Synthesis and antibody-mediated detection of oligonucleotides containing multiple 2,4-dinitrophenyl reporter groups

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Received March 10, 1993; Revised and Accepted September 23, 1993

ABSTRACT

A series of non-nucleoside-based 2,4-dinitrophenyl (DNP) phosphoramidites have been prepared and used in the multiple labelling of oligonucleotides during solid-phase synthesis. The length of spacer arm between the DNP label and the oligonucleotide phosphate backbone, and the number of attached DNP groups have both been varied in order to determine the optimum conditions for anti-DNP antibody binding. Detection using enzyme-linked colorimetric techniques showed sensitivity equivalent to that obtainable using biotinylated oligonucleotides.

INTRODUCTION

Detection of oligonucleotide probes can be achieved by the addition of non-radioactive reporter groups, thereby precluding the hazardous, expensive and time consuming introduction of radiolabels such as $^3$H, $^{32}$P, $^{35}$S and $^{125}$I. Despite these advantages, non-radioactive labelling of oligonucleotides has been limited by its poor sensitivity relative to standard radiolabelling techniques. A solution to this problem is provided by the attachment of multiple labels and this has facilitated the visualisation of oligonucleotide probes in quantities previously obtainable only by radiolabelling (1,2). The requirements for the multiple attachment of non-radioactive labels are: (i) the aqueous solubility of the oligonucleotide must be unaffected by the introduction of lipophilic reporter groups; (ii) the reporter groups must not interfere with oligonucleotide hybridisation; (iii) the addition of reporter groups should be controllable to allow the attachment of a defined number of labels; and (iv) the labelling procedure should be simple and the materials inexpensive.

The most commonly used non-radioactive labelling group is biotin, which has been incorporated into oligonucleotides enzymically (3,4), photochemically (5), by reaction with amino-functionalised oligonucleotides (6–10), and by incorporation of biotinylated phosphoramidites during solid-phase synthesis (11–14). The main drawbacks are that biotin and its derivatives are expensive, the synthesis of biotinylated phosphoramidites is made difficult by the poor solubility of biotin, and endogenous biotin occurs at high levels in certain tissues, making it unsuitable for some types of in situ hybridisation. Detection of biotinylated oligonucleotides commonly employs enzyme linked immunosorbent assay (ELISA) methodology, with colorimetric or chemiluminescent visualisation via avidin or streptavidin conjugated to reporter enzymes such as alkaline phosphatase or horseradish peroxidase. Oligonucleotides can also be labelled with these enzymes directly, although the oligonucleotide-enzyme conjugate synthesis and purification procedures are time consuming (15) and such conjugates are unsuitable for use in techniques such as P.C.R. Fluorescent labelling of amino-modified oligonucleotides using fluorescein isothiocyanate (FITC) and other fluorescent dyes is now routine (15–19). However, the detection of oligonucleotides labelled in this way requires specialised equipment.

Recently, the hapten digoxigenin has been used as a non-radioactive reporter group (20–22). The labelled oligonucleotides are detected by digoxigenin-specific polyclonal sheep antibodies conjugated to alkaline phosphatase. Labelling with digoxigenin involves the enzymic incorporation of digoxigenin-labelled deoxyuridine-triphosphate into DNA, or via the reaction of a digoxigenin active ester with 5'-amino modified oligonucleotides. However the chemical labelling procedure is expensive and allows only the introduction of a single label whereas the enzymic method does not allow the controlled insertion of a specific number of labels.

An inexpensive label which can be detected immunogenically is the 2,4-dinitrophenyl group (DNP group). The DNP group has been introduced into oligonucleotides via the action of deoxyribonucleotidyl terminal transferase or DNA polymerase on a DNP-aminohexyl derivative of ATP, and by the reaction of Sanger's reagent (2,4-dinitrofluorobenzene) with oligonucleotides containing an aminohexyl derivative of adenosine (23). DNP groups have been introduced photochemically (24) and by reaction with brominated bases (25). Both single and multiple

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DNP groups have been incorporated into oligonucleotides using DNP phosphoramidites during solid phase synthesis, although in both examples of multiple addition the DNP group was unstable in aqueous ammonia under normal base-catalysed deprotection conditions (12,26).

Oligonucleotides labelled with a multiple addition DNP phosphoramidite have been previously synthesised by this group. These oligonucleotides, which had the DNP groups directly linked to the nitrogen atoms of a 3,6-diazaoctane backbone gave much lower sensitivity on detection with a monoclonal IgG mouse anti-DNP antibody than those labelled with a single addition DNP phosphoramidite separated from the DNA backbone, by a hexyl spacer (26). This suggested to us that a spacer arm is necessary for maximum sensitivity using the above antibody. The aim of this work was to develop a range of simple DNP phosphoramidites with various spacer arms for the poly-labelling of oligonucleotides during standard solid phase synthesis.

RESULTS AND DISCUSSION

The first step in the synthesis of a 2,4-dinitrophenyl multiple addition phosphoramidite is the construction of the backbone onto which the DNP label, a dimethoxytrityl group and a phosphoramidite group can be attached. There are two basic requirements for this backbone: (i) Two hydroxyl functions, preferably one primary and one secondary to give the necessary specificity for the sequential addition of the dimethoxytrityl and phosphoramidite moieties, (ii) An amino group separated from the backbone by a linker to allow attachment of the DNP group. Misiura et al (1) made use of a 3-carbon glycerol type backbone in the construction of a biotin phosphoramidite, while Nelson et al (27) have used the readily available 3-amino-1,2-propanediol to introduce multiple primary amino groups to the 5' end of oligonucleotides. We describe here the synthesis of a range of DNP phosphoramidites with various spacer arms for the poly-labelling of oligonucleotides during standard solid phase synthesis.

Synthesis of DNP phosphoramidites

DNP phosphoramidite [1c]. (Scheme 1) 3-Amino-1,2-propanediol was treated with 2,4-dinitrofluorobenzene to give, after column chromatography the DNP-diol derivative in 82% yield. Reaction of the diol with 4,4'-dimethoxytrityl chloride gave the monomer precursor [1b] in 90% yield and subsequent reaction of [1b] with 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite afforded the DNP phosphoramidite [1c] in 47% yield.

DNP phosphoramidite [2g]. (Scheme 2) 1,2,6-Trihydroxyhexane was converted to its acetonide derivative [2a] in a yield of 85% by reaction with anhydrous acetone in the presence of anhydrous sodium sulphate and concentrated aqueous HCl. Compound [2a] was converted to the tosylate [2b] and the tosyl group was displaced by the action of potassium phthalimide in DMF to give the phthalimide [2c] in 96% yield. Subsequent removal of the phthaloyl group with hydrazine hydrate in methanol gave, after distillation, the amine [2d] in 51% yield. This amine was reacted with 2,4-dinitrofluorobenzene in methanol in the presence of triethylamine to give, after wet flash column chromatography the DNP derivative [2e] in quantitative yield. The isopropylidine group was removed by acidic treatment and the resulting diol was reacted with 4,4'-dimethoxytrityl chloride in anhydrous pyridine to give compound [2f], after chromatography, in 72% yield. This was then phosphitylated in the normal way to give the DNP-phosphoramidite [2g].

DNP phosphoramidite [3c]. (Scheme 3) This compound has a 1-O-(3-aminopropyl) glycerol backbone, originally developed by Misiura et al (1) for use in the construction of multiple addition biotin and phosphotyrosyl phosphoramidites. No alterations were made to the original experimental procedure to prepare 3-aminopropyl solketal. This was treated with 2,4-dinitrofluorobenzene to give, after wet flash column chromatography the DNP derivative [3a] in 94% yield. Subsequent removal of the...
isopropylidene protecting group followed by addition of a dimethoxytrityl group gave the DNP monomer precursor [3b] in 79% yield. Reaction of [3b] with 2-cyanoethyl-N,N-

disopropyl phosphoramidochloridite gave the DNP phosphoramidite [3c] in 83% yield.

**DNP phosphoramidite [4f]**. (Scheme 4) 6-Aminohexanoic acid was reacted with ethyl trifluoroacetate to give a near quantitative yield of the trifluoroacetyl (TFA) protected amino acid [4a]. This was converted to the 2-nitrophenyl active ester [4b] which was reacted with 3-aminopropyl solketal to give the TFA-acetonide [4c] in quantitative yield. Heating this compound in conc. aqueous ammonia in a sealed pressure tube at 70°C cleanly removed the TFA protecting group and subsequent reaction of the crude free amine with 2,4-dinitrofluorobenzene in methanol and triethylamine gave the DNP-acetonide [4d]. Removal of the isopropylidene group by acidic treatment followed by the standard tritylation and phosphitylation procedures, gave the DNP phosphoramidite [4f] in an overall yield of 78%.

**DNP-labelling of oligonucleotides**

**Synthesis of DNP-labelled oligonucleotides.** The solid phase synthesis of DNP-labelled oligonucleotides was carried out using DNP phosphoramidites [1c], [2g], [3c] and [4f] as 0.15M solutions in anhydrous acetonitrile. A standard 0.2-μmol-scale synthesis cycle was used in all cases. All phosphoramidites gave excellent coupling efficiencies under standard conditions and no modifications to either solvent or oligonucleotide synthesis cycle were required. However, after a ‘trityl-off’ end procedure oligonucleotides labelled with each of these phosphoramidites were substantially degraded in conc. ammonia at 55°C after 8 hours (H.P.L.C. analysis). These observations are in accordance with those of Misiura et al [1], who suggested that attack of the free glyceryl hydroxyl group on the adjacent phosphate linkage during ammonia treatment might give rise to elimination of the terminal glyceryl unit. Fortunately multiple DNP labelled oligonucleotides synthesised ‘trityl-on’ are completely stable to normal deprotection conditions, so this procedure was adopted throughout.

**Purification of DNP labelled oligonucleotides.** The reversed phase H.P.L.C. purification of multiple DNP labelled oligonucleotides is very easy as the lipophilic DNP groups cause labelled oligonucleotides to elute much later than failure sequences. Additional lipophilicity is gained by the presence of the terminal DMTr-group and this was left attached to the oligonucleotide during H.P.L.C. purification. The effect of increasing the number of DNP groups on oligonucleotide retention times is shown in Figure (1). After H.P.L.C. purification the oligonucleotides were detritylated with acetic acid and desalted by gel-filtration on Sephadex G-25. DNP labelled oligonucleotides are bright yellow in colour.

**DNP label detection.** The detection of DNP labelled oligonucleotides was carried out simply and conveniently using the following procedure:

(i) Known amounts of DNP labelled 24-mer probes were fixed

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**Figure 1.** Reversed phase H.P.L.C. chromatogram of a mixed injection of purified 5'-DMTr-multiple DNP labelled 24-mers and unlabelled reference sequence: From left to right: unmodified 24-mer, (DNP)2-24mer, (DNP)3-24mer. DNP labels were introduced via phosphoramidite [3c].
Figure 2. Colorimetric detection of 24-mer probes end-labelled with 1, 3 and 5 DNP groups. DNP labels introduced via phosphoramidite [3c]. Oligonucleotides were immobilised on nylon membrane.

Figure 3. DNA synthesis phosphoramidite monomer used to introduce a tetraethylene glycol spacer into the DNA backbone.

Figure 4. Colorimetric detection of DNP labels attached to 24mer oligonucleotide probes by a tetraethylene glycol linker arm. One and three DNP labels were introduced using phosphoramidite 3c.

Figure 5. Colorimetric detection of the DNP labels in 3'-DNP-5'-biotin labelled 24-mer oligonucleotide probes. One, three and five DNP labels were introduced via phosphoramidite [3c].

Figure 6. Colorimetric detection of the biotin labels in 3'-DNP-5'-biotin labelled 24-mer probes. Biotin labels were introduced via a commercially available single addition biotin phosphoramidite (Cruachem Ltd).

It is clear from Figure 2 that three DNP labels gave better sensitivity than a single DNP group. However, increasing the number of labels from three to five did not give any further improvement (Figures 2, 5, 7). Why do three DNP groups give optimum sensitivity? Clearly, it is unlikely that more than one antibody molecule can bind simultaneously to a short string of DNP groups due to the huge size of an IgG molecule relative to the DNP monomer units. In addition, the space between the two FAB regions of an IgG molecule is very large so it is not possible for both arms of the antibody to bind to a single short oligonucleotide.

Does the steric bulk of the oligonucleotide hinder the interaction between some of the DNP groups and the antibody? To investigate this a tetraethylene glycol monomer was prepared (Figure 3) and incorporated during oligonucleotide synthesis between the 5'-end of the oligonucleotide and the DNP labelling groups. Although the tetraethylene glycol spacer arm separates the DNP groups from the oligonucleotide it does not produce any enhancement in colorimetric detection (Figure 4). As tetraethylene glycol is similar in length to two DNP monomer units and is stericly undemanding, the lack of increased signal with this spacer suggest that the steric bulk of the oligonucleotide does not hinder the antibody-hapten interaction. It is possible that the increased sensitivity achieved by three DNP groups relative to one arises from co-operativity between the DNP groups. When one DNP group occupies the antibody binding site, its neighbours all lie very close to the site. Thus there is a high local concentration of haptens around the antibody binding site. Under these circumstances it is reasonable to assume that three DNP groups may be as effective as five.

Significantly, labelling with three or more DNP groups gave detection limits comparable with those of biotin labelled oligonucleotides (Figures 5 & 6). A much lower background was obtained with no significant loss of signal using a commercially available anti-DNP polyclonal antibody-horseradish peroxidase conjugate (Dakopats, results not shown).

All four phosphoramidite monomers 1c, 2g, 3c and 4f were attached singly and multiply to synthetic oligonucleotides to investigate the relationship between sensitivity of detection and
spacer arm length. Figure 7 shows that there is no significant difference in detection sensitivity between any of the monomers. Therefore, our results show that the optimum DNP oligonucleotide-labelling system will consist of three DNP labels attached to an oligonucleotide probe via a spacer arm of any reasonable length.

Conclusion
These results augur well for the establishment of the DNP hapten as a useful alternative to currently available non-radioactive labels such as biotin or digoxigenin. The combined advantages of minimal background signal, ease of introduction and low cost offer a system of practical and commercial viability. The uses of DNP labelled oligonucleotides as probes in applications such as the detection of Polymerase Chain Reaction products and in situ hybridisation are currently under investigation. The results of these studies, which are so far extremely encouraging, will be published elsewhere.

EXPERIMENTAL

Anhydrous solvents: Dichloromethane was distilled over CaH₂; tetrahydrofuran and diethyl ether were distilled over sodium/benzophenone; N,N-dimethylformamide was fractionally distilled under reduced pressure over 4A molecular sieves; pyridine was distilled over CaH₂; acetonitrile was distilled over anhydrous CaSO₄ (Sikkon Fluka Universal Desiccant); hexane was dried over Na wire; triethylamine and N,N-diisopropylethylamine were dried over CaH₂; anhydrous acetonitrile was purchased from Applied Biosystems Inc. (ABI). Pyrogen-free, distilled under reduced pressure over 4A molecular sieves; was dried over Na wire; triethylamine and N,N-diisopropylethylamine and 3-nitrobenzyl alcohol (3-NOBA) matrix. Anhydrous solvents: 2D Dichloromethane was distilled over CaH₂ using 31P-NMR spectra were recorded on a Brucker WP-200 spectrometer (90MHz). Positive Fast Atom Bombardment (FAB) analysis and purification. All other reagents were supplied by Aldrich or Fluka. 'H-NMR spectra were recorded on a Brucker WP-80 spectrometer (80MHz) and a Brucker WP-200 spectrometer (200.130MHz); 31P-NMR spectra were recorded on a Brucker WP-200 spectrometer (81MHz) or on a Jeol FX90Q spectrometer (90MHz). Positive Fast Atom Bombardment (FAB) mass spectra were recorded on a Kratos MS50 TC spectrometer using a thioglycerol or 3-nitrobenzyl alcohol (3-NOBA) matrix.

Wet flash column chromatography was carried out using silica gel 60 (Fluka). Thin layer chromatography (tlc) was carried out on aluminium sheets, silica 60 F₂₅₄, 0.2mm layer (Merck) using the following solvent systems:

(A) Dichloromethane-methanol (90:10, v/v)
(B) Toluene-ethyl acetate (4:1, v/v)
(C) Ethyl acetate-methanol-NH₄OH (5:1:1, v/v)
(D) Hexane-ethyl acetate (1:1, v/v)
(E) Dichloromethane-ethyl acetate (1:1, v/v)
(F) Dichloromethane-methanol (99:1, v/v)

0.1ml of triethylamine was added to 10ml of each of the above when compounds bearing a dimethoxytrityl group were examined by tlc.

Supplementary data. Syntheses of all compounds in schemes 2 and 3 are described in detail. Full experimental details for the synthesis of the compounds in schemes 1 and 4 and Figure 7 are available on request. 13C n.m.r. and i.r. spectral data are also available for most compounds.

5,6-Isopropylidene dioxyhex-1-ol (2a). To a solution of 1,2,6-trihydroxyhexane (37g; 0.27mol) in anhydrous acetone (250ml) was added anhydrous sodium sulphate (100g) and conc. aqueous HCl (5ml). The reaction was stirred overnight at 20°C. Lead (II) carbonate (basic form) (100g) and Na₂CO₃ (1g) were added and the reaction was set aside for 48 hrs. Solids were then filtered off and the solvent was evaporated in vacuo to give a pale yellow liquid. Na₂CO₃ (1g) was added to the liquid to maintain basic conditions and the product was purified by kugelrohr distillation to give a clear liquid (b.pt. 120°C at 0.038 mm Hg), (39.92g,85%). Rf 0.46 (solvent A). 1H n.m.r. spectra were recorded on a Brucker WP-200 spectrometer (81MHz) or on a Jeol FX90Q spectrometer (200.130MHz) and a Brucker WP-200 spectrometer (81MHz) or on a Jeol FX90Q spectrometer (90MHz). Positive Fast Atom Bombardment (FAB) mass spectra were recorded on a Kratos MS50 TC spectrometer using a thioglycerol or 3-nitrobenzyl alcohol (3-NOBA) matrix.

Wet flash column chromatography was carried out using silica gel 60 (Fluka). Thin layer chromatography (tlc) was carried out on aluminium sheets, silica 60 F₂₅₄, 0.2mm layer (Merck) using the following solvent systems:
10% ethyl acetate in toluene, to give a waxy white solid (3.037g, 96%). Rf 0.37 (solvent B). 1H n.m.r. data (CDCl3): δH 1.24 (s, 3H, CH3), 1.29 (s, 3H, CH3), 1.30 - 1.71 (m, 2H, CH2), 3.37 - 3.46 (m, 1H, CH), 3.60 (t, 2H, N-CH2), J = 7.1Hz, 3.89 - 4.01 (m, 2H, CH2), 7.60 - 7.78 (m, 4H, Ar-CH); F.a.b. mass spectrum: m/z 304.15488 calc. for C17H22NO4 (M+H)+, m/z 304.15487.

5,6-Isopropylidene dioxycleylamine [2d]. To a solution of [2c] (2.501g; 8.25mmol) in methanol (10ml) was added hydrazine hydrate (9.08mmol; 0.44ml). The reaction was stirred for 16hrs. at 20°C, during which time a white precipitate formed. The solution was evaporated to dryness, and the residue was dissolved in diethyl ether and washed with 2M NaOH (30ml). The aqueous phase was extracted with diethyl ether (3 x 20ml) and the combined organic phase was dried (MgSO4), filtered and evaporated in vacuo to give a white solid. The product was purified by kugelrohr distillation to give a clear liquid (b.p. 100°C at 0.0076 mmHg), (0.720g, 51%). Rf 0.36 (solvent C). 1H n.m.r. data (CDCl3): δH 1.27 (s, 3H, CH3), 1.32 (s, 3H, CH3), 1.17 - 1.63 (m, 6H, CH2), 1.49 (s, 2H, NH2), 2.62 (t, 2H, N-CH2), J = 6.6Hz), 3.32 - 3.46 (m, 1H, CH), 3.92 - 4.03 (m, 2H, CH2), 7.60 - 7.78 (m, 4H, Ar-CH), 8.54 (bt, 1H, NH), 9.07 (d, 1H, DNP H-3, J = 2.6Hz). F.a.b. mass spectrum: m/z 356.14579.

1-(2,4-Dinitrophenylamino)-5,6-isopropylidene dioxycleyxane [2e]. To a solution of 2,4-dinitrofluorobenzene (1.88g, 10mmol) was added triethylamine (3.0ml, 22mmol) in methanol (10ml) and the reaction mixture was stirred at room temperature for 2 hours. The reaction mixture was quenched with methanol (10ml), and excess hydrazine hydrate (3.0ml) was added to a solution of 3-aminopropyl solketal (2.30g, 12mmol) in methanol (10ml) and the resultant oil was dried by coevaporation (4 x 20ml), the organic phase dried (MgSO4), and the residue was purified by wet flash column chromatography, eluting with a gradient of 40% ethyl acetate in pentane. The result was washed with sat. aqueous KCl (4 x 20ml), and the residue was purified in vacuo, and the product was purified by wet flash column chromatography, eluting with a gradient of 0 to 3% methanol in dichloromethane methanol. The appropriate fractions were evaporated in vacuo to give the title compound as a yellow oil (3.42g, 94%). Rf 0.56 (solvent F). 1H n.m.r. data (CDCl3): δH 1.27 (s, 3H, CH3), 1.33 (s, 3H, CH3), 1.99 (quintet, J = 5.1Hz, 2H, CH2), 3.42 - 3.56 (m, 4H, CH2, CH, CH3), 3.98 - 4.12 (m, 2H, CH2), 6.89 (d, 1H, DNP H-6, J = 9.5Hz), 8.22 (dd, 1H, DNP H-5, J = 9.5Hz, J = 2.6Hz), 8.54 (bt, 1H, NH), 9.07 (d, 1H, DNP H-3, J = 2.6Hz). F.a.b. mass spectrum: m/z 340.15088 calc. for C17H16N2O6 (M+H)+, m/z 340.15085.

1-(4,4'-Dimethoxytrityloxy)-4-oxa-6,7-isopropylidene dioxycleyxane [2f]. To a solution of [2e] (1.00g; 2.95mmol) in anhydrous pyridine (10ml) was added 2-cyanoethyl N,N-diisopropyl phosphoramidite (1.19mmol; 0.266ml). After 1 hour the reaction was quenched by the addition of ethyl acetate (100ml). The resulting solution was washed with sat. aqueous KCl (4 x 20ml), the organic phase dried (MgSO4), filtered and evaporated in vacuo. The crude product was applied to a silica gel column which had been pre-equilibrated with hexane containing 40% ethyl acetate and 1% triethylamine. Elution with 40% ethyl acetate in hexane gave an orange oil (0.85g, 89%). Rf 0.54 (solvent D). 31P n.m.r. data (CD3CN solvent) δP147.57(s), 147.74 (s). F.a.b. mass spectrum: m/z 803.36588 calc. for C23H36N3O8P (M+H)+, m/z 803.36589.

1-(4,4'-Dimethoxytrityloxy)-4-oxa-6,7-isopropylidene dioxycleyxepane [3a]. 2,4-Dinitrofluorobenzene (1.88g, 10mmol) was added to a solution of 3-aminopropyl solketal (2.30g, 12mmol) and triethylamine (3.0ml, 22mmol) in methanol (10ml) and the solution was stirred at room temperature. After 24 hours the reaction mixture was evaporated in vacuo, and the product was purified by wet flash column chromatography, eluting with a gradient of 0 to 3% methanol in dichloromethane methanol. The appropriate fractions were evaporated in vacuo to give the title compound as a yellow oil (3.42g, 94%). Rf 0.56 (solvent F). 1H n.m.r. data (CDCl3): δH 1.27 (s, 3H, CH3), 1.33 (s, 3H, CH3), 1.99 (quintet, J = 5.1Hz, 2H, CH2), 3.42 - 3.56 (m, 4H, 2 x CH2), 3.60 - 3.73 (m, 3H, CH, CH3), 4.02 (dd, J = 8.3Hz, J = 6.4Hz, 1H, CH), 4.27 (quintet, J = 6.2Hz, 1H, CH), 6.91 (d, J = 9.5Hz, 1H, DNP H-6), 8.14 (dd, J = 2.6Hz, 1H, J = 9.5Hz, 1H, DNP H-5), 8.77 (bt, H, DNP-NH), 8.96 (d, J = 2.6Hz, 1H, DNP H-3). F.a.b. mass spectrum: m/z 356.14576 calc. for C17H36N3O8P (M+H)+, m/z 356.14579.
in hexane containing 2% triethylamine. The appropriate fractions were collected and evaporated in vacuo to give the title compound as a yellow foam (1.52g, 79%). Rf 0.23 (solvent D). 1H n.m.r. data (CDCl3): δH 2.01 (quintet, J=5.2Hz, 2H, CH2), 2.63 (bs, 1H, OH), 3.20 (d, J=5.4Hz, 2H, CH2), 3.43 - 3.85 (m, 12H, 3xCH2, 2xOCH3), 3.98 - 4.12 (m, 1H, CH), 6.76 - 6.88 (m, 4H, 4 aromatic CH), 7.13 - 7.43 (m, 10H, 10 aromatic CH), 8.21 (dd, J=2.8Hz, J=9.4Hz, 1H, DNP-H-5), 8.83 (bt, 1H, DNP-NH), 9.08 (d, J=2.8Hz, 1H, DNP-H-5). F.a.b. mass spectrum: m/z 617.23731 calc. for C33H33N3O9 (M+H)+., m/z 617.23750.

2-Cyanoethyl [(4,4'-dimethoxytrityloxy)-4-oxa-6-(2,4-dinitrophenylamino) hept-2-yl] N,N-diisopropylamino phosphoramide (3c). Compound [3b] (0.510g, 0.8mmol, 1eq) was coevaporated with anhydrous THF (3x5ml) and dissolved in anhydrous THF (5ml). To this solution was added N,N-diisopropylethylamine (0.5ml, 3mmol) and 2-cyanoethyl-N,N-diisopropylphosphonochloridit (0.31ml, 1.4mmol) and the mixture was stirred at room temperature for 3 hours. The reaction was quenched with ethyl acetate (50ml), washed with sat. aqueous NaHCO3 (2x50ml), the organic layer dried (Na2SO4), and then evaporated in vacuo. The crude product was purified by wet flash column chromatography on a short column of silica gel which was pre-equilibrated with sonication in hexane/triethylamine (99:1v/v). The product was applied to the column in the minimum volume of hexane/ethyl acetate (1:1v/v) and eluted with ethyl acetate. The appropriate fractions were evaporated in vacuo to give the title compound as a yellow foam (0.564g, 83%). Rf 0.37 and 0.44 (diastereomers), (solvent D). 31P n.m.r. data (CDCl3): δP 149.83 (s) and 150.00 (s). F.a.b. mass spectrum: m/z 818.35305 calc. for C42H33N3O10P (M+H)+., m/z 818.35298.

**Oligonucleotide synthesis.** Oligonucleotide synthesis was performed using cyanoethyl phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer. All DNA synthesis reagents and cyanoethyl-phosphoramidite monomers were supplied by Cruachem. DNP phosphoramidites were used as equivalents for di- and tri-functionalisation reactions. 'Trityl-on' synthesis was carried out throughout and all oligonucleotides were deprotected in cone, aqueous ammonia for 5 hours at 60°C.

**Measurement of phosphoramidite coupling efficiencies.** Fractions from successive detritylations were diluted to 25ml with 0.1M 4-toluene-sulphonic acid in acetonitrile and coupling efficiencies were measured by comparison of absorbances at 498nm.

**Oligonucleotide analysis and purification.** HPLC analysis and purification of 'trityl-on' oligonucleotides was carried out on a Gilson model 306 using a Brownlee Aquapore Octyl reverse phase column (10 mm x 250 mm) with a flow rate of 3ml/minute and the following gradient:

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Buffer A: 0.1M NH4OAc. Buffer B: 0.1M NH4OAc with 50% acetonitrile. The desired 'trityl-on' products eluted significantly later than failure sequences.

**Dethritylation of 'trityl-on' oligonucleotides.** After HPLC purification, 'trityl-on' oligonucleotides were evaporated to dryness and dissolved in 3% aqueous acetic acid (10ml). After 30mins at 20°C the solutions were evaporated to dryness in vacuo and oligonucleotides were desalted on NAP-10 columns (Sephadex G25, Pharmacia) following the manufacturer's instructions.

**Dot blot experiments.** Solutions used:
1. PBS/EDTA/EGTA: Phosphate-buffered saline solution (Sigma) containing 1mM EDTA and 1mM EGTA.
2. PBS/EDTA/EGTA/Tween: Phosphate-buffered saline solution (Sigma) containing 1mM EDTA and 1mM EGTA and 0.5% Tween-20.
3. Blocking solution: 10% solution of fat-free skimmed milk in PBS/EDTA/EGTA.
4. Mouse anti-DNP antibody ascitic fluid: containing approx. 7mg.ml⁻¹ of monoclonal anti-DNP antibody.
5. Avidin-HRP conjugate solution: a 0.2mg.ml⁻¹ solution of avidin-horseradish peroxidase conjugate (Sigma) in PBS.
6. DAB solution: a 0.5mg.ml⁻¹ solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 50mM Tris-HCl, pH 7.6. Hydrogen peroxide (0.06% final conc.) added just before use.

**Solutions of DNP and DNP/biotin labelled oligonucleotides of the appropriate concentrations were made up and 0.5µl of each solution was spotted onto nylon (Hybond N) filters (Amersham). The oligonucleotides were fixed to the membrane by UV irradiation for 5 min. The filters were blocked by incubation with blocking solution for 1h and washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min).

**DNP detection.** The filters were incubated for 1h with a 1:200 dilution of anti-DNP antibody ascitic fluid in PBS/EDTA/EGTA, then washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min). The filters were then incubated with a 1:1000 dilution of goat-anti-mouse IgG solution (Sigma) for 1h, then washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min).

**Biotin detection.** The filters were incubated for 1h with a 1:1000 dilution of avidin-HRP conjugate solution in PBS/EDTA/EGTA then washed with PBS/EDTA/EGTA/Tween (3 x 2min) and PBS/EDTA/EGTA (1 x 2min).

**Colour reaction.** The filters were incubated with DAB solution for 10—30 min. The reaction was stopped by washing the filter several times with water.

**ACKNOWLEDGEMENTS**

We are grateful to SERC for an earmarked studentship to D.W.W. and Cruachem Ltd. for a C.A.S.E. studentship to J.G.

**REFERENCES**