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Efficient methods for attaching non-radioactive labels to the 5′ ends of synthetic oligodeoxyribonucleotides

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ABSTRACT

The syntheses are described of two types of linker molecule useful for the specific attachment of non-radioactive labels such as biotin and fluorophores to the 5' terminus of synthetic oligodeoxyribonucleotides. The linkers are designed such that they can be coupled to the oligonucleotide as a final step in solid-phase synthesis using commercial DNA synthesis machines. Increased sensitivity of biotin detection was possible using an anti-biotin hybridoma/peroxidase detection system.

INTRODUCTION

Synthetic oligonucleotides are widely used as probes for the detection and isolation of specific genes and as primers for DNA sequencing.[1] The most common labelling technique for oligonucleotides is the incorporation of the radio-isotope ^{32}P in the form of a 5'-terminal phosphate using the enzyme T4 polynucleotide kinase.[2] Although high sensitivites of detection can often be obtained, the use of oligonucleotides labelled in this way is limited by their instability and by their hazardous nature, which precludes their use in many clinical and diagnostic applications. Recently much effort has gone into the development of methods for labelling oligonucleotides with non-radioactive tags, such as biotin or fluorophores. Biotin has a strong affinity ($K_D \propto 10^{-15}$M) for egg-white avidin or bacterially derived streptavidin. This interaction forms the basis of enzyme linked detection kits that are commercially available and thus biotin-labelled oligonucleotides have been shown to be useful as gene probes.[3,4] In applications of DNA sequencing it is more convenient for labelled DNA to be detected by direct means. For example, oligonucleotides tagged with fluorescent groups can be detected in polyacrylamide gels used for DNA sequencing by means of an argon ion laser and a fluorescence detection system[5].

Several methods have been described recently for incorporation of

biotin into DNA probes. One procedure involves using an unlabelled oligonucleotide as a primer on a complementary DNA template with the enzyme DNA polymerase to incorporate at the 3' end of the primer analogues of dUTP containing a biotin covalently bound to the 5 position of the pyrimidine ring.[4,6] Another method involves use of a photo-activatable analogue of biotin for the random labelling of DNA [7], although the procedure is not particularly useful for short oligonucleotide probes since modifications to the heterocyclic bases can occur giving rise to a substantial reduction in the melting temperature of such probes.

The attachment of biotin to the 5' end of an oligonucleotide offers considerable advantages. Such a location for the biotin causes little or no destabilisation of the hybridised oligonucleotide compared to the unlabelled form [8,9] and the 3' end is also free for use of the probe as a primer in enzyme-mediated extension reactions. Labelling of oligonucleotides with fluorescent groups is also preferable at the 5' termini for similar reasons.[5]

Of the various published procedures for attachment of labels to the 5' of oligonucleotides none appear to be without drawback. The method described by Chu and Orgel[9] and subsequently used by Chollet and Kawashima[10] requires 4 separate reactions (enzymatic phosphorylation, imidazole treatment, reaction with 1,2-diaminoethane or 1,6-diaminohexane and reaction with an activated label such as N-hydroxysuccinimido biotin) and two chromatographic purification steps. Attachment of a label via a 2-aminoethylphosphate linkage as described by Kempe et al[8] is a chemically attractive route. However the method chosen to achieve this is laborious in that it requires that each label required be functionalised first to form a 2-aminoethyl-p-chlorophenyl phosphate before attachment in a non-aqueous chemical coupling reaction. Moreover, the label is exposed to both coupling and deprotection reactions required for synthesis of the oligonucleotide. The route may therefore not be suitable for the incorporation of sensitive labels.

The procedure described by Smith et al[5] for the attachment of fluorescent labels is more suitable in that a linker molecule (5'-amino-5'-deoxythymidine) is attached to the oligonucleotide as a 5'-protected 3'-O-phosphoramidite in a final coupling reaction compatible with solid-phase machine-aided DNA synthesis. A free amino group is liberated during deprotection of the oligonucleotide and can be used for specific attachment of a variety of labels. However the synthesis of the

linker unit requires 5 steps starting from thymidine and also an extra nucleotide base is added to the probe that is in a position to base pair. In rare cases this might cause mis-hybridisation of the oligonucleotide.

Connoly and Rider[11] have very recently described preparation of a linker molecule that can also be incorporated as an extra step following solid-phase oligonucleotide synthesis. After deprotection, the 5' end of the oligonucleotide contains a 2-mercaptoethyl, 3-mercaptopropyl or 6-mercaptohexyl phosphate to which thiol specific labels can be attached such as iodoacetates or maleiimides. However linkages via thiol groups have a limited pH range of stability. Many of the popular labelling agents are designed for reaction with amino groups or have nucleophilic groups suitable for attachment to electrophilic centres giving rise to more stable amide or similar linkages. With such needs in mind we now describe the synthesis of two types of linker molecule useful for specific attachment of biotin and fluorescent labels to the 5'- end of synthetic oligonucleotides. The linker molecules are attached to the 5' end of an oligonucleotide chain as an extra step in solid-phase synthesis and the procedures are compatible with commercial solid-phase DNA synthesisers.

RESULTS AND DISCUSSION

A routinely useful 5'-labelling method for synthetic oligonucleotides would have the following features. The labelling agent should be attached to a pre-assembled oligonucleotide chain under mild aqueous conditions that do not affect the heterocyclic bases. The procedure should be suitable for use with a wide range of commercially available labelling agents and the linker molecule used to join the oligonucleotide to the labelling agent should be easily prepared and capable of being used in commercial solid-phase DNA synthesis machines without the need for additional reagents. Above all the procedure should be rapid, simple to carry out and should give high yields.

Many commonly used labelling reagents are designed for attachment either to amino groups (eg in proteins) or to aldehydes (eg in 3'-oxidised RNA). We have developed therefore two types of linker molecule that are suitable for use with such labelling reagents and that can be joined to an oligodeoxyribonucleotide at its 5' end as a final step in solid-phase DNA synthesis.

The first procedure is based on well known chemistry for the 3'-labelling of RNA.[12] Sodium periodate smoothly and quantitively oxidises the 3' terminal ribonucleoside residue of RNA to its corresponding 2',

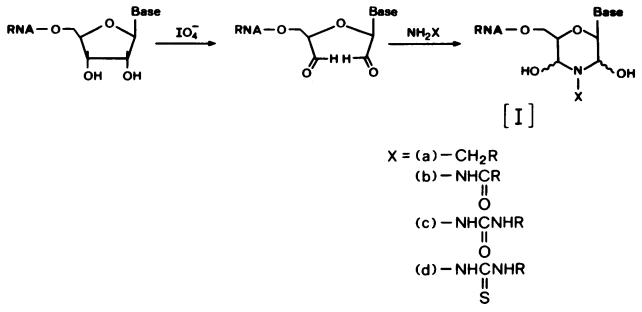

Figure 1 General scheme for 3'-labelling of RNA.

3'-dialdehyde derivative. The dialdehyde can now react with a variety of labelling reagents containing primary amino groups, [13,14] semi-carbazides[14] or thio semi-carbazides[15] to give adducts of cyclic structure (Fig Ia-d). Both oxidation and labelling reactions proceed under mild aqueous conditions.

In order to adapt this chemistry for labelling DNA it is necessary to add an appropriately protected ribonucleoside unit to the 5' position. For the synthesis of 5'-phosphorylated oligodeoxyribonucleotides Nadeau et al recently reported [16] a procedure that involved the preparation of a ribonucleoside unit phosphorylated at the 5'-position. As an extra step in a solution synthesis approach the ribonucleoside unit was coupled to the 5'-terminus of a growing oligodeoxyribonucleotide chain to give a 5'-5' linked product. After removal of protecting groups the terminal ribonucleoside was oxidised to the 2',3'-dialdehyde with periodate and then treated with cyclohexylamine in a β-elimination reaction to liberate the oligodeoxyribonucleotide as its 5'-phosphate derivative.

Using a similar approach we have prepared two protected uridine monomer units suitable for coupling to the 5' position of protected oligodeoxyribonucleotide chains by the phosphotriester route and phosphoramidite routes respectively. To this end 5'-O-acetyl uridine[17] (II,Fig 2) was converted to 2',3'-di-O-pixyl uridine (III) by treatment with 2.2 molar equivalents of pixyl chloride[18] in pyridine followed by removal of the acetyl group with methanolic ammonia. For the preparation of monomer V used for phosphotriester synthesis, compound III was phosphorylated by a similar procedure to that used by Wreesman et al for the preparation of 5'-O-methylphosphates.[19] Hence III was reacted with 1.5 molar equivalents of 2-chlorophenyl bis-(1-benzotriazolyl)phosphate in pyridine and then 2 molar equivalents of p-nitrobenzylalcohol to afford

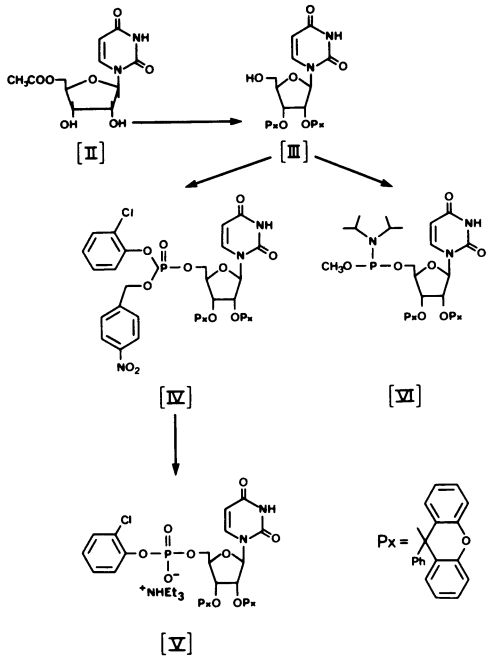

Figure 2 Scheme for synthesis of uridyl monomers V and VI.

after work-up and purification the triester IV in 47% yield. The nitrobenzyl group was then specifically removed[20] from triester IV using p-toluene thiol and triethylamine in acetonitrite to afford the 2-chlorophenyl phosphodiester monomer V in 81% yield. For the preparation of the phosphoramidite monomer VI, compound III was treated with 1.5 molar equivalents of bis-(diisopropylamino)methoxy phosphine[21] in the presence of the acidic catalyst diisopropylammonium tetrazolide to give after work-up monomer VI in 79% yield.

To demonstrate the utility of monomers V and VI, the synthesis of the oligonucleotide rU-(5'-5')-d[GTAAAACGACGGCCAGT] [U-(5'-5')-17] was carried out by both phosphotriester and phosphoramidite routes. This oligonucleotide contains the "universal" DNA sequencing primer for M13 templates.[22] The assembly by the phosphotriester route was carried out on a manual flow system using a controlled pore glass support and deoxynucleoside 2-chlorophenyl phosphate monomer units as previously described [23,24] except that in the final coupling step the ribonucleoside V monomer was used. After standard deprotection reactions[24] (NB the pixyl groups are removed under the same conditions as for 5'-O dimethoxytrityl groups) the crude oligonucleotide product was purified by ion exchange hplc and isolated in

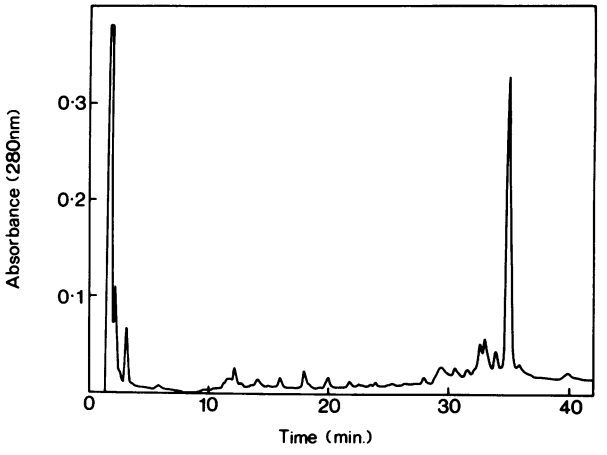

Figure 3 Ion exchange hplc trace of reaction products in assembly of rU-(5'-5')d[GTAAAACGACGGCCAGT], IX, by the phosphoramidite method (for conditions - see Experimental Section).

5.5% overall yield. The assembly by the phosphoramidite route was carried out by automated synthesis using deoxynucleoside 3'-O-methyl-N,N-diisopropyl phosphoramidites and standard procedures.[25] In the final coupling step the ribonucleotide monomer VI was used. After complete deprotection

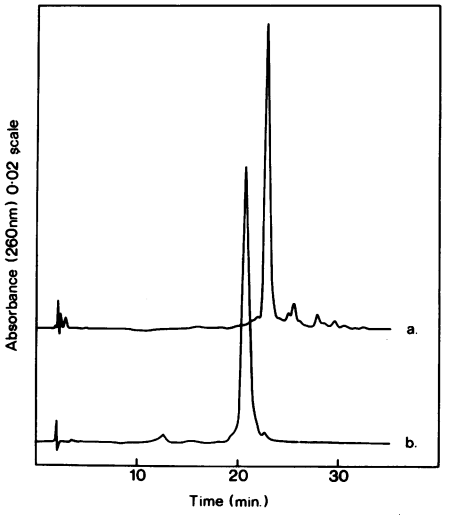

Figure 4 Reversed phase hplc traces of uridyl oligonucleotide IX (a) before oxidation and (b) after oxidation by sodium periodate and purification (for conditions - see Experimental Section).

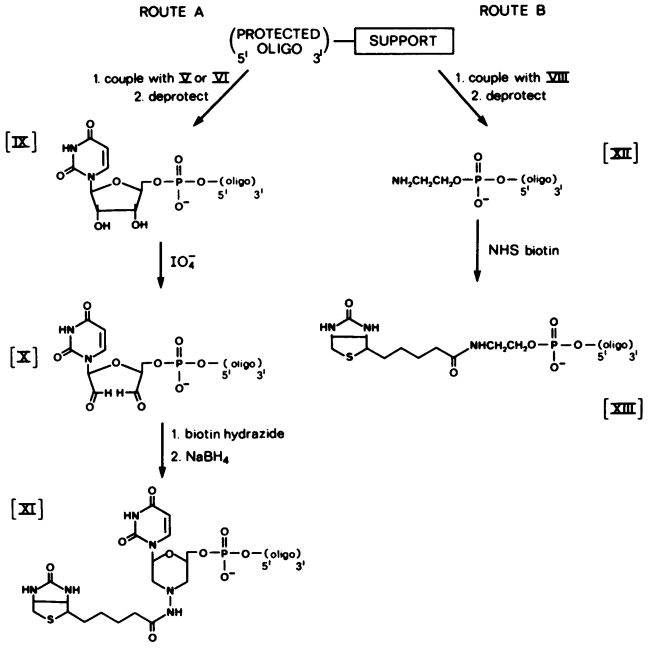

Figure 5 Scheme for synthesis and attachment of biotin to synthetic oligonucleotides.
Route a: reaction of uridyl oligonucleotide IX with periodate followed by biotin hydrazide and reduction with sodium borohydride.
Route b: reaction of amino oligonucleotide XII (NH_2-17) with NHS biotin.

the crude oligonucleotide mixture was purified by ion exchange hplc (Figure 3) and isolated in 17.6% overall yield. The better yield in the case of the phosphoramidite route reflects higher stepwise yields throughout the synthesis rather than purely the final coupling of the ribonucleotide, which in both cases appeared to couple with similar efficiency to that of the deoxynucleotide monomers. Analytical reversed phase hplc of oligonucleotides obtained in the two syntheses showed in each case predominantly a single peak with the same elution time (Figure 4a shows the chromatogram from the phosphoramidite synthesis). The elution time was slightly later than that of the corresponding 17-mer oligonucleotide without the terminal uridyl residue, whose synthesis was described previously[22] (data not shown).

The route for attachment of the non-radioactive label biotin is shown in Figure 5 (Route a). Oxidation of the uridyl oligonucleotide IX with

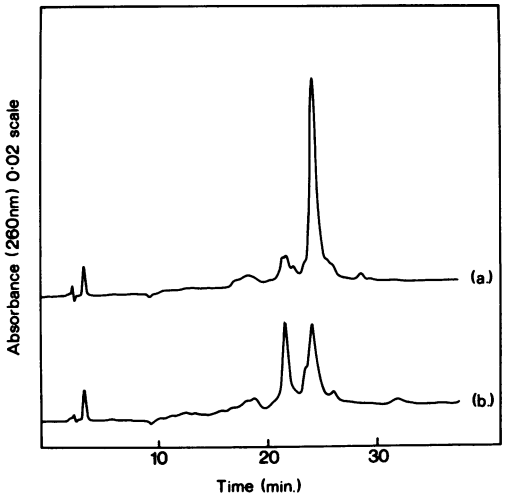

Figure 6 Reversed phase hplc traces of (a) reaction product of uridyl oligonucleotide dialdehyde X with biotin hydrazide and (b) same after heating at 65° for 2.5h in water.

sodium periodate at 4° for 1h in 0.1M sodium acetate (pH 5) was quantitative as judged by its conversion to an earlier eluting peak on hplc (Figure 4b). It was convenient to purify the dialdehyde X by hplc at this stage, since some impurities in the original batch of IX were still present. Subsequently we have found this step not to be necessary with more highly

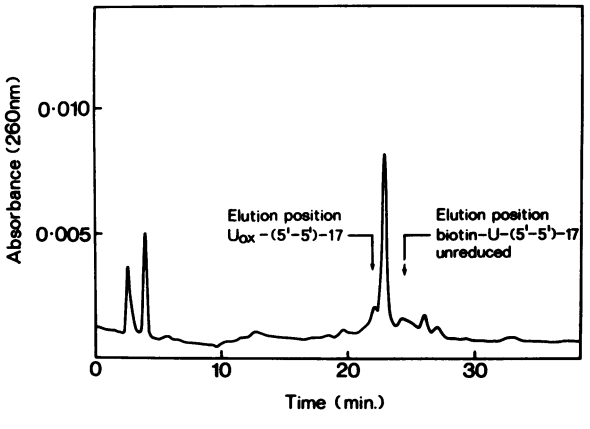

Figure 7 Reversed phase hplc trace of reaction product of uridyl oligonucleotide dialdehyde X with biotin hydrazide followed by reduction with sodium borohydride.

purified oligonucleotides (see later). The dialdehyde X (80-85% yield) was then reacted with biotin hydrazide in 50mM sodium acetate buffer (pH 5) in methoxyethanol/water (1:2) for 2h at 37°. After dialysis or gel filtration to remove unattached biotin hydrazide, hplc showed that a new product had been formed in greater than 85% yield (Figure 6a). However, the product was unstable and when heated to 65° for 2.5h in aqueous solution was 50% converted back to dialdehyde X (Figure 6b). Heat instability of such adducts has been observed previously.[14]

To stabilise the linkage further the product of the reaction of biotin hydrazide and dialdehyde X was reduced, without intermediate isolation, using sodium borohydride at 20° for 3h. After work-up, hplc showed the formation of a new product with elution time intermediate between that of the unreduced biotin adduct and dialdehyde X (Figure 7). The reduced biotinylated product XI was purified by hplc and isolated in 77% yield. XI was completely stable at 65° for 2.5h in aqueous solution as judged by hplc assay.

Biotinylated oligonucleotide XI was found to be able to detect M13 DNA (both single and double-stranded) immobilised on nitrocellulose filters. Using either of two standard commercial streptavidin/alkaline phosphatase detection kits, the sensitivity of detection was about 1 fmole (1-3ng), which is similar to that reported by Kawashima and Chollet.[10] However, using an anti-biotin hybridoma/peroxidase detection system [32,33] the sensitivity was considerably improved (0.1 fmole, 0.1-0.3ng).

Reactions of the dialdehyde X with the fluorescent derivatives, fluorescein thiosemicarbazide and Texas Red hydrazide, were disappointing. In both cases analytical hplc showed predominantly unchanged dialdehyde X together with only minor quantities of later eluting products (data not shown). The poor reactivity of these fluorescent reagents may be due to their increased bulk compared to biotin hydrazide, or possibly to decreased stability of the cyclic adducts. This problem might be solved by use of a longer spacer arm between the fluorophore and oligonucleotide. Further investigation of this route was discontinued however following the success of the alternative labelling route described below (Figure 5, Route b).

A linker molecule suitable for attachment of amino-specific labelling agents was prepared by a convenient two step procedure. Ethanolamine was reacted with a slight excess of 9-fluorenylmethylchloroformate in aqueous acetone containing sodium carbonate to afford 2-(9-fluorenylmethoxycarbonyl) aminoethanol (VII, Figure 8) which was isolated in 90% yield. Compound VII

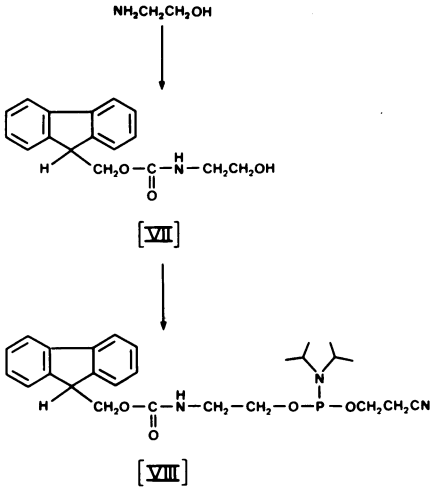

Figure 8 Scheme for synthesis of linker phosphoramidite VIII.

was then reacted with 2-cyanoethyl-N-N-diisopropylamino chlorophosphite[26] in dichloromethane solution in the presence of diisopropylethylamine to afford after work-up 2-(9-fluorenylmethoxycarbonyl)aminoethyl-2-cyanoethyl-N-N-diisopropylamino phosphite (VIII) as an oil. Although this oil could not be induced to crystallise it showed predominantly a single peak by ^{31}P-NMR and could be stored stably in a sealed flask at -20°.

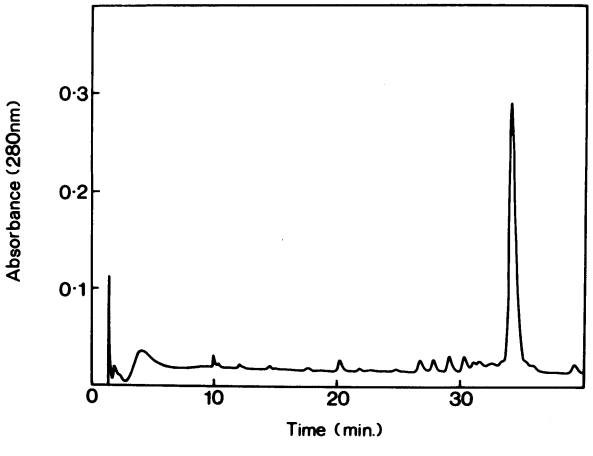

Figure 9 Ion exchange hplc trace of reaction products in assembly of NH_2-$(CH_2)_2$O-d[pGTAAACGACGGCCAGT], XII [NH_2-17] by the phosphoramidite method.

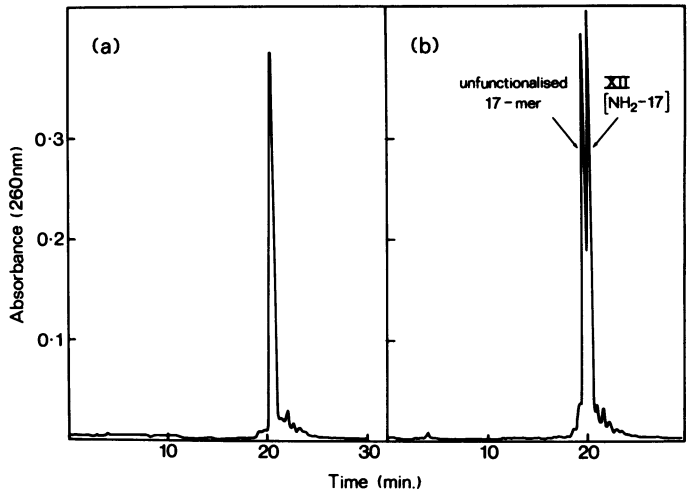

Figure 10 Reversed phase hplc traces of (a) amino oligonucleotide XII [NH_2-17] and (b) co-injection of XII with unfunctionalised 17-mer oligonucleotide prepared previously (Reference 22).

The linker phosphoramidite VIII was used in the final step of assembly of the same oligonucleotide sequencing primer,d[GTAAAACGACGGCCAGT], carried out on an automated DNA synthesiser but using deoxynucleoside-2-cyano-ethyl-N-N-diisopropylamino phosphites[26] instead of the methyl derivatives. After complete deprotection (NB The 9-fluorenylmethoxycarbonyl group is removed during ammonia treatment) the product was purified by anion exchange hplc (Figure 9) and isolated in 24.5% overall yield. The product [XII in Figure 5] was homogeneous as analysed by reversed phase hplc (Figure 10a) and had a later elution time to that of the unfunctionalised 17-mer sequencing primer[22] (Figure 10b).

Amino oligonucleotide XII was reacted with N-hydroxysuccinimidobiotin in 0.125M Tris.HCl buffer (pH 7.4) in DMF/water (1:1) for 18h at 20°. After hplc purification the biotinylated product, XIII was isolated in 79% yield. It was found to be completely stable at 65° for 2.5h in both water and in standard buffers used for hybridisation analysis (NB the linkage in XIII is the same as that described by Kempe et al,[8] but obtained by an entirely different route). Biotinylated oligonucleotide XIII was found to be able to detect M13 DNA (both single and double stranded) immobilised on nitrocellulose filters, similarly to oligonucleotide XI. Once again the sensitivity was about 1 fmole (1-3ng) using either of two commercial streptavidin/alkaline phosphate kits, but using the anti-biotin hybridoma/

TABLE 1

OLIGONUCLEOTIDE		RETENTION TIME (MIN)	
NH_2-17 [XII]		23.0	
biotin-NH_2-17 [XIII]		25.4	
FITC-NH_2-17	A	29.8	(90%)
	B	28.5	(10%)
NBD-NH_2-17		24.2	
TMRITC-NH_2-17	A	27.3	(39%)
	B	30.8	(47%)
	C	33.5	(14%)

Retention times of labelled oligonucleotides by reversed phase hplc (for conditions - see Experimental Section).

peroxidase detection system the sensitivity reached 0.05-0.1 fmole. Chu and Orgell have reported[8] that increased sensitivity of detection can be achieved using a longer spacer arm to link biotin to an oligonucleotide. Such a linker could be achieved here by use of a linker phosphite similar to VIII but with a C_5 or C_6 spacer, or by use of ε-caproylamidobiotin-N-hydroxysuccinimide ester as biotinylating agent. Such possibilities are currently under investigation.

Amino oligonucleotide XII was also reacted with amino-specific fluorophores. Smooth reactions occurred at room temperature with fluorescein isothiocyanate (FITC), 4-fluoro-7-nitrobenzofurazan (NBDF) and tetramethylrhodamine isothiocyanate (TMRITC) at pH 9. Hplc analysis after 18h showed greater than 85% conversion of XII to more retained oligonucleotide products. Whereas the product of the NBDF reaction was a single fluorescent component, reaction with the commercial sample of FITC (isomer I) gave two fluorescent products in the ratio of 9:1 and reaction with TMRITC (isomer R) gave the three products. Relative elution times are given in Table 1. All products exhibited UV absorbance spectra consistent with incorporation of a fluorescent label. Preliminary results suggest that these primers will be useful for non-radioactive sequencing of DNA (B. Barrell and A. Bankier, unpublished) by methods similar to those previously described.[5]

EXPERIMENTAL SECTION

Unless otherwise mentioned reagents and materials are as previously described.[24] Fluorescein isothiocyanate (FITC) isomer I, 4-fluoro-7-nitrobenzofurazan (NBDF) and tetramethylrhodamine isothiocyanate

isomer R (TMRITC) were obtained from Sigma. Texas Red hydrazide was obtained from Molecular Probes (Junction City, Oregon, USA). Fluorescein thiosemicarbazide was prepared by reaction of FITC (25mg, 64μmole) and hydrazine hydrate (3.4mg, 67μmoles) in DMF (2ml) for 30 min at room temperature. DMF was removed by evaporation, the product washed with cold ethanol (1ml) and crystallised from ethanol (yield 92%).

N-hydroxysuccinimidobiotin (NHS-biotin) and biotin hydrazide are available from Sigma. However, because of variable quality, the compounds were also prepared as follows. NHS-biotin was prepared from biotin (Sigma) by the method of Becker et al.[27] Biotin hydrazide was prepared essentially by the method of Bayer et al[28] except that NHS-biotin was used instead of biotinyl chloride. Hence NHS-biotin (100mg, 0.29mmole) was dissolved in DMF (3ml) and hydrazine hydrate (16mg, 0.32mmole) added. After stirring for 15 min the DMF was removed by evaporation, the product washed with diethyl ether and crystallised from DMF (66% yield, mp 242-243°).

Oligonucleotide Synthesis was carried out by the phosphotriester method using an Omnifit (Cambridge, UK) manually operated flow system using synthesis materials obtained from Cruachan Chemicals, Livingstone, Scotland as previously described,[23,24] and by the phosphoramidite method using an Applied Biosystems 380B DNA synthesiser using methyl phosphoramidites (Applied Biosystems) or 2-cyanoethyl phosphoramidites (B.D.H., Poole, UK) following manufacturers recommendations.

Hplc was carried out using a Waters gradient hplc-system consisting of two model 510 pumps, a 680 gradient programmer, 481 variable wavelength UV detector, 730 data module and a Rheodyne 7125 injector. Columns were of the Radial PAK cartridge type of μ-Bondapak C18 (reversed phase) or Whatman 10-SAX (ion exchange) for use with a Z module system.[29] Chromatography was carried out at ambient temperature at a flow rate of 3ml min^{-1}. For ion exchange hplc the buffers were prepared from a stock solution of 1M KH_2PO_4 adjusted to pH 6.3 with KOH to give (A) 1mM and (B) 0.3M both containing 60% formamide, Gradient was 0% 4', 0-90% 45'. For reversed phase hplc the buffer was 0.1M ammonium acetate (pH unadjusted) containing (A) 0% CH_3CN and (B) 80% CH_3CN. Gradient was 0% B 2', 0-8% B 5', 8-18% B 30'.

Tlc was carried out on kieselgel 60 F254 plates (aluminium backed, Merck 5554) using solvent (a) chloroform (acid free)/methanol (9:1) or (b) dichloromethane/ethylacetate/triethylamine (45:45:10). For the detection of biotin and derivatives on tlc plates the p-dimethylaminocinnamaldehyde spray reagent[30] gave pink spots on a white background.

NMR ^{1}H spectra were recorded relative to TMS on a Varian T60 spectrometer. ^{31}P spectra were recorded relative to H_3PO_4 on a Bruker 90 or 250 MHz spectrometer.

2',3'-di-O-pixyl uridine (III)

To 5'-O-acetyl uridine[17] (2.5g, 8.73mmoles) in dry pyridine (25ml) was added pixyl chloride (5.62g, 19.2mmoles) in pyridine (25ml) dropwise while stirring. After reaction at room temperature overnight (15hrs) tlc in solvent (a) showed complete conversion to a product of higher Rf. The reaction mixture was evaporated to a gum, dissolved in 8M methanolic ammonia (25ml) and left at room temperature for 30 min. The reaction mixture was evaporated to dryness, co-evaporated with toluene and ethanol and chromatographed on a silica gel column eluting with chloroform and then methanol/chloroform (5:95). Pure factions were collected and evaporated to dryness. The product was crystallised from toluene with a small amount of methanol added. Yield 2.5g (38%), mp 170-172°; tlc homogeneous in solvent a.

2',3'-di-O-pixyl uridine 5'-O-(2-chlorophenyl, p-nitrobenzyl)phosphate (IV)

To 1-Hydroxybenzotriazole (3mmoles), 40g) dissolved in THF (10ml) was added 2-chlorophenyl phosphorodichloridate (1.5mmoles), 0.37g) and anhydrous pyridine (3.3moles, 0.261g). After stirring for 40 min 2',3'-di-O-pixyl uridine (1mmol, 0.76g) in pyridine (5ml) was added and the reaction mixture stirred for 40 min. p-Nitrobenzylalcohol (2mmole, 0.306g) in pyridine (2ml) was added and the reaction mixture stirred for a further 2hrs. The product was evaporated to small volume, partitioned between chloroform (30ml) and saturated sodium bicarbonate solution (30ml). The chloroform layer was washed again with sodium bicarbonate solution and the combined aqueous layers washed once with chloroform (30ml). The combined chloroform extracts (60ml) were dried over magnesium sulphate and evaporated to a foam. The product was chromatographed on a silica gel column eluting with chloroform and then ethanol/chloroform (2:98). Product was precipitated from chloroform into n-pentane. Yield 0.504g (47%). ^{31}P NMR(δ)(DMSO) 4.96, 5.03 (two diasteromers).

Triethylammonium 2',3'-di-O-pixyluridine-5'-O-(2-chlorophenyl)phosphate] V

Compound IV (0.25mmoles), 0.271g) was dissolved in dry acetonitrile (6.25ml) and p-toluenethiol (2mmoles, 0.248g) dissolved in acetonitrile (6.25ml) added. To the stirred reaction mixture was added dry triethylamine (1.8mmoles, 0.182g). TLC (solvent a) after 10 min showed complete conversion to base-line material. The mixture was evaporated to dryness, dissolved in chloroform (10ml) and precipitated from cyclohexane followed by reprecipitation from ether and finally from n-pentane. Yield

0.212g (81%) ^{31}P NMR(δ)(DMSO) 4.57.

2',3'-di-O-pixyluridine-5'-O-(methyl,N-N-diisopropylamino)phosphite VI

To a mixture of 2',3'-di-O-pixyl uridine, (0.757g, 1mmole) and diisopropylammonium tetrazolide (0.086g, 0.5mmole) dissolved in dry dichloromethane (5ml) was added under N_2 bis(diisopropylamino)-methoxyphosphine (0.394g, 1.5mmoles). After 1.5h the reaction mixture was poured into 20ml of saturated aqueous sodium bicarbonate and extracted with dichloromethane (20ml). The organic phase was washed with brine (2 x 20ml) and dried over sodium sulphate. Solvent was removed by evaporation to yield a foam. Product was precipitated from dichloromethane solution into n-pentane. Yield 0.725g (79%) ^{31}P NMR(δ)(DMSO) 148.1, 149.4.

Assembly of rU-(5'-5')d[GTAAAACGACGGCCAGT] (IX)

a) Phosphotriester Route: 1μmole scale (CPG glass support) by standard method.[23,24] In the final coupling step monomer V dissolved in pyridine was used. After deprotection the product was dissolved in water (2.5ml) and purified by ion exchange hplc in 5 injections. After dialysis against distilled water 10.7A_{260} units was obtained (5.5% yield).

b) Phosphoramidite Route: 1μmole scale (CPG glass support) using ABS DNA synthesiser and deoxynucleoside methyl-N,N-diisopropyl phosphoramidites. In the final coupling step monomer VI dissolved in anhydrous acetonitrile was used. After deprotection the product was dissolved in water (2ml) and purified by ion exchange hplc in 8 injections. (Figure 3 shows analytical hplc). After dialysis against distilled water 34 A_{260} units were obtained (17.6% yield). Reversed phase hplc - Figure 4(a).

Attachment of Biotin to IX

a) Oxidation with periodate: 5-20 A_{260} units of IX was dissolved in 0.5ml 0.1M sodium acetate buffer (pH 5) and 0.5ml of freshly prepared sodium periodate solution (10mM) added. After incubation at 4° for 1h the product was purified by reversed phase hplc (3 A_{260} units per injection) and dialysed against water. The product (X) was homogeneous by hplc (Figure 4(b)). Yield: 80-85%. (NB hplc purification is not necessary if the starting oligonucleotide is highly purified).

b) Reaction of X with Biotin Hydrazide: 1-5 A_{260} units of X was dissolved in 250μl of 100mM sodium acetate buffer (pH 5) and 250μl of 15mM biotin hydrazide solution in methoxymethanol/water (1:1) added. After incubation at 37° for 2h the product was either dialysed against distilled water at 4° overnight in the dark or passed through a Sephadex G25 column eluting with ethanol/water (2:8). Reversed phase hplc (Figure 6a) showed a major peak

more retained than dialdehyde X. 0.5 A_{260} units of this product heated at 65° for 2.5h showed by hplc (Figure 6b) ca 50% conversion back to dialdehyde X.

c) Reaction of X with Biotin Hydrazide Followed by Reduction: To the reaction mixture obtained from b) (before dialysis) was added at room temperature 0.5ml of 100mM Tris/HCl buffer (pH9). Sodium borohydride, freshly dissolved in water, was added in increments at 30 min intervals to achieve a final concentration of 0.1M. Incubation was continued at 20° for 3h. The pH was adjusted to 5 by addition of sodium acetate buffer and the mixture dialysed against water at 4° overnight in the dark or passed through a Sephadex G25 column as in b). Reversed phase hplc of the product (XI) (Figure 7) showed one major component which was purified by hplc. After dialysis against water the yield was 77%. No change in hplc pattern was observed after heating XI at 65° for 2.5h in water.

2-(9-fluorenylmethoxycarbonyl)aminoethanol (VII)

To ethanolamine (10% aqueous W/V, 22.5mmoles) was added aqueous sodium carbonate (30% solution W/V, 59mmoles) and acetone (20ml). A solution of 9-fluorenylmethyl chloroformate (23.65mmoles) in acetone (15ml) was added over 15 min with stirring. A white solid separated out, which was filtered off, washed with ice cold acetone-water (25ml), 1:1) and dried overnight in a vacuum dessicator to give VII 5.96g (20.1mmoles, 89.5% yield). Mp 144°. ^{1}H NMR(δ)(DMSO) 2.2 (s(b),OH,1H); 3.4 (m,CH_2O,2H); 3.6 (t,CH_2N,2H); 4.2-4.5 (m,CH_2OCO + H(C9), 3H); 5.2 (s(b), NH,1H); 7.2-7.9 (m, aromatic, 8H).

2-cyanoethyl N,N-diisopropylaminophosphochloridite

2-Cyanoethylphosphodichloridite[31] (15g, 0.087mol) was dissolved in anhydrous diethyl ether (70ml) cooled to 15° (ice-water bath) and diisopropylamine (24.5ml, 0.175mol) added dropwise under N_2 over 1.5h. Diisopropylamminium chloride was removed by filtration and the solution evaporated to an oil. The oil was redissolved in ether and a slight precipitate removed by filtration through a fine porosity sinter. Evaporation to dryness yielded an oil (17g, 83% yield). ^{1}H NMR(δ)($CDCl_3$) 1.4 (d,CH_3,12H); 2.7-2.9 (t,CH_2CN,2H); 3.5-4.2 (m, CH_2O + CH(N),4H).

2-(9-fluorenylmethoxycarbonyl)aminoethyl (2-cyanoethyl,N,N-diisopropylamino)phosphite (VIII)

To VII (0.239g, 1mmole) suspended in anhydrous dichloromethane (3,ml) was added diisopropylethylamine (0.78ml, 4.6mmole) and the mixture stirred at room temperature. 2-Cyanoethyl, N,N-diisopropylaminophosphochloridite (0.473g, 2mmole) was added and after 15 min at room temperature ethyl acetate (30ml) was added. This was washed with cold saturated brine (2 x

25ml) and the organic phase dried over sodium sulphate. The solvent was removed by evaporation to yield a colourless oil, 470mg (99%). ^{31}P NMR(δ) (DMSO) 146.4

Assembly of $NH_2(CH_2)_2O$-d[pGTAAAACGACGGCCAGT]

Oligonucleotide synthesis was carried out by the phosphoramidite route using deoxynucleoside 2-cyanoethyl, N,N-diisopropyl phosphoramidites. In the final step the linker molecule VIII dissolved in acetonitrile was used. After deprotection the product was dissolved in water (1ml) and purified by ion exchange hplc in 5 injections (Figure 9 shows an analytical run). After dialysis against distilled water 47.4 A_{260} units (24.5% yield) were obtained. Reversed phase hplc - Figure 10(a) and coinjection with unfunctionalised primer - Figure 10(b).

Attachment of Biotin to XII

1.25 A_{260} units of XII was dissolved in 150µl of 0.25M Tris/HCl buffer (pH 7.6), 150µl of 15mM NHS-biotin in DMF added and the mixture left at room temperature for 18h. Analytical reversed phase hplc showed greater than 85% conversion to a more retained peak (Table 1). The product was purified by reversed phase hplc and the material in the major peak dialysed against distilled water to yield 0.91 A_{260} units (79% yield).

Attachment of Fluorescent Labels to XII

To 1.5-2.5 A_{260} units of XII in 150µl water was added 1mg of fluorescent agent (NBDF, FITC or TMRITC) dissolved in 150µl of either 1M sodium carbonate-bicarbonate pH 9/water/acetonitrile (5:3:2) [for NBDF] or 1M sodium carbonate-bicarbonate pH 9/water/DMF (5:8:2) [for FITC] or 0.1M sodium carbonate-bicarbonate pH 9/water/DMF (7.5:2.5:5) [for TMRITC]. The mixture was left at room temperature in the dark for 18h and the products examined by hplc. (Table 1). In all cases reaction had proceded to greater-than 85%. The reaction mixture was applied to a Sephadex G25 (fine) column [17 x 1.5cm] and eluted with ethanol/water (2.8). The material appearing in the excluded volume (in the case of TMRITC reaction the gel filtration had to be repeated in order to remove small amounts of contaminating dye) was further purified by reversed phase hplc. Yields 47-75% as single or multiple peaks (Table 1). UV Spectra were carried out in water (NB extinction coefficients vary substantially with solvent). FITC-NH_2-17 λmax 260, 481; isomer A 260/481 : 9.4; isomer B 260/481 : 9.6. NBD-NH_2-17 : λmax 260, 486; 260/486 : 6.6. TMRITC-NH_2-17 λ max 260,560 : isomer A 260/560 : 5.6; isomer B 260/560 : 4.0; isomer C 260/560 : 5.6.

Hybridisation Assay for Biotinylated Oligonucleotides

To 100ng of M13 DNA (single or double stranded) in 20µl of 10mM Tris.HCl (pH 7.4), 0.1mM EDTA was added 1.5µl of 4M NaOH and the mixture heated at 60-70° for 30 min. After cooling the solution was neutralised with 20µl of 2M ammonium acetate solution and in serial dilutions spotted on a prewashed nitrocellulose membrane filter (Schliecher and Schuell, 0.45µ). The filter was baked at 80° for 1h, washed in 6 x SSC for 5 min and then prehybridised at 67° for 5 min in 6 x SSC, 10 x Denhardt's (0.2% BSA, 0.2% polyvinylpyrrollidone PVP 40, 0.2% Ficoll, 0.2% SDS). After brief rinsing in 6 x SSC, the biotinylated oligonucleotide (25ng/ml) was added and the hybridisation continued for 1h at 37°. The filter was washed with 6 x SSC at 25° for 3 x 1 min and biotin detected on duplicate filters by a) streptavidin/alkaline phosphatase kit (520-8239SA) from Gibco B.R.L. or b) streptavidin/alkaline phosphatase kit (520-8279SA, BluGene) from Gibco B.R.L. or c) an anti-biotin hybridoma/peroxidase system as follows. Filters were blocked in 5% foetal calf serum in PBS for 2h at room temperature or overnight at 4° and then incubated with a rat anti-biotin monoclonal antibody [33] for 30 min at room temperature and washed twice with 5% FCS/PBS containing 0.05% Triton X-100. The filter was then incubated with rabbit anti-rat IgG peroxidase conjugate (Miles-Yeda) for 30 min at room temperature. After washing the filter twice as before the filter was developed in 4-chloro-1-naphthol to give dark blue spots on a white background.

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