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The synthesis of oligonucleotides that contain 2,4-dinitrophenyl reporter groups*

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ABSTRACT

The synthesis of non-nucleoside-based phosphoramidites that bear the 2,4-dinitrophenyl group is reported. These labelled phosphoramidites, which have been used in solid-phase oligonucleotide synthesis to attach single and multiple dinitrophenyl groups to the 5′-end of oligonucleotides, are entirely compatible with the normal oligonucleotide synthesis cycle. Multiple labelling can be performed readily in high yield and the resulting oligonucleotides can be purified readily by reversed-phase h.p.l.c. The labelled oligonucleotides have been detected using monoclonal and polyclonal anti-dinitrophenyl antibodies.

INTRODUCTION

Non-radioactive labelling of oligonucleotides has attracted much interest in recent years as the number of biological and biomedical techniques that require labelled oligonucleotides has increased. The major drawback of non-radioactive detection has been its poor sensitivity relative to standard radiolabel-detection techniques. A solution to this problem may lie in the attachment of multiple labelling groups to the oligonucleotide which allow amplification of the detection signal. The attachment of multiple labels must not affect the hybridisation properties and solubility of the oligonucleotide, and it should be controllable in order to allow the optimum mumber of labels to be attached. The method used for multiple labelling should also be inexpensive and simple.

The most commonly used non-radioactive labelling group is biotin, which has been incorporated into oligonucleotides enzymically[1,2], by reaction with 5′-amino-functionalised oligonucleotides[3–7], and by incorporation of biotinylated phosphoramidites during solid-phase synthesis[8–10]. Only the last technique allows the introduction of multiple labelling groups in a controlled manner. The major disadvantages are that biotin and its analogues are expensive, the synthesis of biotinylated phosphoramidite monomers is made difficult by the poor solubility of biotin, and the presence of high levels of endogenous biotin in certain tissues makes it unsuitable for certain types of hybridisation *in situ*. Enzymic labelling of oligonucleotides with digoxigenin is also possible[11], but this technique is expensive and uncontrolled.

* Dedicated to Professor Grant Buchanan on the occasion of his 65th birthday.
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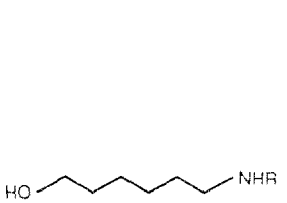
Labelling of oligonucleotides with fluorescent groups has also been achieved[9,12–15]. However, detection of oligonucleotides labelled in this way requires specialised equipment, which is not required in the enzyme-linked immunosorbent assay (ELISA) or chemiluminescent detection of immunogenically labelled oligonucleotides.

An inexpensive labelling group 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 deoxynucleotidyl terminal transferase or DNA polymerase on a DNP-aminohexyl derivative of ATP, and by reaction of Sanger's reagent (1-fluoro-2,4-dinitrobenzene) with oligonucleotides that contain an aminohexyl derivative of adenosine[16]. DNP groups have been introduced photochemically[17] and by reaction with brominated bases[18]. Multiple DNP groups have been incorporated into oligonucleotides in a more controlled manner using a DNP nucleoside phosphoramidite during solid-phase synthesis[9]. The DNP group is unstable under the normal conditions for base-catalysed deprotection, but this problem can be overcome by using labile base-protected monomers for DNA synthesis.

The aim of the present work is to develop simple DNP phosphoramidites for the mono- or poly-labelling of oligonucleotides during solid-phase synthesis. The strategy used involves inexpensive, readily available, non-nucleoside starting materials that can be functionalised in the minimum number of steps to give the desired DNP phosphoramidite, which can then be used in standard procedures to give the desired mono- or poly-labelled oligonucleotides.

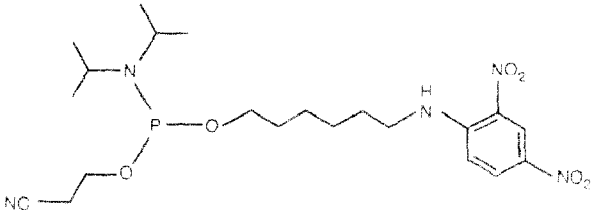
RESULTS AND DISCUSSION

Synthesis of a mono-labelling DNP phosphoramidite. – The DNP phosphoramidite **3**, which allows the attachment of a single DNP group to the 5'-end of oligonucleotides during solid-phase synthesis, was obtained as follows. 6-Aminohexan-1-ol (**1**) was treated with Sanger's reagent in methanol to give the mono-DNP derivative **2**. The reaction of **2** with 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite then gave **3** (80%).



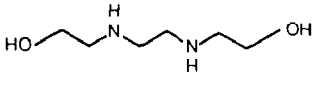
1 R = H

2 R = DNP



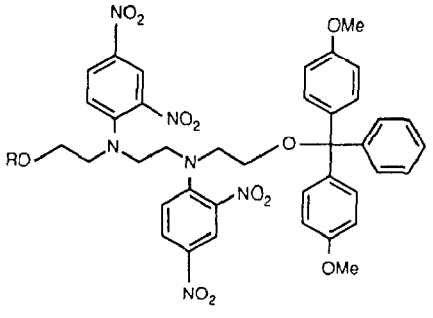
3

Synthesis of a poly-labelling DNP phosphoramidite. — The DNP phosphoramidite **7**, which allows the attachment of multiple DNP groups to oligonucleotides during solid-phase synthesis, was obtained as follows. 3,6-Diazaoctane-1,8-diol (**4**) was treated with Sanger's reagent in methanol and the product (**5**, 81%) yielded crystals that were suitable for X-ray diffraction analysis. The X-ray crystal structure of **5** showed that the DNP groups were arranged "*anti*" with respect to each other[19]. If the same conformation is retained in solution, this may be advantageous, allowing the binding of an antibody to each of the two DNP groups, thus enhancing the detection sensitivity. The reaction of **5** with 4,4'-dimethoxytrityl chloride in pyridine gave **6a** (31%). The poor yield was due to the equivalence of the two primary hydroxyl functions present. This reaction was not optimised, but **5** could be recovered by treatment of the bis(4,4'-dimethoxytrityl) side-product **6b** with acetic acid. Reaction of **6a** with 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite gave **7** (84%). One advantage of **7** is that it allows the attachment of two DNP groups to the oligonucleotide per synthesis cycle.



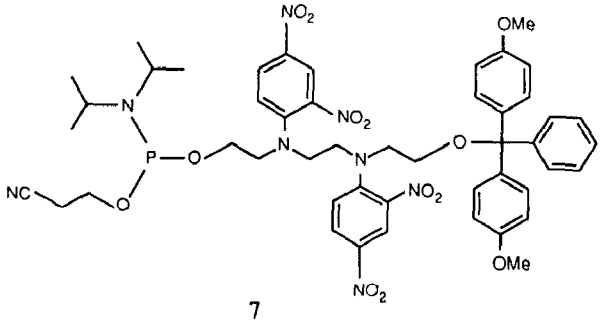
4 R = H

5 R = DNP



6a R = H

6b R = 4,4'-dimethoxytrityl



7

Synthesis of DNP-labelled oligonucleotides. — The DNP phosphoramidites **3** and **7** were used as 0.15M solutions in anhydrous acetonitrile and anhydrous dichloromethane, respectively, for the solid-phase synthesis of labelled oligonucleotides. A standard 0.2-μmol-scale synthesis cycle was used. The coupling efficiency of **7** was >97% in all syntheses. The coupling efficiency of **3** could not be measured directly, but was estimated by h.p.l.c. analysis to be >>90%. Oligonucleotides that had up to ten DNP groups (*i.e.*, five couplings of **7**) were synthesised, although, theoretically, any even number of DNP groups could be attached in this way.

Stability of the labelling to basic deprotection conditions. — The single DNP label introduced by the coupling of **3** to the 5′-end of the oligonucleotide was found by h.p.l.c. to be stable to normal base-catalysed deprotection (5 h, 55°, conc. NH_4OH). However, the multiple labels introduced by the coupling of **7** to the 5′-end of oligonucleotides were unstable under these conditions. H.p.l.c. showed that there was only one degradation product formed, which was neither 2,4-dinitroaniline nor 2,4-dinitrophenol. This instability problem was overcome by the use of commercially available phosphoramidites for DNA synthesis (Pharmacia "PAC"-amidites or ABI "FOD"-amidites), which bear base protecting groups that are removed under milder conditions (5 h in conc. NH_4OH at 20°). Under such conditions, the DNP label was degraded only to a very small extent, and no problems arose in the purification and isolation of the labelled oligonucleotide in high yield.

Purification of DNP-labelled oligonucleotides. — The highly lipophilic nature of the DNP group results in DNP-labelled oligonucleotides being eluted later than unlabelled failure sequences on reversed-phase h.p.l.c. This difference makes the purification of DNP-labelled oligonucleotides extremely easy, as the labelled oligonucleotide behaves similarly to a conventional "trityl-on" sequence. The effect of increasing the number of DNP labels on the time of elution is shown in Fig. 1. The labelled oligonucleotides are bright yellow in colour, which allows them to be distinguished readily from unlabelled sequences. Traces of the low-molecular-weight degradation product were removed when the sample was passed through a column of Sephadex G25 during the normal purification procedure.

Detection of DNP-labelled oligonucleotides. — Preliminary experiments confirmed that anti-DNP antibodies bind to DNP-labelled oligonucleotides, and that the antibodies can be detected both on the basis of radioactivity and by an ELISA method. Oligonucleotides labelled with one, two, six, and ten DNP groups were fixed on nylon filters in duplicate by u.v. irradiation. The filters were blocked using bovine serum albumin with EDTA to inhibit any DNAase activity, washed, and incubated with a 1:200 dilution of K3 monoclonal anti-DNP antibody for 1 h. After further washing, one filter was treated with ^{125}I-Protein A and then washed, and the signal was detected using X-ray film. The other filter was incubated with an anti-mouse-IgG-peroxidase conjugate for 1 h, washed, and detected by 4-chloro-1-naphthol and H_2O_2, which gave a blue precipitate. Further experiments are in progress.

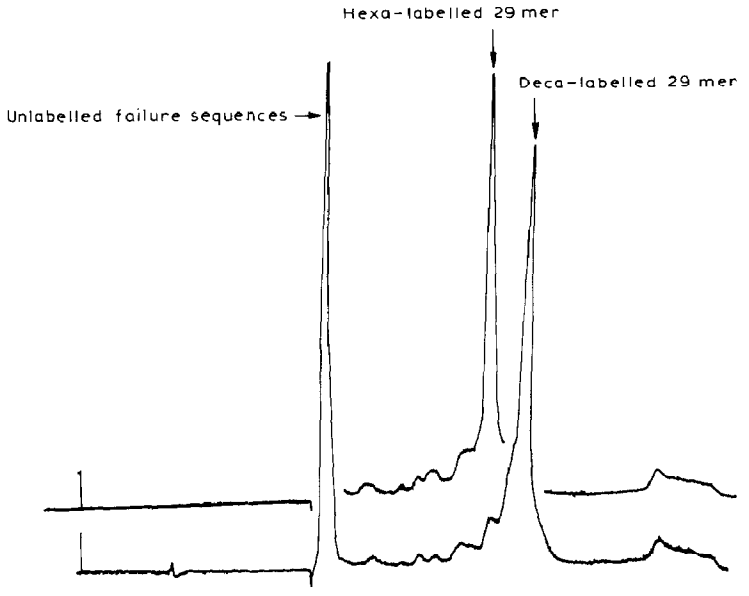


Fig. 1. Superimposed traces from h.p.l.c. of identical 29mers labelled with six (upper trace) and ten (lower trace) DNP groups, showing the effect of the lipophilicity of the DNP groups on elution time. Buffer *A*, 0.1M NH_4OAc; buffer *B*, 0.1M NH_4OAc/60% of acetonitrile.

EXPERIMENTAL

All solvents were of analytical grade. Alcohol-free anhydrous dichloromethane was prepared by distillation from CaH_2, and anhydrous tetrahydrofuran by distillation from sodium–benzophenone. Hexane was dried over sodium wire. Anhydrous acetonitrile was supplied by Applied Biosystems Inc. ^{1}H-N.m.r. spectra were recorded with Bruker WP-80 (80 MHz) and WP-200 (200 MHz) spectrometers. ^{31}P-N.m.r. spectra (81 MHz) were recorded with the latter spectrometer. F.a.b. mass spectra (positive ion, thioglycerol matrix) were recorded on a Kratos MS50 TC spectrometer. Oligonucleotide synthesis was performed with an Applied Biosystems 381A DNA synthesiser, and ending procedures with an ABI 380B DNA synthesiser. 2-Cyanoethyl phosphoramidite monomers for DNA synthesis were supplied by Applied Biosystems and Pharmacia. Sephadex G-25 N.A.P. (nucleic acid purification) columns were supplied by Pharmacia. Flash-column chromatography was carried out on Silica Gel 60 (Fluka), and t.l.c. on Silica Gel 60 F_{254} (Merck), using *A*, toluene–ethyl acetate (1:1), 1% of triethylamine; *B*, toluene–ethyl acetate (4:1), 1% of triethylamine; *C*, dichloromethane–methanol (9:1), 1% of triethylamine; *D*, dichloromethane–methanol (99:1), 1% of triethylamine; *E*, dichloromethane–2-propanol (95:5), 1% of triethylamine; *F*, dichloromethane–ethyl acetate (1:1), 1% of triethylamine.

6-(2,4-Dinitrophenylamino)hexan-1-ol (**2**). — To a solution of 6-aminohexan-1-ol (0.8 g, 6.8 mmol) in methanol (20 mL) was added 1-fluoro-2,4-dinitrobenzene (1.3 g, 1 equiv.). After 12 h, the solvent was evaporated *in vacuo* and the resulting oil was washed with ether to leave crude **2** (1.3 g, 70%). Recrystallisation from ether–methanol gave **2** (1.04 g, 54%), m.p. 75°, R_f 0.52 (solvent *C*). F.a.b. mass spectrum: *m/z* 284.12462 [calc. for $C_{12}H_{18}N_3O_5$: ($M^+ + H$) *m/z* 284.12463]. ^{1}H-N.m.r. data ($CDCl_3$): δ 1.1–2.0 (m, 9 H, 4 CH_2 and OH), 3.25–3.5 (m, 2 H, H-1,1), 3.64 (t, 2 H, *J* 6.2 Hz, H-6,6), 6.9 (d, 1 H, Ar H-6), 8.25 (dd, 1 H, Ar H-5), 8.5 (bs, 1 H, NH), 9.1 (d, 1 H, Ar H-3).

Anal. Calc. for $C_{12}H_{17}N_3O_5$: C, 50.88; H, 6.01; N, 14.84. Found: C, 50.40; H, 5.78; N, 14.70.

2-Cyanoethyl 6-(2,4-dinitrophenylamino)hexyl N,N-di-isopropylphosphoramidite (**3**). — To a solution of **2** (140 mg, 0.495 mmol) in anhydrous tetrahydrofuran (5 mL) was added anhydrous *N,N*-di-isopropylethylamine (0.1 mL, 1 equiv.) and 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite (0.132 mL, 1.2 equiv.). After 1 h, the reaction was quenched by the addition of ethyl acetate (20 mL), and the solution was washed with aqueous $NaHCO_3$ (2 × 5 mL) and brine (2 × 5 mL), dried (Na_2SO_4), filtered, and evaporated *in vacuo*. Flash-column chromatography (dichloromethane–ethyl acetate, 1:1) of the residue gave an oil which was dissolved in dichloromethane and precipitated with anhydrous hexane to give **3** (100 mg, 80%) as an oil, R_f 0.8 (solvent *F*). ^{31}P-N.m.r. data: δ 147.54 (s).

1-(4,4'-Dimethoxytrityloxy)-6-(2,4-dinitrophenylamino)hexane. — To a solution of **2** (1 g, 3.53 mmol) in anhydrous pyridine (15 mL) was added 4,4'-dimethoxytrityl chloride (1 equiv., 1.2 g). The mixture was protected from moisture and the reaction was complete after 1 h at 20°. Water (150 mL) was added and the product was extracted into chloroform (3 × 30 mL). The combined extracts were dried (Na_2SO_4) and filtered, and the solvent was evaporated *in vacuo*. The product was purified by flash-column chromatography (dichloromethane + 2% of triethylamine) to give the title compound isolated as an oil (1.24 g, 60%), R_f 0.78 (solvent *C*); F.a.b. mass spectrum: *m/z* 585.24744 (calc. for $C_{33}H_{33}N_3O_7$: *m/z* 585.24748). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.0–3.5 (m, 12 H, 6 CH_2), 3.75 (s, 6 H, 2 OMe), 6.8 (m, 4 H, Ar), 7.2–7.5 (m, 9 H, Ar and DNP H-6), 8.25 (d, 1 H, DNP H-5), 8.5 (bs, 1 H, NH), 9.1 (d, 1 H, DNP H-3).

3,6-Bis(2,4-dinitrophenyl)-3,6-diazaoctane-1,8-diol (**5**). — To a solution of 3,6-diazaoctane-1,8-diol (**4**; 1.5 g, 10 mmol) in methanol (30 mL) was added triethylamine (2.5 equiv., 2.53 g, 3.48 mL, 25 mmol) and 1-fluoro-2,4-dinitrobenzene (2 equiv., 3.72 g, 20 mmol). After 3.5 h, the mixture was cooled to 0°, and the orange precipitate was collected, washed with methanol (30 mL) and ether (3 × 30 mL), and dried *in vacuo* to give **5** (3.89 g, 81%). The product was recrystallised three times from methanol to give **5** with m.p. 149–150°, R_f 0.38 (solvent *C*). F.a.b. mass spectrum: *m/z* 481. ^{1}H-N.m.r. data ($CDCl_3$): δ 3.0–4.0 (m, 12 H, 6 CH_2), 4.7 (t, 2 H, 2 OH), 7.33 (d, 2 H, $J_{5,6}$ 9.5 Hz, DNP H-6), 8.13 (dd, 2 H, $J_{5,3}$ 2.8 Hz, DNP H-5), 8.47 (d, 2 H, DNP H-3).

Anal. Calc. for $C_{18}H_{20}N_6O_{10}$: C, 45.0; H, 4.16; N, 17.5. Found: C, 44.8; H, 4.18; N, 17.4.

8-(4,4'-Dimethoxytrityloxy)-3,6-bis(2,4-dinitrophenyl)-3,6-diazaoctan-1-ol (**6a**). — Anhydrous pyridine (3 × 30 mL) was evaporated from **5** (3.30 g, 6.88 mmol), which was then dissolved in anhydrous pyridine (60 mL). To this solution was added dropwise, during 30 min, a solution of 4,4'-dimethoxytrityl chloride (1.3 equiv., 3.03 g, 8.94 mmol) in anhydrous pyridine (100 mL). After 2 h, more 4,4'-dimethoxytrityl chloride (0.2 equiv., 0.47 g, 1.38 mmol) in anhydrous pyridine (20 mL) was added dropwise during 20 min and, after a further 30 min, the reaction was quenched with methanol (20 mL) and the solvent was evaporated *in vacuo*. A solution of the residue in dichloromethane (250 mL) was washed with saturated aqueous $NaHCO_3$ (50 mL) and water (3 × 50 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Column chromatography (solvent *A*) of the residue gave amorphous **6a** (1.68 g, 31%), R_f 0.29 (solvent *D*), 0.58 (solvent *E*), and 0.35 (solvent *A*). F.a.b. mass spectrum: *m/z* 783.26259 [calc. for $C_{39}H_{39}N_6O_{12}$: (M^+ + H) *m/z* 783.26257]. ^{1}H-N.m.r. data ($CDCl_3$): δ 2.0–2.25 (bs, 1 H, OH), 3.25–3.72 (m, 12 H, 6 CH_2), 3.73 (s, 6 H, 2 OCH_3), 6.65–7.23 (m, 15 H, Ar and DNP H-6), 8.06 (dd, 1 H, $J_{5,6}$ 9.3, $J_{3,5}$ 2.7 Hz, DNP H-5), 8.13 (dd, 1 H, DNP H-5), 8.52 (d, 1 H, DNP H-3), 8.55 (d, 1 H, DNP H-3).

2-Cyanoethyl [8-(4,4'-dimethoxytrityloxy)-3,6-bis(2,4-dinitrophenyl)-3,6-diazaoctyl] N,N-*di-isopropylphosphoramidite* (**7**). — Anhydrous tetrahydrofuran (3 × 20 mL) was evaporated from **6a** (0.643 g, 0.822 mmol) which was then dissolved in anhydrous tetrahydrofuran (30 mL). To this solution was added *N,N*-di-isopropylethylamine (4 equiv., 3.29 mmol, 0.425 g, 0.573 mL) and 2-cyanoethyl *N,N*-di-isopropylphosphoramidochloridite (1.5 equiv., 1.23 mmol, 0.291g, 0.276 mL). The solution was stirred at 20° for 15 min, quenched with ethyl acetate (30 mL), washed with brine (4 × 100 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Column chromatography (solvent *B*) of the residue gave **7** as an oil, a solution of which, in the minimum volume of anhydrous dichloromethane, was added dropwise to anhydrous hexane (750 mL) at −78°. The orange precipitate which formed was collected and washed with anhydrous hexane to give **7** (0.678 g, 84%), R_f 0.50 (solvent *B*). F.a.b. mass spectrum: *m/z* 983.37044 [calc. for $C_{48}H_{56}N_8O_{13}P$: (M^+ + H) *m/z* 983.37042]. ^{31}P-N.m.r. data ($CDCl_3$): δ 149.12 (s).

Synthesis of DNP-labelled oligonucleotides. — Oligonucleotide synthesis was performed with an Applied Biosystems 381A DNA synthesiser, using the standard 0.2-μmol-scale synthesis cycle. For the additions to the 5'-end of oligonucleotides, a 0.15M solution of **3** in anhydrous acetonitrile and a 0.15M solution of **7** in anhydrous dichloromethane were used. Ending procedures were carried out on an Applied Biosystems 380B DNA synthesiser.

Degradation of 7-CTC GAG TAT GCC GAG ACC CCT AAT with ammonia. — A solution of 6 o.d. units (A_{264}) of the title compound in distilled water (200 μL) was added to conc. ammonia (1.8 mL) in a sealed vial. Every hour, an aliquot (200 μL) was removed, diluted with 0.1M NH_4OAc (700 μL), and analysed by h.p.l.c.

Measurement of monomer coupling efficiencies. — Coupling efficiencies were measured by comparison of the absorbance at 498 nm of the 4,4'-dimethoxytrityl cations produced in the deprotection steps of successive synthesis cycles. Each fraction was diluted to 25 mL with 0.1M toluene-4-sulphonic acid in acetonitrile.

H.p.l.c. — A Gilson 303 and a Perkin–Elmer 410 system were used with ABI aquapore octyl reversed-phase columns. The buffer systems were varied according to the number of DNP labels attached to the oligonucleotide. The general buffer systems were *A*, 0.1M NH_4OAc; and *B*, 0.1M NH_4OAc/X% of acetonitrile where $30\% < X < 70\%$. The greater the number of DNP groups present, the higher the percentage of acetonitrile used in buffer *B*. These buffers were used (flow rate, 3 mL/min) in combination with the following gradient to achieve the optimum separation for the sequence being examined:

Time (min)	0	3	4	26	28	29	32
Buffer *B* (%)	0	0	15	100	100	0	0

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