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A new linkage for solid phase synthesis of oligodeoxyribonucleotides

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ABSTRACT

An aryl diisocyanate has been used to attach an appropriately protected 2'-deoxyribonucleoside bearing a free 3'-hydroxyl group, to a long chain alkylamine controlled pore glass support via a urethane moiety, in a simple two step procedure. This obviates the need for the preparation and short column chromatographic purification of the 2'-deoxyribonucleoside-3'-O-succinates required for preparation of the widely used succinyl linked supports. The greater stability of the urethane bond compared to an ester bond led to substantially higher yields of oligodeoxyribonucleotides prepared by the solid phase phosphotriester method. More than twenty oligodeoxyribonucleotides have already been synthesized on the glass support bearing the new linkage.

INTRODUCTION

The most commonly used linkage for oligodeoxyribonucleotide synthesis by phosphotriester and phosphite methods has been the succinate linkage (1,2,3,4,5) although an alternative ester linkage has been recently proposed (6) it is not in widespread usage. Moreover, some special β-eliminating linkages (7,8) have been prepared enabling fully protected oligodeoxyribonucleotide fragments, bearing a 3'-terminal phosphodiester moiety, to be cleaved from the support under very mild conditions.

The succinate linkage is not ideal as traces of basic impurities in solvents such as N,N-dimethylformamide, pyridine, and acetonitrile cause cleavage of the ester bond in the 3'-O-succinyl moiety via neighbouring group participation of the amide moiety. Thus as the assembly of the oligodeoxyribonucleotide proceeds a few percent (probably 1-2) of support bound material is cleaved per cycle. Moreover, using the phosphotriester method of assembly, this undesired cleavage was even greater before

controlled pore glass (9) replaced the Kieselguhr-polydimethylacrylamide support (10), simply because N,N-dimethylformamide was used in the wash cycle to quench the trichloroacetic acid used in the detritylation step.

Additionally, preparation of the succinyl linked supports necessitated the preparation of the four protected 2'-deoxyribonucleoside-3'-O-succinates, which had to be purified by short column chromatography. These compounds were then activated either by conversion to symmetrical anhydrides using DCCI (4) or by conversion to active esters (3) in order to attach them to the primary amino groups on the solid support. It was also necessary to use a substantial excess of these reagents over the amino loading of the support.

It seemed reasonable to replace the rather too labile succinyl linkage with a linkage in which the 3'-terminal 2'-deoxyribonucleoside was attached via a urethane moiety viz. 3'-O-CO-NH- which would be much less susceptible to unwanted cleavage during oligodeoxyribonucleotide assembly. In order to attach an appropriately protected 2'-deoxyribonucleoside bearing a free 3'-hydroxyl group to the amino group of a solid support, e.g. long chain alkylamine controlled pore glass (LCAA/CPG), a reactive bifunctional reagent bearing at least one isocyanate group was sought. Tolylene-2,6-diisocyanate was a commercially available reagent of that type and was used with great success. Initial experiments performed with 1,6-diisocyanatohexane were not very successful and showed that a more rigid and less reactive diisocyanate was required.

The efficient synthesis of a 19-mer using monomers is described using the new urethane linked support and the standard succinate linked support for direct comparison.

DISCUSSION AND RESULTS

The support

The preparation of the support is illustrated in Figure 1. Initially the appropriately protected (5'-OH and base $-NH_2$ groups) 2'-deoxyribonucleoside (I) is reacted with one equivalent of tolylene-2,6-diisocyanate (II) in the presence of one equivalent of N-ethyldiisopropylamine as catalyst in

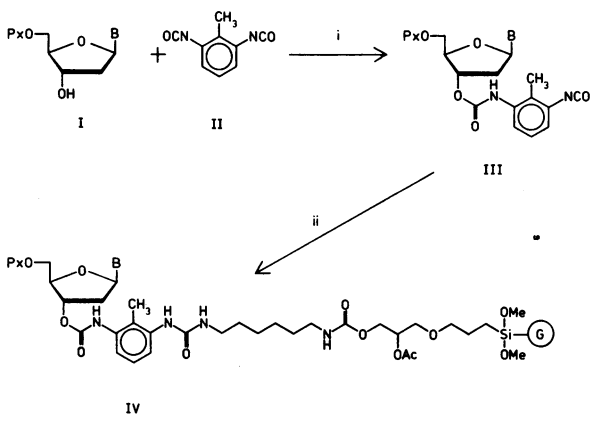

Figure 1. Scheme for the preparation of the urethane linked support. Reagents: i, N-ethyldiisopropylamine in pyridine / 1,2-dichloroethane; ii, long chain alkylamine controlled pore glass. (Px = 9-phenylxanthen-9-yl; B is an appropriately protected nucleoside base).

pyridine/1,2-dichloroethane to generate the monoisocyanate (III). This is not isolated but added directly to one equivalent of long chain alkylamine controlled pore glass to generate the fully functionalised support (IV). After washing and a capping step the support is ready for use. The preparation is easily carried out on a scale of several g of support and is relatively inexpensive. Loadings of 2'-deoxyribonucleosides of 22-30 μmol g^{-1} of support were achieved by this method. For comparison purposes the structure of the standard succinyl linkage to LCAA/CPG is illustrated in Figure 2. The urethane linkage is cleaved by prolonged treatment with concentrated aqueous ammonia at 50-60°C.

Oligodeoxyribonucleotide synthesis

A sequencing primer for the T4 RNA ligase gene viz.

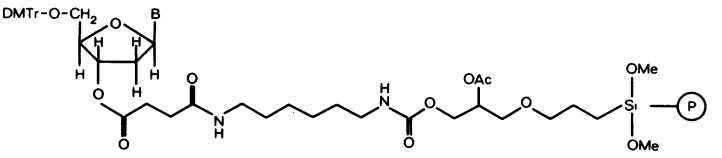

Figure 2. Structure of a protected deoxyribonucleoside 3'-O-succinate linked to long chain alkylamine controlled pore glass. (DMTr = 4,4'-dimethoxytriphenylmethyl).

d[CACAGCAACATAGCCTTCG] was prepared using the LCAA/CPG support with the new linkage and also with the standard succinyl linkage for direct comparison. The improved phosphotriester chemistry described previously (9) was used for assembly of the oligodeoxyribonucleotide with a few alterations. Monomers were used bearing 5'-O-pixyl protection (11) in place of 5'-O-dimethoxytrityl protection and the N^6-benzoyl protection for 2'-deoxyadenosine was replaced by the N^6-phthaloyl group (12,13) to reduce the amount of depurination.

The wash and deprotection cycle used was as below (for 25 mg of support), with a flow rate of about 2 ml min^{-1} using the Omnifit system:

pyridine wash	2 min
1,2-dichloroethane wash	2 min
3% v/v dichloroacetic acid in	0.5-0.75 min
1,2-dichloroethane wash	depending on 5'-base
1,2-dichloroethane wash	2 min
pyridine wash	2 min
coupling	15 min

When the 19-mer was prepared on the LCAA/CPG support with the two different linkages the following results were obtained. The new linkage yielded 28.6% of 19-mer after preparative ion-exchange h.p.l.c. (see Figure 3) and desalting, corresponding to a coupling yield of 93.3% per cycle. However, when prepared on the normal succinyl linked support the isolated yield was only 20.7%, corresponding to a coupling yield of 91.6% per cycle (the ion-exchange h.p.l.c. trace was essentially identical to that shown in Figure 3). The ion-exchange h.p.l.c. purified 19-mer prepared on LCAA/CPG with the new linkage was further purified by preparative reversed phase h.p.l.c. (see Figure 4). The reversed phase h.p.l.c. of the ion-exchange h.p.l.c. purified 19-mer prepared on the succinyl linked support was virtually identical to the trace shown in Figure 4.

The final isolated yield of d[CACAGCAACATAGCCTTCG] prepared on the urethane linked LCAA/CPG support was 16.8% after preparative ion-exchange h.p.l.c., desalting, preparative reversed phase h.p.l.c., and a further desalting.

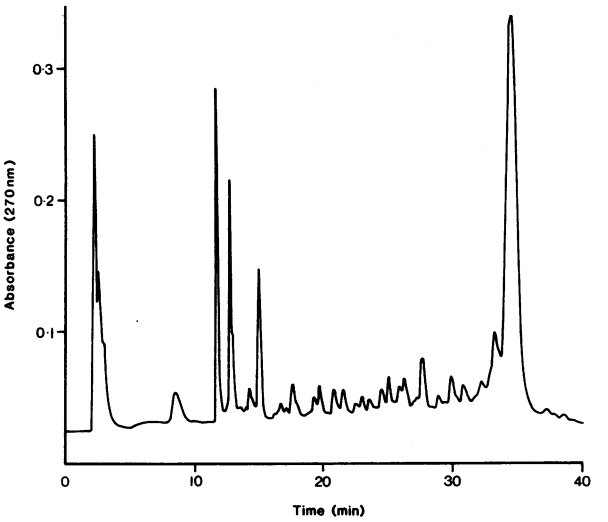

Figure 3. Ion-exchange h.p.l.c. elution profile of crude d[CACAGCAACATAGCCTTCG] on Partisil 10SAX using a gradient of KH_2PO_4 (pH 6.3) in formamide / water (6:4 v/v).

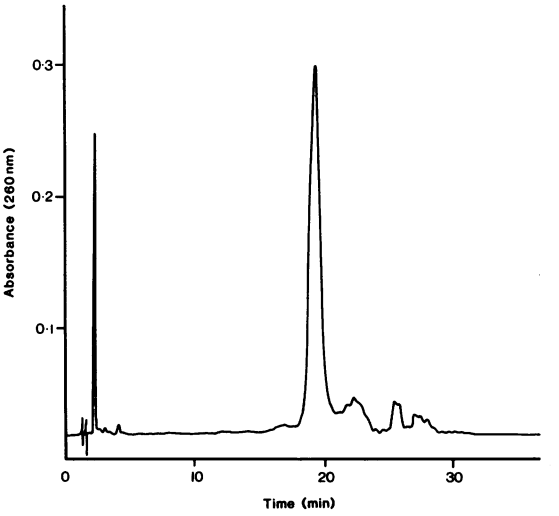

Figure 4. Reversed phase h.p.l.c. elution profile of ion-exchange purified d[CACAGCAACATAGCCTTCG] on μ-Bondapak C_{18} using a gradient of acetonitrile in 0.1 M aqueous ammonium acetate.

EXPERIMENTAL PROCEDURES

Functionalisation of supports

The appropriately protected 2'-deoxyribonucleoside (0.2 mmol, viz. 99.7 mg of 5'-O-pixyl-thymidine, 117.5 mg of 5'-O-pixyl-N^4-benzoyl-2'-deoxycytidine, 129.4 mg of 5'-O-pixyl-N^6-di-n-butylaminomethylene-2'-deoxyadenosine [14], or 118.7 mg of 5'-O-pixyl-N^2-isobutyryl-2'-deoxyguanosine) is dried in vacuo, in a septum sealed vial fitted with a small syringe needle, over P_2O_5 and KOH for several h. The vacuum is released with dry argon (or nitrogen), the needle removed, and the solid is dissolved in dry 1,2-dichloroethane/pyridine (1 ml, 1:1 v/v). Redistilled N-ethyldiisopropylamine (34.2 µl, 0.2 mmol) and tolylene-2,6-diisocyanate (28.4 µl, 0.2 mmol; obtained from Aldrich. Caution: SEVERE POISON) are then injected into the vial using pre-dried gas-tight syringes. The solution is then kept in the dark under anhydrous conditions for 6 h at room temperature.

The solution is then withdrawn into a dry gas-tight syringe and injected into a septum sealed vial (ca. 7 ml capacity) containing dried long chain alkylamine controlled pore glass (2 g, ca. 0.2 mmol of amino groups; supplied by Pierce, 500 Å pore diameter, particle size 125-177 µm) suspended in dry 1,2-dichloroethane (4.5 ml) under argon (or nitrogen). The mixture is agitated briefly to ensure thorough mixing and then left in the dark under anhydrous conditions for 24 h. The glass beads are then filtered off on a sintered glass funnel, washed with 1,2-dichloroethane (2x10 ml), pyridine (5x10 ml), and diethyl ether (5x10 ml). The beads are then suspended in pyridine/water (10 ml, 8:2 v/v) and left for 2 h at room temperature in order to hydrolyse any residual isocyanate groups. The beads are filtered off, washed with dry pyridine (5x10 ml) and then treated with a capping solution of pyridine/acetic anhydride/1-methylimidazole (10 ml, 8: 1: 1 v/v/v) under anhydrous conditions for 30 min at room temperature, in order to cap off any residual amino groups. The glass beads are again filtered off, washed with pyridine (5x10 ml), 1,2-dichloroethane (5x10 ml) and then diethyl ether (5x10 ml), and are finally dried in vacuo. The dried glass beads are then stored at -20°C.

The beads give a negative ninhydrin test as expected, however

prior to capping the ninhydrin test (15) is always positive. The loading of the beads is then determined by treating a small weighed portion with 70% aqueous perchloric acid/ethanol (3: 2 v/v) and measuring spectrophotometrically the amount of pixyl cation released; ε_{376nm} = 31.8 cm^2 μmol^{-1}. In typical experiments the following loadings were obtained:

T support - 30 μmol nucleoside g^{-1}
C support - 23.4 μmol nucleoside g^{-1}
A support - 24.6 μmol nucleoside g^{-1}
G support - 21.7 μmol nucleoside g^{-1}

Oligodeoxyribonucleotide assembly procedure

The long chain alkylamine controlled pore glass supports (25 mg of each, viz. 0.54 μmol of the urethane linked support and 0.64 μmol of the succinate linked support functionalised with 5'-O-pixyl-N^2-isobutyryl-2'-deoxyguanosine and 5'-O-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine respectively) were packed into two Omnifit columns. The following wash, deprotection, and coupling cycle was then used:

pyridine wash	2 min
1,2-dichloroethane wash	2 min
3% v/v dichloroacetic acid in 1,2-dichloroethane wash	0.5-0.75 min depending on 5'-base
1,2-dichloroethane wash	2 min
pyridine wash	2 min
coupling	15 min

The coupling mixture for each column consisted of 13.3 μmol of 5'-O-pixyl protected monomer (as the triethylammonium salt of the 3'-O-[2-chlorophenyl phosphate]), 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (20 mg, 67.5 μmol), dry redistilled 1-methyl imidazole (10 μl, 126 μmol) and dry pyridine (100 μl) and was made up just prior to injection onto the columns. The full procedure is described elsewhere (16). At the end of the assemblies the supports were washed with pyridine, 1,2-dichloroethane, then removed from the columns and washed with 1,2-dichloroethane followed by dry diethyl ether on small sintered glass funnels.

Deprotection and purification

Prepared 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). Treated each support with 0.5 ml of this solution overnight (about 16 h) at room temperature. The following workups are then slightly different since only the succinate linkage is rapidly cleaved by the oximate reagent. The urethane linkage is only partially cleaved during the oximate treatment so some material is still attached to the support.

(i) Succinate linked support. After oximate treatment the beads were filtered off and washed with dioxan/water (a few ml, 1: 1 v/v). The combined filtrate and washings were evaporated to dryness in vacuo to leave a glass. The glass was dissolved in concentrated aqueous ammonia (5 ml, 35%) and the solution was kept in a tightly sealed vessel for 6 h at 50°C and then overnight at room temperature, in order to remove the nucleoside base protecting groups. The solution was then evaporated to dryness in vacuo.

(ii) Urethane linked support. After the oximate treatment, solvent was removed in vacuo from the supernatant plus beads. Concentrated aqueous ammonia (5 ml, 35%) was added and the mixture was kept sealed at 56°C for 48 h (this time can be reduced substantially if the amount of product required is not a prime consideration, since the urethane bond is approximately 70% cleaved by concentrated aqueous ammonia in 24 h at 56°C). When cool the supernatant was removed, the beads were washed with a little water, and the combined supernatant and washings were evaporated to dryness in vacuo.

Both residues were then dissolved in 80% acetic acid (5 ml) and the solutions were kept at room temperature for 25 min to effect the loss of the 5'-terminal pixyl protecting group. Added water (5 ml) and washed each solution with diethyl ether (5x10 ml). The aqueous layers were filtered and evaporated to dryness in vacuo. Residual acetic acid was removed by coevaporation with water in vacuo. The products were dissolved in water (1 ml), analysed and purified by ion-exchange h.p.l.c. on Partisil 10SAX as described previously (10,16). After desalting, the two 19-mer samples were further analysed and purified by reversed phase h.p.l.c. on µ-Bondapak C_{18} using a Waters Z-module system.

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