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Highly efficient oligodeoxyribonucleotide synthesis using fully base protected phosphodiester building blocks carrying 2-(1-methylimidazol-2-yl)phenyl protection of the phosphate

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ABSTRACT

Four fully base protected phosphodiester building blocks have been synthesised and fully characterised. The phosphate protecting group used was the 2-(1-methylimidazol-2-yl)phenyl group, enabling intramolecular catalysis of the condensation step in oligodeoxyribonucleotide synthesis by the solid phase phosphotriester method. Cycle times of about 12 min could thus be achieved. Moreover, the used of extra protecting groups on deoxythymidine and 2'-deoxyguanosine resulted in much cleaner oligodeoxyribonucleotides as evidenced by ion-exchange and reversed phase h.p.l.c.

INTRODUCTION

A variety of methods have been used for accelerating the reaction between a phosphodiester and the 5'-hydroxyl group of a polymer supported oligodeoxyribonucleotide in the presence of 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) or mesitylenesulphonyl chloride. These include the use of 1-methylimidazole (1-3) and more recently a variety of 4-substituted pyridine-1-oxides (4) as nucleophilic catalysts. In the absence of any catalysis, condensation times using MSNT in pyridine were about 40 min (5). Addition of 1-methylimidazole reduced the condensation time to 10-15 min depending on the solvent (2). 4-Substituted pyridine-1-oxides, in particular the 4-dimethylamino and 4-morpholino derivatives enable condensation times of about 4min to be used (4), thus giving cycle times of about 10 min which is comparable to the cycle time in the phosphoramidite procedure. At the moment pyridine is the solvent of choice when MSNT is employed as the condensing agent, and unfortunately these pyridine-1-oxides function much better in solvents other than pyridine.

A recently proposed idea of intramolecular catalysis (6) by incorporation of the 1-methylimidazole moiety in the phosphate protecting group was highly promising, giving a condensation time of 5 min, however only the T monomer could be prepared due to the synthetic route chosen.

In order to produce oligodeoxyribonucleotides of sufficient purity for n.m.r. work and crystallography it was decided to incorporate additional base protecting groups on 2'-deoxyguanosine and 2'-deoxythymidine. These bases are known to undergo modification during oligodeoxyribonucleotide synthesis by the phosphotriester method (7-14). A number of protecting groups have been described for additional protection of dT (15-19) and dG (15,16,19-25) and we chose to use O^4-phenyl protection for dT (19) and O^6-diphenylcarbamoyl protection for dG (23). It has also been recently shown that modification of thymine (26) and guanine bases (27) takes place during the phosphoramidite procedure. A relatively easy and efficient synthesis of the four phosphodiester building blocks is described, and all the compounds were fully characterised by standard methods (t.l.c., n.m.r. and mass spectrometry).

The highly efficient synthesis of two 25-mers using these new monomers and phosphotriester chemistry on a urethane linked (28) long chain alkylamine controlled pore glass (LCAA/CPG) support is described.

DISCUSSION AND RESULTS

The monomers

The 9-phenylxanthen-9-yl (pixyl or Px) group was used as the 5'-hydroxyl protecting group (29) as it is slightly more acid labile than the dimethoxytrityl group. We chose to protect the thymidine moiety with an O^4-phenyl group (19) since 5'-O-pixyl-O^4-phenylthymidine was readily prepared on a large scale and could be easily purified by crystallisation from ethyl acetate. The standard benzoyl group was used to protect the exocyclic 4-amino group of 2'-deoxycytidine. Protection of the 6-amino group of 2'-deoxyadenosine by the di-n-butylaminomethylidene group (30) was used to reduce depurination. The O^6-position of 2'-deoxyguanosine was protected by a diphenylcarbamoyl group

Table 1

Molecular weights and silica gel t.l.c. data for protected 2′-deoxyribonucleosides

Derivative[1]	Molecular weight	R_f in solvent A[2]	R_f in solvent B[3]
A	646.79	0.59	0.74
G	774.83	0.67	0.80
C	587.63	0.56	0.70
T	574.63	0.66	0.78

[1]A is 5′-O-pixyl-N^6-di-n-butylaminomethylidene 2′-deoxyadenosine; G is 5′-O-pixyl-O^6-diphenylcarbamoyl-N^2-propionyl-2′-deoxyguanosine; C is 5′-O-pixyl-N^4-benzoyl-2′-deoxycytidine; T is 5′-O-pixyl-O^4-phenylthymidine.
[2]Solvent A is ethanol/chloroform (1:9 v/v) containing 0.5% pyridine.
[3]Solvent B is ethyl acetate/acetone/water (5: 10: 1 v/v) containing 0.5% pyridine.

(23) which could be readily cleaved by aqueous ammonia. 5'-O-Pixyl-O^6-diphenylcarbamoyl-N^2-propionyl-2'-deoxyguanosine was readily prepared on a large scale without any intermediate purification steps, the final yield after preparative liquid chromatography was 49% based on 2'-deoxyguanosine. All four protected 2'-deoxyribonucleosides gave single spots on silica gel t.l.c. in two solvent systems (refer to Table 1 for R_f values).

The preparation of the monomers is illustrated in Figure 1. It was decided to prepare the 5'-O-pixyl base protected 2'-deoxyribonucleoside-3'-O-[2,5-dichlorophenyl,2-(1-methylimidazol-2-yl)phenyl phosphate] and to then selectively cleave the 2,5-dichlorophenyl moiety with an oximate reagent (25) to yield the desired phosphodiester. In the earlier paper (6) removal of a 2,4-dichlorophenyl moiety was done with concentrated aqueous ammonia/dioxan (1:1 v/v) for 12 h at 60°C, conditions which cleave the acyl protecting groups from the exocyclic amino groups in A, G and C; hence only the T monomer was prepared. In the procedure outlined in Figure 1, 2,5-

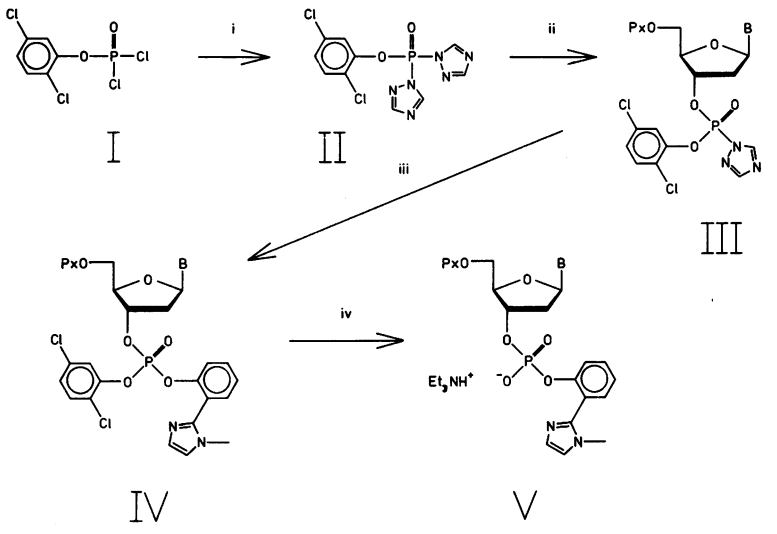

Figure 1. Scheme for the preparation of phosphodiester building blocks. Reagents: i, 1,2,4-triazole and triethylamine in dioxan; ii, 5′-O-pixyl base protected 2′-deoxyribonucleoside; iii, 2-(1-methylimidazol-2-yl) phenol; iv, pyridine-2-carbaldoxime in triethylamine/water/dioxan (1:1:1 v/v).
B is O^4-phenylthymin-1-yl, N^4-benzoyl-cytosin-1-yl, N^6-di-n-butylaminomethylidene-adenin-9-yl, or O^6-diphenylcarbamoyl-N^2-propionyl-guanin-9-yl.

dichlorophenyl phosphorodichloridate (I) was converted to its ditriazolide (II) in dry dioxan by the standard procedure (31). Dioxan was found to be superior to tetrahydrofuran for these reactions. The phosphoroditriazolide smoothly and cleanly converted an appropriately protected 2'-deoxyribonucleoside bearing a 3'-hydroxyl group into the phosphotriazolide diester (III). The remaining triazolide moiety was easily displaced by the hydroxyl group of 2-(1-methylimidazol-2-yl)phenol, prepared by a standard procedure (32), within 1 h without additional catalysis. Silica gel t.l.c. of the intermediate phosphotriester (IV) showed a very clean product. Purification of the intermediate phosphotriester was deemed unnecessary. Alkyl diaryl phosphates are rather sensitive to silica gel chromatography purification; in particular 5'-O-pixyl-O^4-phenylthymidine-3'-O-(2-chlorophenyl,2,4,5-trichlorophenyl phosphate) was recovered in 52% yield after

Table 2

Yields and selected physical data for the new monomers

Monomer	yield (%)	^{31}P (p.p.m.)[1]	molecular weight	molecular ion[2] and formula
A	48	-9.08	984.14	881, $C_{48}H_{50}N_8O_7P$
G	42	-8.68	1112.19	1009, $C_{55}H_{46}N_8O_{10}P$
C	80	-8.80	924.98	822, $C_{45}H_{37}N_5O_9P$
T	30.5	-8.57	911.98	809, $C_{45}H_{38}N_4O_9P$

[1] ^{31}P n.m.r. spectrum recorded in $CDCl_3$ solution with broad band proton decoupling. Chemical shift, δ, relative to external trimethyl phosphate.

[2] Determined by negative ion fast atom bombardment mass spectrometry. Formula is for the molecular ion.

chromatography and the very labile 5'-O-pixyl-N^6-pivaloyl-2'-deoxyadenosine-3'-O-(2-chlorophenyl,2,3,4,5,6-penta-fluorophenyl phosphate) was only obtained in 20% yield. The 2,5-dichlorophenyl group was expected to be much more labile towards displacement by oximate than the imidazolyl phenyl group and this proved to be the case. Triester (IV) was converted quantitatively (as judged by t.l.c.) into the desired phosphodiester (V) within 10 min, possibly with assistance from the imidazolylphenyl group. Purification of the monomers as their triethylammonium salts was achieved by preparative liquid chromatography on silica. The C monomer was obtained in 80% yield, whereas the A nd G monomers were obtained in 48% and 42% yield respectively. The yield of T monomer was rather low (30%) and is attributed to some loss of the O^4-phenyl group during the oximate treatment. The use of an N^3-anisoyl or benzoyl protected T derivative should overcome this problem.

All four monomers were fully characterised by t.l.c., ^1H, ^{13}C, and ^{31}P n.m.r. spectroscopy and negative ion fast atom bombardment mass spectrometry (FAB MS) and some of the data is summarised in Table 2. ^{31}P n.m.r. spectroscopy showed the presence of an impurity (about 1%) in C and T monomers and about 5% in the G and A monomers) which was identified as 2,5-dichlorophenyl,2-(1-methylimidazol-2-yl)phenyl phosphate by FAB

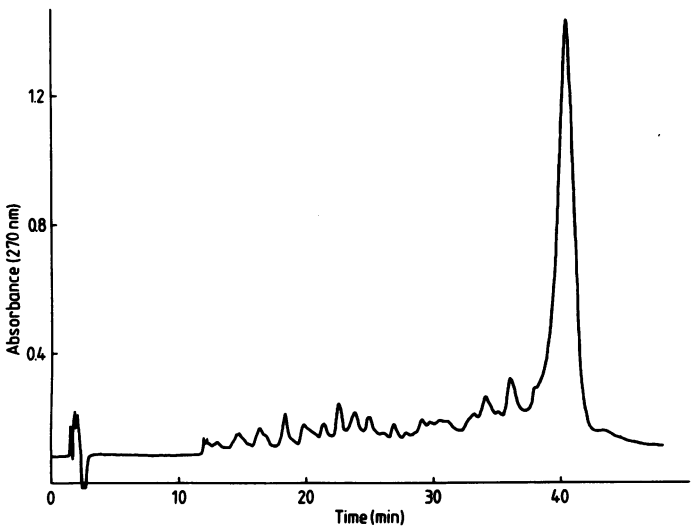

Figure 2. Ion-exchange h.p.l.c. elution profile of desalted crude d [TTCCCAGTCACGACGTTGTAAAACG] on Partisil 10SAX using a gradient of KH_2PO_4 (pH 6.3) in formamide/water (6:4 v/v).

MS. However the presence of this impurity did not markedly impair the efficiency of the condensation reactions, when these monomers were used in solid phase oligodeoxyribonucleotide synthesis.

The 2-(1-methylimidazol-2-yl)phenyl protecting group could be removed from fully protected oligodeoxyribonucleotides by 12 h oximate treatment at 60°C (6). It was expected that a 4-chloro-2- (1-methylimidazol-2-yl)phenyl group would be removed rather more easily. However, when the synthesis of the phenol was attempted from 5-chlorosalicylic acid methyl ester and N-methylethylenediamine it proved impossible to dehydrogenate the intermediate imidazoline to the desired imidazole using palladium on charcoal. Alternative routes to the desired phenol are being sought.

Oligodeoxyribonucleotide synthesis

The new monomers were used very successfully for the rapid synthesis of oligodeoxyribonucleotides by the improved solid phase phosphotriester method (28,33), as exemplified by the synthesis of two 25-mers, viz. d [TTCCCAGTCACGACGTTGTAAAACG] a new M13 sequencing primer, and d [AGGGACCAGGCAGCACAGGCCTGCC] a

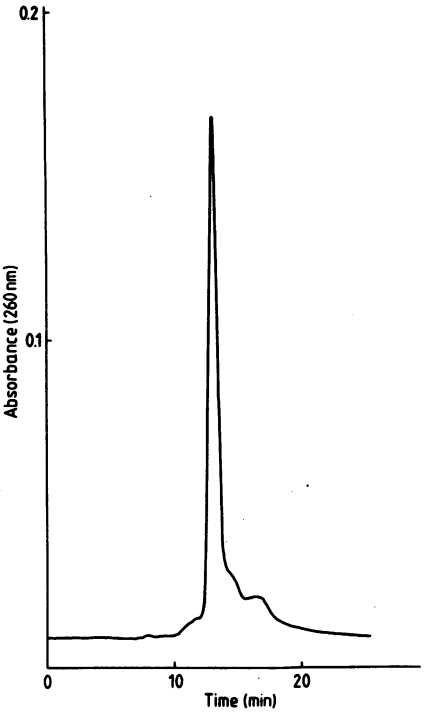

Figure 3. Reversed phase h.p.l.c. elution profile of ion-exchange purified d[TTCCCAGTCACGACGTTGTAAAACG] on μ-Bondapak C_{18} using a gradient of acetonitrile in 0.1 M aqueous triethylammonium acetate pH 7.

probe for the second exon of human α_1 antitrypsin gene. Condensation times as short as 5 min (6) were not very successful with A and G monomers due to steric hindrance to reaction by the additional bulky protecting groups in the new monomers. Condensation times of 5 min for pyrimidine monomers and 7 min for purine monomers gave excellent results.

The M13 primer was obtained in 15.6% yield after purification by ion-exchange h.p.l.c. followed by desalting (see Figure 2). The reversed phase h.p.l.c. trace of the ion-exchange h.p.l.c. purified material is shown in Figure 3. The yield of the other sequence was here 8.7% and the ion-exchange and reversed phase h.p.l.c. traces are shown in Figures 4 and 5 respectively. The reversed phase h.p.l.c. traces were very good and losses of product were minimal.

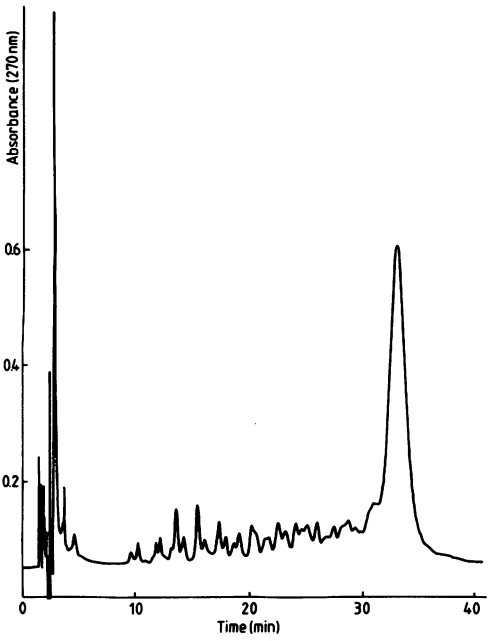

Figure 4. Ion-exchange h.p.l.c. elution profile of crude d [AGGGACCAGGCAGCACAGGCCTGCC] on Partisil 10SAX at 56°C using a linear concentration gradient of KH_2PO_4 (pH 6.3) in formamide/water (6:4 v/v).

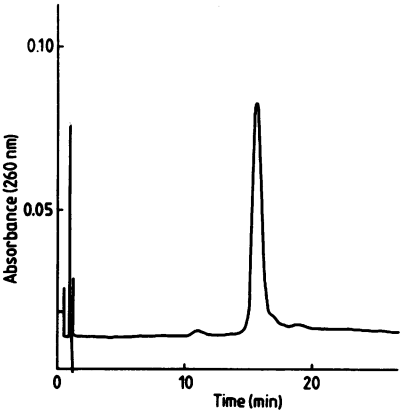

Figure 5. Reversed phase h.p.l.c. elution profile of ion-exchange purified d[AGGGACCAGGCAGCACAGGCCTGCC] on μ-Bondapak C_{18} using a gradient of acetonitrile in 0.1 M aqueous triethylammonium acetate pH 7.

Improvements to the phosphotriester methodology mean that large scale syntheses to produce sufficiently pure material for crystallography can be made on solid phase. The advantage of the phosphotriester method is that the excess monomeric units can be recovered, and this is not possible using the phosphoramidite procedure. The alternative method for large scale synthesis of oligodeoxyribonucleotides is the solution method developed by Reese, the so called filtration method (34).

EXPERIMENTAL

General Materials and Procedures

Dioxan was passed through basic alumina to remove peroxides and then distilled from sodium plus benzophenone. Triethylamine was dried by distillation from potassium hydroxide pellets. Pyridine was distilled from ninhydrin and then from barium oxide. 1,2-Dichloroethane was passed through basic alumina and then distilled from phosphorus pentoxide.

1-Mesitylenesulphonyl-3-nitro-1,2,4-triazole was prepared as described previously (33). 2-(1-Methylimidazol-2-yl)phenol was prepared as described (32) and recrystallised from carbon tetrachloride/diethyl ether (1:1 v/v). 5'-O-Pixyl-O^4-phenyl-thymidine was prepared by the procedure of Reese (19) and purified by recrystallisation from ethyl acetate containing a little pyridine. 5'-O-Pixyl-N^4-benzoyl-2'-deoxycytidine and 5'-O-pixyl-N^6-di-n-butylaminomethylidene-2'-deoxyadenosine were prepared as described previously (29,33) and purified by preparative liquid chromatography on silica using ethanol/dichloromethane (5: 95) containing 0.2% pyridine as eluent. O^6-Diphenylcarbamoyl-N^2-propionyl-2'-deoxyguanosine was prepared as described (23) and the crude product was pixylated in the usual way (29). The product was purified by preparative liquid chromatography on silica using ethyl acetate/dichloromethane (4:1 v/v) containing 0.2% pyridine as eluent. Preparative LC purified protected nucleosides and monomers were obtained as powders by dropwise addition of a dichloromethane solution into a large volume of vigorously stirred dry petroleum benzene or pentane containing 1% triethylamine. Precipitated material was then filtered off and

dried. In the case of 5'-O-pixyl-N^4-benzoyl-2'-deoxycytidine the product crystallised out of dichloromethane, and substantial losses occurred during chromatography due to solubility difficulties.

Preparative liquid chromatography was performed on silica cartridges using a Waters Prep 500A system with refractive index detector.

Silica gel t.l.c. was performed on aluminium foil supported silica containing a fluor at 254 nm, in the ascending mode using ethanol/chloroform (1:9 v/v) containing 0.5% pyridine as eluent. Pixyl containing materials gave a yellow spot on spraying with 70% perchloric acid/ethanol (3:2 v/v).

N.m.r. spectra were recorded on a Bruker AM250 spectrometer. ^{31}P chemical shifts are reported relative to external trimethyl phosphate (positive shifts are downfield from the reference).

Preparation of monomers

Dry 1,2,4-triazole (1.38 g, 20 mmol) was dissolved in dry dioxan (50 ml) in a 250 ml capacity glass vessel (33), fitted with a ground glass joint at the top and a glass sinter at the bottom plus an outlet tube and tap. Dry triethylamine (2.43 ml, 17.5 mmol) was added, followed by 2,5-dichlorophenyl phosphorodichloridate (1.31 ml, 7.5 mmol). The mixture was shaken briefly and kept under anhydrous conditions for 30 min. The phosphoroditriazolide (II) was transferred under dry nitrogen pressure (the precipitate of triethylamine hydrochloride remained on the glass sinter) into a flask containing dried 5'-O-pixyl base protected 2'-deoxyribonucleoside (5 mmol, dried by evaporation of anhydrous pyridine to leave a viscous syrup). The clear solution was then kept in the dark under anhydrous conditions. Silica gel t.l.c. showed complete reaction after 60-90 min (a u.v. and pixyl positive spot of R_f 0.1-0.18 is observed), giving the phosphorotriazolide (III).

Dry 2-(1-methylimidazol-2-yl)phenol (2.18 g, 12.5 mmol) was then added and the pale yellow solution was kept in the dark under anhydrous conditions. The reaction was monitored by t.l.c. and was complete after 60-90 min (two intense u.v. and pixyl

positive spots of R_f about 0.60 and 0.56 due to the chiral phosphorus atom). The solution containing the intermediate phosphotriester (IV) was then evaporated in vacuo to leave a syrup, which was dissolved in dichloromethane (200 ml). This solution was washed with aqueous sodium dihydrogen phosphate (3x200 ml, 0.1 M, pH 7) followed by water (250 ml) and then dried (anhydrous sodium sulphate). After filtration and evaporation of the solvent in vacuo in the presence of a few ml of triethylamine, crude phosphotriester (IV) was obtained as a foam (generally pale yellow).

The 2,5-dichlorophenyl protecting group was then removed as follows (25): A solution of pyridine-2-carbaldoxime (3.66 g, 30 mmol) in dioxan/water/triethylamine (150 ml, 1:1: 1 v/v) was added to the crude phosphotriester with stirring. Silica gel t.l.c. of the clear solution after 10 min showed that conversion to the desired phosphodiester (V) was complete (u.v. and pixyl positive spot of R_f about 0.1). U.v. positive spots of R_f 0.55 and 0.41 were due to excess 2-(1-methylimidazol-2-yl)phenol and oxime respectively. The solution was evaporated in vacuo to a syrup, which was dissolved in dichloromethane (250 ml) and the solution was washed with 0.5 M aqueous triethylammonium bicarbonate (3x250 ml, pH 7.5) then water (250 ml) and was finally dried (Na_2SO_4). Solvent was removed in vacuo and the residue dried by evaporation of dry toluene. Crude phosphodiester (V) was purified by preparative liquid chromatography on silica using ethanol/dichloromethane/triethylamine (12:87:1 v/v) as eluent at a flow rate of 300 ml min^{-1}. After removal of solvent, the phosphodiester, as its triethylammonium salt, was obtained as a powder as described in "General materials and procedures". The monomers were stored in the dry at -20°C. Data on the four monomers is collected in Table 2.

Oligodeoxyribonucleotide assembly procedure

Long chain alkylamine controlled pore glass (25 mg) bearing the 3'-terminal 2'-deoxyribonucleoside (about 0.75 μmol) was packed into an Omnifit column (33), and the following wash,

deprotection and coupling cycle was used:

pyridine wash	1.5 min
1,2-dichloroethane wash	1 min
3% v/v dichloroacetic acid in 1,2-dichloroethane wash	0.5-0.75 min
1,2-dichloroethane wash	1 min
pyridine wash	1.5 min
coupling (stop flow)	5 min (C and T)
	7 min (A and G monomers)

A flow rate of about 2-3 ml min^{-1} was used. The coupling mixture consisted of 13.3 μmol of the appropriate monomer, MSNT (20 mg, 67.5 μmol) and dry pyridine (100 μl), and was prepared just prior to injection into the column. The full procedure is described elsewhere (33). Deprotection and purification
The support was treated with a solution of pyridine-2-carbaldoxime (51.5 mg, 0.42 mmol) and 1,1,3,3-tetramethylguanidine (50 μl, 0.4 mmol) in dioxan/water (1 ml, 1:1 v/v) overnight at 60°C to cleave the 2- (1-methylimidazol-2-yl)phenyl protecting groups and remove the O^4-phenyl protection from thymine bases. The remaining work up procedure with concentrated aqueous ammonia and then 80% acetic acid was as described previously (28). The crude product was dialysed against water for a few h to remove most of the low molecular weight material, and was then purified by ion-exchange h.p.l.c. on Partisil 10SAX (5,33). After dialysis to remove phosphate and formamide the oligodeoxyribonucleotide was analysed and further purified by reversed phase h.p.l.c. on μ-Bondapak C_{18} using a Waters Z-module system.

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