueous phase was extracted with DCM. The combined organic phases were dried over *anhydrous* sodium sulphate and evaporated to dryness. Silica gel column chromatography (0-7% methanol gradient in DCM containing 2% triethylamine) gave the desired product in the following yields:

Nucleoside H-phosphonate	Yield (%)	^{31}P-NMR (Cl_3CD, 121 MHz)
dA^{Npeoc} (**3a**)	80	3.80 ppm (J_{P-H} 622 Hz)
dC^{Npeoc} (**3b**)	68	3.78 ppm (J_{P-H} 632 Hz)
$dG^{Npe,Npeoc}$ (**3c**)	76	3.68 ppm (J_{P-H} 618 Hz)

Oligonucleotide synthesis and purification.

Sequences A and B were prepared using 20 mg (0.5 µmol) of supports **4a-b**[16] and Npeoc, Npe protected nucleoside H-phosphonate on a home-made manual synthesizer. Syntheses were performed using the protocol described by Froehler et al[21] (coupling reagent : pivaloyl chloride). Coupling efficiencies were 90-92%. Oxidation of H-phosphonate polymers was performed as described[22].

Sequences A - E were assembled using 35 mg (0.9 µmol) of supports **4a-c**[16] and Npeoc, Npe-protected nucleoside phosphoramidites on an automatic DNA synthesizer (Applied Biosystems Mod. 392). Standard 1 µmol scale synthesis cycles were used. Coupling efficiencies were 93-95%.

After the assembly of the sequences the supports were treated with a 0.5 M DBU solution in *anhydrous* pyridine (2-3 ml) at room temperature for 16 hours. Alternatively, sequences C-E were also deprotected adding 5 mg of thymine to the deprotection solution. We also tested other nucleosides and nucleic acid bases including guanosine, adenine, guanine and uracil but none of them was suitable due to their insolubility in the deprotection solution. Deprotection solutions were neutralized with a 50% acetic acid

aqueous solution, filtered and the supports were washed with pyridine and water. The combined filtrates were concentrated to dryness. The residues were dissolved in 20 mM triethylammonium acetate buffer and the solutions were desalted on a Sephadex G-10 column. The oligonucleotide containing fractions were analyzed and purified by HPLC. HPLC conditions were as follows : Column : Nucleosil 120C18 (200 x 4 mm), flow rate 1 ml / min, a 20 min linear gradient from 2 to 25% acetonitrile over 20 mM aqueous triethylammonium acetate. HPLC profiles were shown in FIG. 2-4. Overall (synthesis and purification) yields were 10-15% when thymine was present in the deprotection mixture and 2-5% when no thymine was present. Nucleoside composition was analyzed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis[23].

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REFERENCES

1. Eckstein, F. (Ed.) in "Oligonucleotides and analogues. A practical approach" (1991) Oxford Univerity Press, Oxford.

2. Beaucage, S.L., and Iyer, R.P. (1993) *Tetrahedron*, **49**, 1925-1963.

3. Schulhof, J.C., Molko, D. and Teoule, R. (1987) *Nucleic Acids Res.*, **15**, 397-416.

4. Sinha, N, and Casale, R. (1992) Tenth International Round Table. Nucleosides and Nucleotides and their Biological Applications. Park City, Utah (USA).

5. Vu, H., McColumn, C., Jacobson, K., Theisen, P., Vinayak, R., Speiss, E. and Andrus, A. (1990) *Tetrahedron Lett.*, **31**, 7269-7272.

6. Uznanski, B., Grajkowski, A., and Wilk, A. (1989) *Nucleic Acids Res.* **17**, 4863-4871.

7. Vinogradov, S., Asseline, U., and Thuong, N.T. (1993) *Tetrahedron Lett.* **34**, 5899-5902.

8. Kuijers, W.H.A., Huskens, J., and van Boeckel, C.A.A. (1990) *Tetrahedron Lett.* **31**, 6729-6732.

9. Hayakawa, Y., Wakabayashi, S., Kato, H. and Noyori, R. (1990) *J. Am. Chem. Soc.*, **112**, 1691-1696.

10. Dreef-Tromp, C.H., Hoogerhout, P., van der Marel, G.A., and van Boom, J.H. (1990) *Tetrahedron Lett.*, **31**, 427-430.

11. Koole, L.H., Moody, H.M., Broeders, N., Quaedflieg, P., Kuijpers, W., van Genderen, M., Coenen, A., van der Wal, S. and Buck, H.M. (1989) *J. Org. Chem.*, **54**, 1657-1664.

12. Himmelsbach, F., Schulz, B.S., Trichtinger, T, Charubala, R., and Pfleiderer, W. (1984) *Tetrahedron*, **40**, 59-72.

13. Stengele, K., and Pfleiderer, W. (1990) *Tetrahedron Lett.*, **31**, 2549-2552.

14. a) Pfleiderer, W., Schwarz, M. and Schirmeister, H. (1986) *Chemica Scr.*, **26**, 147-154. b) Pfleiderer, W., Himmelsbach, F., Charubala, R., Schirmeister, H., Beiter, A., Schulz, B., and Trichtinger, T. (1985) *Nucleosides and Nucleotides*, **4**, 81-94.

15. Schirmeister, H., Himmerlsbach, F., and Pfleiderer, W. (1993) *Helv. Chim. Acta*, **76**, 385-401.

16. Eritja, R., Robles, J., Aviñó, A., Albericio, F., and Pedroso, E. (1992) *Tetrahedron*, **48**, 4171-4182.

17. Hamamoto, S., and Takaku, H. (1986) *Chem. Lett.*, 1401-1404.

18. Schwarz, M.W., and Pfleiderer, W. (1985) *Nucleosides and Nucleotides*, **4**, 291-292.

19. Schwarz, M.W., and Pfleiderer, W. (1984) *Tetrahedron Lett.*, **25**, 5513-5516.

20. Beiter, A.H., and Pfleiderer, W. (1984) *Tetrahedron Lett.*, **25**, 1975-1978.

21. Van Aerschot, A., Herdewiijn, P., Janssen, G., and Vanderhaeghe, H. (1988) *Nucleosides and Nucleotides*, **7**, 519-536.

22. Froehler, B.C., Ng, P.G. and Matteucci, M.D. (1986) *Nucleic Acids Res.*, **14**, 5399-5407.

23. Fernandez-Forner, D., Palom, Y., Ikuta, S., Pedroso, E., Eritja, R. (1990) *Nucleic Acids Res.* , **18**, 5729-5734.

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